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Physics of Cancer

Cellular and microenvironmental effects

Claudia Tanja Mierke

VOLUME TWO

SECOND EDITION

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Physics of Cancer, Volume 2 (Second Edition)

Cellular and microenvironmental effects

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Cellular and microenvironmental effects

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Part VI The impact of the mechanical and biochemical interaction of cancer cells with other cells in transendothelial migration

Preface

Dear reader,

My book is about the novel and promising field of the physics of cancer, which has become the focus of many biophysical research groups all over the world. The book aims to present several biophysical approaches used in cancer research. The major findings that contribute significantly to the field are presented from a biophysical point of view. The intended readership includes everyone interested in cancer research, in particular those readers studying biological physics, physics or tumor biology. The book is suitable for upper undergraduate, graduate, doctoral and postdoctoral students, senior scientists, lecturers, principal investigators and professors. I wrote this book because I was kindly invited to by IOP Publishing and because there is currently no such book available in any format. Many parts of it are supported by figures that I have drawn myself or designed. I hope that the book will promote understanding of why physics of cancer is so important for cancer research. All types of readers, including students and researchers, should be guided through the physics of cancer field by it. Every chapter is a complete part, with references and further reading suggestions. The book also has a glossary that will help the reader to follow the book more easily. I hope that the book will help to establish the physics of cancer as an essential part of cancer research.

Preface for second edition

Since the appearance of the first edition of *Physics of Cancer*, I have received many suggestions and very helpful comments. Therefore, I decided to write the second edition of *Physics of Cancer*, which opportunity was offered to me by IOP Publishing. In January 2018 I began to write this second edition in the evenings and at the weekends, again without any support from the University of Leipzig. Specifically, I have completely revised the second edition in terms of language, a great increase in the number of figures, inclusion of four novel chapters and seven partly new chapters, and each chapter has been fully updated. The novel chapters are:

- Actin filaments during matrix invasion
- Microtubules during migration and matrix invasion
- Nuclear deformability during migration and matrix invasion
- The active role of the tumor stroma in regulating cell invasion

Therefore, the size of the book has grown so much that it has made sense to split it into two volumes. The two volumes now contain six parts. I hope that the now broader scope of the two volume book *Physics of Cancer* book will attract even more readers from other fields of research.

Machern, May 2018
Yours sincerely,
Claudia Tanja Mierke

Acknowledgments

I thank Thomas M L Mierke for searching, editing and formating of the references for all chapters and for helping me to keep in the time schedule for writing this book.

Author biography

Claudia Tanja Mierke

Claudia Tanja Mierke studied biology at TU Braunschweig in Germany and received her doctoral degree from the Medical School of Hannover in March 2001; her thesis concerned human endothelial and mast cell cell–cell interactions. Her postdoctoral research was performed in different institutes of the University of Erlangen-Nuremberg, and covered a number of fields: cancer research;
inflammation of endothelial cells and molecular cancer research; and biophysical cancer research. In 2012 she habilitated in biophysics. Her scientific results have been presented at many international conferences and published in leading international peer-reviewed journals in this field. Since 2010, she has worked as a professor at the University of Leipzig and she has published many review articles and book chapters on the subject of physically driven cancer research. As a professor of soft matter physics and biological physics, her research focus is on biophysical cancer research, including cell motility and transmigration through endothelial vessel linings.

With a background in biology and molecular oncology, Professor Mierke is now head of the Biological Physics Division at the University of Leipzig, where she regularly teaches molecular and cell biology, biophysics and soft matter physics to physicists, and is concerned with various research areas that contribute to our understanding of the physical aspects of cancer.

Part IV

The effect of microtubules and the mechanical properties of the nucleus on matrix invasion

Motile cells possess a front–rear polarization of their microtubule framework, which promotes all essential processes, such as providing cellular mechanical properties, intracellular trafficking and signal transduction pathways supporting cell migration. Three-dimensional (3D) cell motility is part of many essential processes such as embryonic development, repair of injured tissues and immune surveillance, and is also part of pathological processes such as the malignant progression of cancer. In 2D cell cultures, the cytoskeleton is well-investigated and has been revealed as a regulator of cell migration. In particular, the microtubule network facilitates the polarized trafficking and signal transduction that are critical for cellular shape and migration in 2D. However, it has been demonstrated that microtubule function in cell morphogenesis and motility can differ strongly in 2D and 3D microenvironments. These differences and their relevance for understanding the role of microtubules in cell migration *in vivo* are presented in part 4. Moreover, a general overview of microtubule structure and functions is given, and it is shown how the cell shape and motility in 3D matrices is controlled by microtubules. In addition, the effect of the nucleus as a mechanical obstacle for cell migration is presented and discussed. The nuclear position and shape can be altered by the surrounding microenvironment and subsequently also gene expression is altered due to changed coupling between the nuclear architecture and the cytoskeleton.

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Chapter 9

Microtubules during migration and matrix invasion

Summary

Chapter 9 introduces briefly the assembly, polymerization and structure of microtubules network in cells. A first focus is on the structure that determines the role of microtubules in the physiological processes such as cell division, cell migration and invasion and in pathological processes such as cancer. Besides the structures of microtubules, the role of interacting proteins can affect the functional properties. A second focus is set on the impact of the microtubules on the cellular mechanical properties and their alterations during malignant cancer progression. Moreover, the microtubules help cancer cells to withstand cancer treatment. Finally, the interaction of microtubules with actin filaments shows how the microtubule cytoskeleton and the actin cytoskeleton are closely associated and hence involved in the process of cellular motility such as for the malignant progression of cancer.

9.1 The structure and assembly of microtubules

The cell's cytoskeleton is composed of three polymers, actin, microtubules and intermediate filaments, that are different in their structure, morphology and function. Moreover, the three cytoskeletal types display specific dynamic properties that determine their individual functions. The three filament types each possess a large number of interacting, so-called accessory proteins, that facilitate the assembly of the monomers to filaments and regulate interactions between the three major filaments. In addition to these interactions, the three filament types can interact with cellular structures such as membranes, cell-membrane receptors, organelles and chromosomes to perform cellular functions. Despite the differences in interacting proteins, filament assembly and dynamical remodeling of the three filaments, these stress filaments are not clearly separated within the cell. They are even polymerized to complex structural networks and these complex structures are required for their interaction. The dynamic

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interactions between all three cytoskeletal filaments are critical for the maintenance of overall integrity, the organization of the cell's cytoplasm and the regulation of different cellular functions. Moreover, these cytoskeletal networks play a key role in pathological processes such as the malignant progression of cancer.

How are microtubules assembled?

Microtubules consist of α- and β-tubulin monomers that form heterodimers in order to polymerize to tubes that show a clear polarization (Desai and Mitchison 1997). They have two different ends that provide a functional polarization of the entire tube. The plus ends of the microtubules display a frequent growth and shrinkage behavior and serve as the binding sites for complexes that consist of so-called plus-end-tracking proteins (+TIPs). These TIPs regulate the polymerization of microtubules and signal transduction processes, and enable the interaction with cellular structures such as membranes (Akhmanova and Steinmetz 2015).

In general, microtubules fulfill roles in the transport of vesicles and macromolecules, cell migration and cytoskeletal reorganization. Additionally, microtubules perform various dynamic roles such as the movement facilitated by kinesin and dynein motor proteins, the beating of flagella or cilia and during the process of mitosis in cell division, the precise segregation and separation of chromosomes. A unique feature of microtubules is their polymerization type (Schek *et al* 2007). Specifically, microtubules build hollow cylinders of about 25 nm in diameter, which are assembled from monomeric subunits of the protein tubulin. First, heterodimers of α- and β-tubulin are created that bind in a head-to-tail manner in order to assemble polar protofilaments. Thirteen of these protofilaments arrange themselves into a closed tube structure (figure 9.1). Thereby, tubulin needs to bind GTP, which enables tubulin to polymerize. The hydrolysis of the bound GTP is critical for the growth and the stability of the microtubules (Desai and Mitchison 1997, Nogales and Wang 2006). Dissimilar to various polymerizing systems such as actin filaments, microtubules perform randomly distributed periods of growth and shrinkage, termed the dynamic instability of microtubules (Mitchison and Kirschner 1984a). In more detail, microtubules grow steadily until a catastrophe occurs, which is a distinct point at which microtubules switch from polymerization to a fast disassembly mode. The disassembly state is followed by rescue events that enable microtubules to return to normal filament polymerization and subsequently microtubule growth.

The alteration of the polymerization conditions can affect the rates of catastrophe and rescue (Walker 1988), however, under a certain condition, the polymerization and depolymerization of microtubules can be detected, which supports the hypothesis that the catastrophe and rescue modes are an intrinsic property of the polymer itself. Until now the mechanism of the phenomenon has not been revealed, although it is closely associated to the GTP hydrolysis event, as the addition of non-hydrolyzable analogs of GTP abolish this specific behavior (Vale *et al* 1994). Through structural analysis it has been demonstrated that the protofilaments polymerized of GTP–tubulin monomers are straight, whereas the protofilaments become curved upon the hydrolysis of GTP to GDP (Melki *et al* 1989, Hyman and Mitchison 1990). Based on these results, it has been hypothesized that there needs to

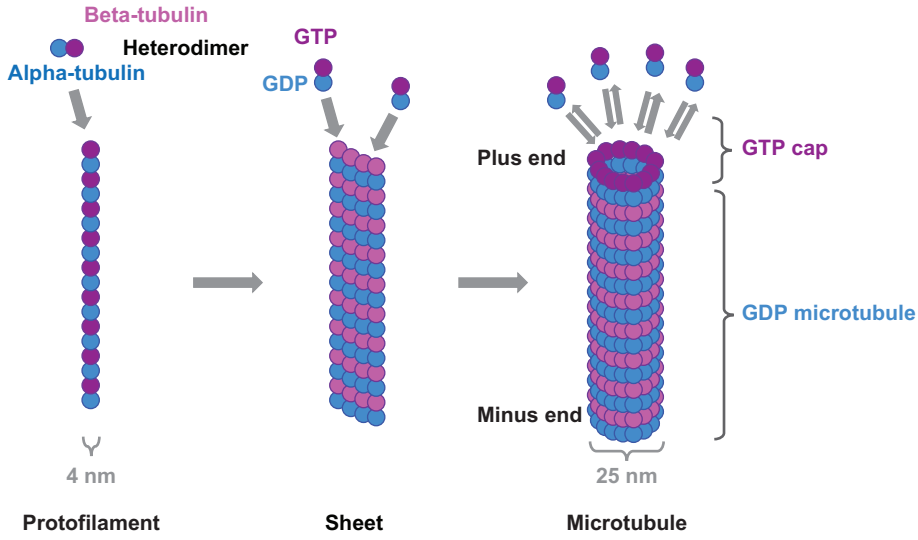

Figure 9.1. Assembly of microtubules. The α- and β-tubulins interact head-to-tail to build a long protofilament. These protofilaments align along at their long axis in a curved sheet. A sheet of 13 protofilaments closes itself to form a hollow tube cylinder, which consists of a GTP cap at its plus end (terminating in β-tubulins) and the GDP containing microtubules is located at the minus end (terminating in α-tubulins). The microtubule elongates by the addition of tubulins to both ends, but preferentially to the plus end.

be a 'cap' of GTP–tubulin at the end of the microtubule causing the stability of the structure and, moreover, this has been used as a prerequisite for the development of a large variety of mechanical and chemical models (Desai and Mitchison 1997). In summary, the essential and basic part of these models is that the loss of the so-called GTP–tubulin cap by GTP hydrolysis or dissociation of the entire subunit puts the microtubule into an unstable state, which switches to microtubule dissociation through a catastrophe, evoking microtubule breakdown.

Several properties of microtubule polymerization contribute to this special kind of polymerization, such as the cylindrical structure of microtubule, the arrangement of exactly 13 protofilaments building the basis for microtubule growth, the fast GTP hydrolysis and their intrinsic instability. Single-molecule studies revealed many advantages for the study of the cell's cytoskeleton, such as precise analysis of the cellular mechanical properties and the movement of specific motor proteins on both microtubules and on actin filaments. Moreover, this single-molecule analysis can be employed for the process of microtubule polymerization, when it is combined with optical tweezers and specific microfabricated barriers, which can help us to obtain significant new insights into the growth process of microtubules. In contrast to standard microscopy techniques with known resolution limits, optical tweezer analysis can reveal insights at the molecular level (Kerssemakers *et al* 2006, Schek and Hunt 2005).

The temporal resolution has been dramatically improved, and measurements can be performed at rates that are 100 times faster than the common video rates used in other conventional microscopic studies (Schek *et al* 2007). In particular, this

enhanced time sampling provides more details about the dynamics of microtubule ends. First, the assembly is driven by the addition of a single subunit, which is in contrast to the conclusion based on previous results that propose the addition of oligomers of three or more dimers to be added to the end (Kerssemakers *et al* 2006). Second, microtubules can perform periods of shortening that can lead to the loss of multiple layers of tubulin dimers. However, this depolymerization mode is dissimilar to a catastrophe mode and the depolymerization mode can switch quickly to a regrowth mode of the polymer. This finding is contrary to other models proposing a small cap of GTP–tubulin or a strict coupling between hydrolysis and polymerization, whereas instead it seems to be possible that GTP–tubulin exists farther away from the growing microtubule end. However, the mechanism is not yet fully understood.

When microtubule dynamics are compared in *in vitro* and *in vivo* systems, the polymerization rates in the cell are approximately five-fold to ten-fold higher than that of purified tubulin in a physiological solution (Cassimeris 1993). These so-called effective polymerization rates inside a cell are averaged over a wide range of dynamics at the growing tip of the microtubule, which also includes the shortening of several tubulin layers (Schek *et al* 2007). The microtubule-associated proteins or some other factors within the cell may reduce the shortening phase, which in turn causes an increase in the overall apparent polymerization rate and may explain the differences between *in vitro* and *in vivo* polymerization experiments. A candidate for such a regulatory protein is doublecortin (Moores *et al* 2006). In addition to the simple assembly dynamics, there are proteins targeting and tracking the growing ends of microtubules, such as a growing family of proteins, including CLIP-170, EB1 and XMAP215 (Akhmanova and Hoogenraad 2005, Howard and Hmyna 2003, Lansbergen and Akhmanova 2006, Schuyler and Pellman 2001), as well as a protein complex called the Dam1 complex that builds a ring structure around the microtubule, which is still bound to it even during microtubule depolymerization (Westermann *et al* 2006). How these proteins bind to the microtubules ends and what specific structural or biochemical features they prefer is still elusive. When the proteins interact with the growing tips of microtubules, they are not continuously bound to them, but instead they bind and unbind during the growth of microtubules and therefore appear to surf. This interaction needs to be characterized in more detail together with the existence of an extended GTP–tubulin cap that can possibly provide interaction cues.

9.2 The assembly of the mitotic spindle during cell division

The overall goal of a somatic cell, when undergoing cell division, is to segregate its genomes precisely and equally to the new daughter cells. In eukaryotic cells, the process of mitotis and subsequently cell division is guided by a self-organized structure termed the mitotic spindle. It is understood that mechanical forces must be applied to the chromosomes, whereas simultaneously, the network of microtubules building the spindle needs to exert forces and also to provide large forces for maintenance of the integrity of the entire spindle apparatus. For measuring the

forces generated by the microtubule network, key proteins have been utilized (Forth and Kapoor 2017). These new findings, such as length-dependent force generation and protein clustering by asymmetric friction and entropic expansion forces, will provide the basis for the development of the advanced force generation models required to explain proper spindle function and maintenance of spindle integrity.

During the cell division of eukaryotes, the mitotic spindle facilitates the distribution of chromosomes into the two daughter cells (Gadde and Heald 2004, Kapoor 2017). In the mitotic spindle the microtubules are arranged as a dense array and their exact positions, orientations, polymerization lengths, overlapping regions and nucleation sites are driven by motor and non-motor proteins. During the process of cell division, mechanical forces are required for the separation of the chromosomes (Nicklas *et al* 1982). Pioneering experiments have been performed using glass microneedles to directly mechanically stimulate the chromosomes of dividing grasshopper spermatocytes. Indeed, the chromosomes in the anaphase of the mitotic phase of the cell cycle are mechanically stimulated to move towards the spindle poles. It has been calculated that forces of approximately 0.1 pN are needed to move chromosomes through a viscous microenvironment. However, the mitotic spindle is able to exert forces up to 700 pN, which has been determined by exerting a counterforce to stall the movement of the chromosomes (Nicklas 1983, 1988). Unexpectedly, this experimental value is several hundreds of times larger than the maximum force of a single motor protein, indicating that the spindle is able to perform more mechanical work by generating pronouncedly higher forces on the micron-length-scale than needed under minimal mechanical work to displace the chromosomes.

When these large forces on chromosomes are applied from the center of the bipolar spindle apparatus towards the outermost spindle poles, how can the spindle still keep its structural integrity? Why does the spindle not collapse under this high tension? An answer seems to be that the microtubule scaffold forming the spindle does not interact with every single microtubule array directly with kinetochores of the chromosomes and hence the ones that are not connected provide opposing forces that counterbalance the forces across the entire bipolar spindle apparatus. In particular, these forces may enable the mitotic spindle to function in a steady state that provides its structural integrity while directing the movement of chromosomes through the dense microtubule scaffold. Several forces, such as overlap length-dependent pushing and braking forces, the frictional forces of proteins and the autonomous clustering of protein ensembles by frictional asymmetry and the entropic forces generated by diffusible crosslinkers, seem to be important for the proper functioning of the mitotic spindle. Finally, a precise force map of the mitotic spindle can be proposed that helps in the conception of supporting experiments.

9.2.1 How are the different subsets of spindle microtubules organized?

The microtubules assembling the mitotic spindle are classified into three distinct groups (figure 9.2). First, there are the kinetochore microtubules (termed k-fibers) that interact directly with the kinetochores of the chromosomes. In higher eukaryotes, the parallel

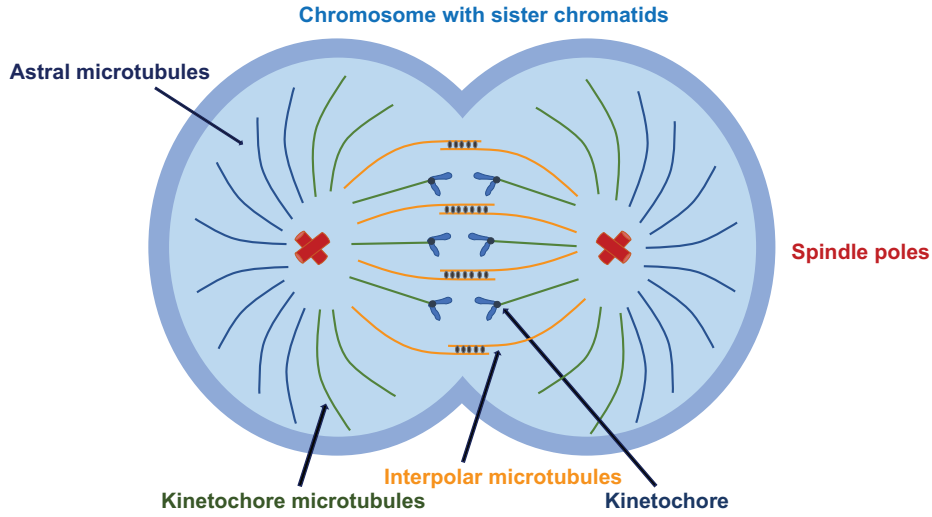

Figure 9.2. The spindle microtubule network. Schematic drawing of the three major classes of microtubules of the mitotic spindle, which are kinetochore microtubules (termed k-fibers), interpolar microtubules and astral microtubules. The k-fibers bind to the kinetochores of the chromosomes to separate and segregate the sister chromatids towards the spindle poles.

bundles of multiple microtubules (approximately 25 in female rat kangaroo *Potorous tridactylus* (Ptk1) kidney epithelial cells) build these k-fibers to interact with the kinetochore on each sister chromatid of the chromosome (McEwen *et al* 1997). Second, there exist astral microtubules originating from the centrosome and elongating towards the cortex of the cell. The astral microtubules carry out the capturing of chromosomes and position the spindle through cortex interactions (Hayden *et al* 1990, Grill *et al* 2003). Third, there are interpolar microtubules present, which are densely arranged in the space between the two opposing spindle poles (Mastronarde *et al* 1993). These interpolar microtubules possess shorter lifetimes than kinetochore microtubules (half-life values of $t_{1/2} = \sim 20$ s) and k-fibers (several minutes) (Saxton *et al* 1984). In particular, these interpolar microtubules are crosslinked at overlaps throughout the spindle apparatus and are mostly arranged antiparallel near the spindle equator and parallel at spindle poles, where they are tightly focused through clustering. In specific systems such as *Xenopus laevis* egg cells, the interpolar microtubules represent approximately 90% of the spindle microtubule population (Dumont and Mitchison 2009). This dense scaffold of microtubules has a mean distance of 30–50 nm between neighboring microtubules (Mastronarde *et al* 1993), but it still needs to fulfill, at first glance, two opposite functions: on the one hand it provides structural integrity to generate and face large forces and on the other hand it needs to support the movement of micron-sized chromosomes through its dense scaffold. Hence, two different kinds of forces seem to play a role, microtubule pushing forces and viscous forces facilitating the sliding of microtubules and the remodeling of the spindle apparatus. How can spindles perform their mechanical function during the process of chromosome segregation?

What kinds of forces, in terms of magnitude, localization and orientation, are required in a network?

9.2.2 What forces are present in the spindle microtubule network?

Microtubules within a spindle produce two kinds of forces, that can be classified into active forces utilizing chemical energy and passive forces, such as friction dissipating energy. Active forces are exerted during dynamical assembly and disassembly and by their associated motor proteins (figure 9.3). The microtubule filaments elongate by addition of GTP-bound tubulin dimers to the filament end and in turn shorten when GDP-bound dimers are dissociated from them. When tubulin dimers are added a filament growth energy of approximately 10 kT is required, however, upon removal of the dimers the filament releases energy that can be converted into mechanical work (Hill and Kirschner 1982). A single dynamic microtubule can exert pushing forces in the range of 2–5 pN, when it grows towards a calibrated load (Dogterom and Yurke 1997) and bundled microtubules can even exert tens of piconewtons of force (Laan *et al* 2008). Moreover, motor proteins utilize the free energy gained from hydrolysis of ATP to perform directional motion on microtubule tracks by producing forces to promote the displacement of cargos or the relative sliding of the microtubules. Using single-molecule force spectroscopy, mitotic motor proteins such as kinesin-5 (Valentine *et al* 2006, Korneev *et al* 2007), dynein (Gennerich *et al* 2007) and kinesin-8 (Jannasch *et al* 2013) have revealed force-dependent stepping behaviors, when it is under a load. Single motor proteins are able to exert maximum forces between 1 to 10 pN. Upon loading with a cargo, the rate and directionality of stepping, the life-time of motor protein–microtubule interactions and the processivity of the motor protein are adapted. Albeit the force generation within microtubule bundles scales with the amount of

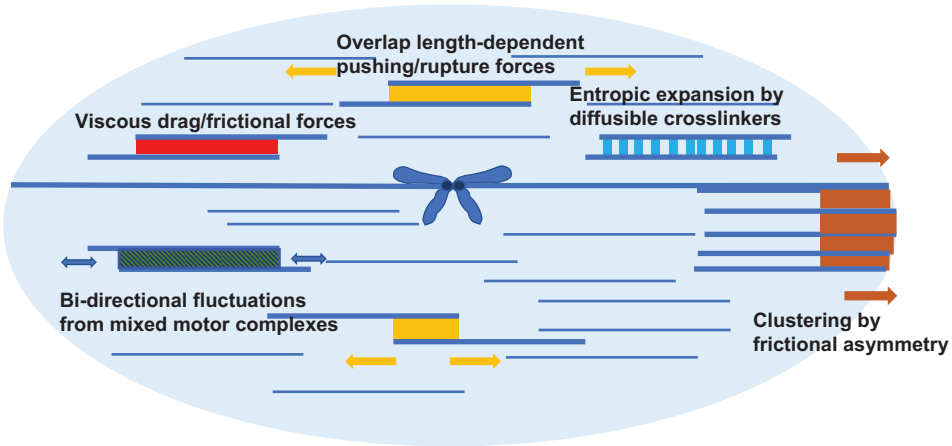

Figure 9.3. Model of the spindle force map. The sources of force production in a dense spindle microtubule apparatus are the overlap length-dependent pushing and rupture forces (orange), viscous frictional forces (red), protein clustering by frictional asymmetry (brown), entropic expansion by diffusible crosslinkers (blue bars) and fluctuations due to mixtures of plus-end- and minus-end-directed motor proteins (blue and green stripes).

motor proteins, multiple motor proteins carrying a load cannot generate ongoing additive forces (Jamison *et al* 2012). Motor protein driven force generation works together with crosslinked filaments, and still requires direct measurement approaches.

Compared to active forces, passive forces such as elastic forces, viscous drags or frictional resistance are much less well studied, but still highly important. The elastic forces include stretching forces exerted on sister kinetochores and the bending forces of stiff microtubule filaments (Gittes *et al* 1993) or bundles (Rubinstein *et al* 2009). Since microtubules are bundled by different protein types possessing different crosslinking densities, the mechanical properties of the microtubules can be altered. When relative filament sliding occurs, the bundle stiffness usually scales with the number of individual microtubules of the bundle. In contrast, in tightly crosslinked bundles, which cannot undergo sliding, the stiffness increases with the square of the microtubule number. Hence, knowledge of the criteria for filament sliding abolishment by crosslinking proteins is essential for describing the mechanical properties of microtubule networks.

In addition, non-motor microtubule-associated proteins (MAPs) that crosslink several microtubules create frictional resistance to the relative sliding within these microtubules. On the molecular level, the resistive and frictional forces occur upon noncovalent bond ruptures between proteins during the relative motions of various spindle factors. The magnitude of these resistive forces may increase directly with the velocity similar to macroscopic Stokes drag force, when an object is moved through viscous environment. What are the time-scales of forces? The spindle is a complex of viscoelastic material that is assembled by dynamically ordered polymer arrays. Although these materials are simple, the mechanical responses rely on whether forces are applied quickly or slowly. In particular, the viscoelastic material behaves as a solid when forces are applied rapidly, but in turn when forces are applied slowly the material displays a more liquid-like behavior. On the molecular level, individual motor proteins can perform moving at rates from tens to hundreds of nanometers per second (Sharp *et al* 2000). In cells, the microtubule plus ends exhibit grow rates of several hundreds of nanometers per second (Rusan *et al* 2001). Thus, both motor proteins and microtubule growth dynamics are capable of a rapid force exertion by performing motor stepping and tubulin polymerization events occurring several times per second at a nanometer scale. Similarly, the frictional forces arise at the same fast time-scales, when the proteins interact with sliding microtubules or the dynamic microtubule ends. Elastic forces such as k-fiber bending or kinetochore stretching across paired chromosomes are present at a time-scale of minutes during the metaphase of the mitotic process. Hence, when presenting a spindle force map, we need to consider the active and passive forces generated by certain individual components of the spindle apparatus and the time-scale on which these forces interact.

How can the mechanical properties of the entire spindle microtubule network be measured?

The spindles assembled in meiotic extract from *Xenopus laevis* eggs can be used as a reliable and easily accessible model system, as these spindles are not embedded by

cellular membranes. The spindle has been probed using calibrated microneedles and hence the time-scale-dependent viscoelastic properties of the metaphase spindle have been analyzed (Shimamoto *et al* 2011). Specifically, two glass needles with a silicone coating that impairs specific binding interactions of these needles with individual spindle components are used (figure 9.4). Both needles are inserted into the spindle apparatus near the metaphase plate. In the next step, perturbations are applied perpendicular to the long axis of the spindle by oscillating one needle, whereas the other needle is used as a force-calibrated readout. The stretching of the spindle on slow (minutes) and fast (seconds) time-scales in the range of micrometer-scale motion revealed that the spindle responded as an elastic material.

However, at intermediate time-scales (tens of seconds), the spindle displays viscous properties and hence behaves as a liquid. These time-scale-dependent mechanical properties are described by the Zener-type viscoelastic solid model (synonymously termed the standard linear solid model) (figure 9.5). The model is based on two springs and a dashpot. One of the two spring-like elements, in which a stretching force induces a distinct amount of displacement, seems to be connected to the bending stiffness of interpolar microtubules. In serial arrangement with this spring is a dashpot element, serving as a viscous frictional damper reducing the motion, which seems to be connected to the lifetimes and strengths of microtubule crosslinks. Both serial elements together are arranged in parallel with a stiffer spring-like element corresponding to k-fiber bending (figure 9.5). Using specific biochemical

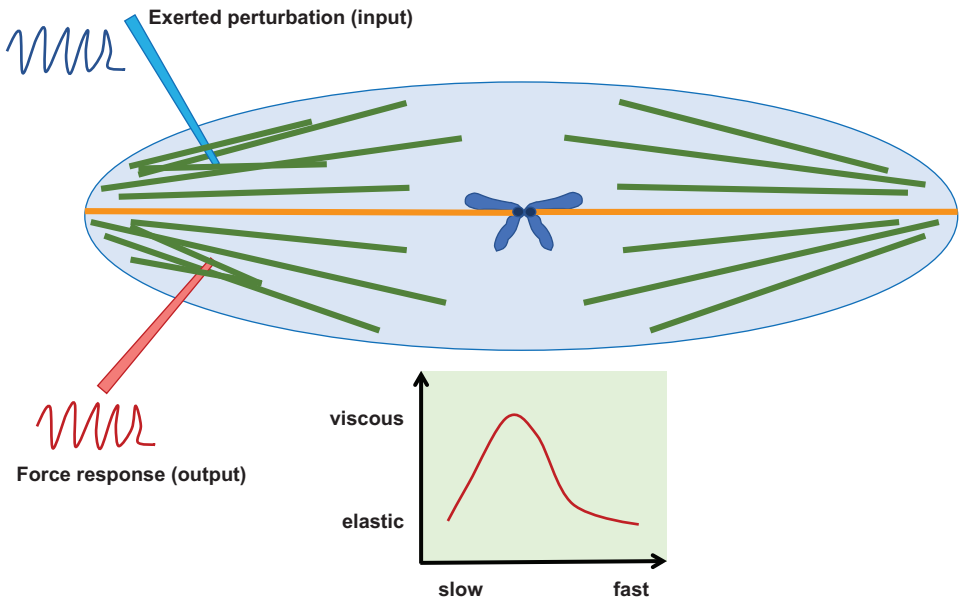

Figure 9.4. Experimental force measurement setup. Two microneedles are inserted into a spindle. One of these needles is used to apply mechanical perturbations perpendicular to the long axis of the spindle. The second needle serves as probe, which is perturbed upon force stimulation by the first needle. Within seconds (on a fast time-scale), the spindle shows more elastic behavior, whereas with minutes (on a slow time-scale), the spindle displays more viscous behavior.

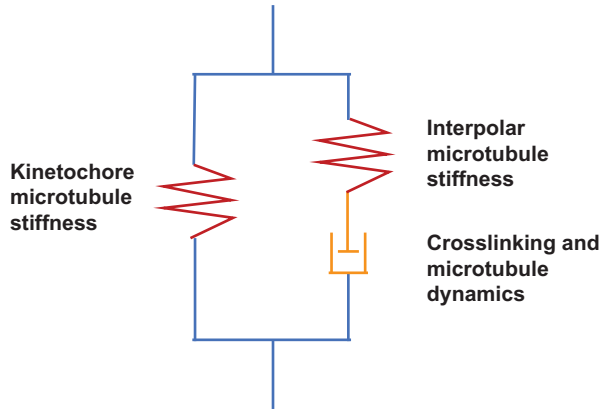

Figure 9.5. Zener-type model for a viscoelastic solid. The elastic spring-like elements depict the stiffness of the k-fibers and interpolar microtubules, whereas the viscous dashpot type is associated with crosslinking dynamics.

inhibitors for spindle component disrupturing or specific microtubule populations, the contribution of each individual component to the mechanical phenotype can be determined. The application of compressive forces with microneedles at the spindle equator decreases the spindle width by approximately 10%, which leads to a compensatory alteration in spindle length, indicating that the spindle behaves as a viscoelastic solid material (Itabashi *et al* 2009). Moreover, when forces are directly applied to the spindle poles that stretch the spindle outward along the long axis of the spindle, a restoring force is generated that persists over several minutes (Takagi *et al* 2014). The spindle volume and the density of tubulin is conserved during the stretch application. In summary, the time-dependent viscoelastic properties of the spindle apparatus with the dynamics of interpolar microtubules, the long-lived and stiff kinetochore fibers and protein-facilitated microtubule crosslinking, provide a strong network facilitating the chromosome segregation by remodeling itself.

9.2.3 How are the forces altered by crosslinked microtubule pairs?

Reconstituted microtubule networks of purified proteins have revealed mechanical properties of the individual components and can thereby lead to new principles and mechanisms.

9.2.3.1 The active force generation in microtubule networks

Kinesin-5 was identified as the first motor protein that binds to and acts on antiparallel microtubules in order to slide them apart by exerting a force on them (Kapitein *et al* 2005). Specifically, it assembles as a coiled-coil homotetramer structure and adapts a bipolar shape by placing the N-terminal motor domains at their opposite ends (Kashina *et al* 1996, Scholey *et al* 2014). The processive directional motion is provided by crosslinking of microtubules and thereby microtubule sliding is enhanced by a non-motor microtubule-binding domain of kinesin-5 at its C-terminus (Weinger *et al* 2011). Hence, the ability to push apart antiparallel

microtubules depends on the functions of kinesin-5, such as the emergence and maintenance of the spindle bipolarity and the regulation of the microtubules flux to the spindle poles in the metaphase (Ferenz *et al* 2010). Moreover, the sliding of microtubule pairs has identified for at least two minus-end motor proteins. An example is kinesin-14 (Ncd), a microtubule minus-end-directed kinesin with a C-terminal motor domain and an N-terminal non-motor microtubule-binding domain (enhancing its processivity), which enables kinesin-14 to bundle and slide antiparallel microtubules (Fink *et al* 2009). Another minus-end-directed motor protein is cytoplasmic dynein, which has been shown to slide apart two microtubules in an *in vitro* microtubule bundle assay (Tanenbaum *et al* 2013).

Moreover, it has been proposed that these motor proteins exert forces to provide microtubule sliding, which has been experimentally confirmed for kinesin-5 (Shimamoto *et al* 2015). Using single-molecule assays, specific proteins can be coated directly on a trapping bead that is attached to one of two microtubules, whereas the second microtubule is tightly associated to a surface. After connection of kinesin-5 to these microtubules, the force generated by the sliding microtubule pairs can be determined, and when the number of kinesin-5 molecules and the length of the interaction between the two microtubules are known, the force generation can be analyzed. Indeed, the analysis of the force exertion by kinesin-5 complexes acting on crosslinked microtubule pairs has revealed that the magnitudes of the pushing and braking forces correlate positively with the overlap length of microtubules (Shimamoto *et al* 2015). Moreover, it has been found through analysis of cargo carrying kinesin-1 complexes that the force did not persistently scale with the number of motor proteins attached to the microtubules' surface (Jamison *et al* 2012). Crosstalk between two or more kinesins may evoke fast detachment if one kinesin impairs the mechanical force exertion of another (Furuta *et al* 2013). In particular, when a motor protein steps on the microtubules, a strain is exerted on other proteins within this motor protein complex and then may lead to unbinding of this protein, in the case that it is sensitive to force. Subsequently, a persistent force cannot be generated. In contrast, during the process of sliding of antiparallel microtubules by kinesin-5, the magnitude of the sliding force scales with the microtubule overlap length and with the number of crosslinking motor proteins (Shimamoto *et al* 2015). Thus, kinesin-5 molecules do not interfere with the stepping of an adjacent kinesin-5 in a force-dependent manner without a microtubule unbinding event or the abolishment of crosslinking. When the microtubules are moving faster than the intrinsic kinesin-5 stepping rate, a resistive braking force can be generated scaling with the microtubule overlap length (Shimamoto *et al* 2015). In addition to antiparallel microtubule overlaps, parallel microtubule overlaps crosslinked by kinesin-5 complexes revealed a directional microtubule sliding with back-and-forth force fluctuations displaying amplitudes of ~2 pN, which are independent of the overlap length (Shimamoto *et al* 2015). Similarly, length- and velocity-dependent braking forces resisting microtubule sliding have been determined for these parallel overlaps. In summary, based on the findings for both antiparallel and parallel crosslinking geometries, kinesin-5 complexes act as a regulator of microtubule sliding velocity within the spindle by generating larger forces when the antiparallel overlaps are long, smaller forces when they are

short and braking forces against the relative sliding of antiparallel microtubules that move faster than the natural stepping rate of kinesin-5 and parallel microtubules moving across a wide range of velocities. In particular, motor proteins with opposing directional preferences are able to bind to the same overlapping region in microtubule bundles of the spindles. Thus an antagonistic behavior can be seen, as one protein slides the plus ends of the microtubules apart, whereas another protein slides the minus ends apart. Indeed, microtubule bundles crosslinked by kinesin-5 and kinesin-14 *in vitro* displayed a stable force balance. When the fractional amounts are similar between both proteins, the fluctuation of microtubule motion is revealed to be unstable. In particular, if one motor protein is titrated more than the other, this leads to directional motion (Hentrich and Surrey 2010). The magnitude of unstable fluctuations can be decreased by adding an artificial microtubule crosslinking construct without any motor activity. The combined action of active force generators and passive brakes that act on a distinct microtubule network create stable overlaps persisting for several minutes. However, in order to reveal more insights about the mechanism, a detailed investigation of passive microtubule crosslinkers under controlled load is suggested for defined time-scales and analysis of the microtubule orientation.

9.2.3.2 The passive force generation in microtubule networks

Molecular friction exerted by the kinesin-8 motor protein Kip3 has been analyzed (Bormuth *et al* 2009). In particular, it has been revealed that single molecules of three different non-motor MAPs fulfill distinct mitotic functions and generate frictional resistance when they are dragged along the lattice (Forth *et al* 2014). Among these motor proteins are: NuMA, a large protein bundling microtubules to direct them to the metaphase spindle pole; EB1, a plus-end-binding protein marks the growing tips of microtubules; and PRC1, a homodimeric protein in the MAP65 family crosslinking antiparallel microtubules that are positioned to the spindle midzone in anaphase. All these proteins create frictional resistance with the magnitude of the frictional forces depending nonlinearly on the dragging speed. Additionally, differences in the microtubule-binding structural motifs affect the strengths of the frictional interactions. Can a single molecule generate sufficient friction to alter the speed of a motor protein? When moving with velocities of 1 μm s^{-1} (the speed of one dynein), each of the non-motor MAPs is able to create frictional forces of 0.1–0.2 pN (Forth *et al* 2014). Thus, a single MAP interacting with a microtubule cannot provide substantial resistance against the motor protein-generated forces, however, clustering of MAPs may lead to an increase of the total resistive force. In particular, a complex of only ten MAPs bound to a single microtubule can generate piconewtons of resistive load decreasing or even stalling the stepping velocity of a motor protein.

Diverse frictional asymmetries have been detected. Two examples are Kip3 and EB1 that produced less frictional resistance when dragged towards the plus ends of a microtubule (Bormuth *et al* 2009, Forth *et al* 2014). However, NuMA causes less frictional resistance when it moves towards the minus end that is congruous with its localization at the spindle poles, where the minus ends are clustered (Forth *et al* 2014).

In contrast, PRC1 displayed no directional preference, as it exhibits equal frictional forces independent of the microtubule polarity. How is the frictional asymmetry provided within the active microtubule? When a NuMA-dimer induces crosslinks of parallel microtubules and forces are applied on these microtubules, the NuMA moves toward the minus ends (Forth *et al* 2014). Based on the intrinsic mechanical anisotropy, directional motion of distinct MAPs can be observed within this actively fluctuating network in the absence of motor proteins. However, these fluctuations seem to be based on the competition between plus-end- and minus-end-directed motor proteins crosslinking the two microtubules (Hentrich and Surrey 2010). As frictional asymmetry possibly develops from the directional dependence of force-facilitated bond breaking at the atomic level, this special kind of autonomous directed motion can also be found in other active polymer networks in living cells.

In addition to the production of frictional resistance and thus acting as a brake against motion (Braun *et al* 2011), a new mechanism of force generation has been revealed for Ase1 (synonymously known as yeast MAP65). The Ase1 crosslinking of two microtubules has been compared to a compressible gas that generates entropic forces, and hence further confinement through growing microtubule overlaps evokes an increase in entropic forces as the diffusible molecules of Ase1 are compacted into smaller and smaller overlap regions (Lansky *et al* 2015). In more detail, these non-motor crosslinking proteins perform thermally driven diffusion along the lattice of each of the microtubule surfaces they are contacting. When the number of available binding sites is low relative to the amount of Ase1 molecules, the system will expand to elevate the number of binding sites and hence maximize the number of microscopic states, which can be reached by the sliding of microtubules in a one-dimensional system to increase the overlap length. The magnitude of the entropic force has been observed to be around several piconewtons in the presence of overlaps with Ase1 molecules, which leads to the hypothesis that the entropic force is able to stall weak motor protein motions (figure 9.6) (Lansky *et al* 2015). This entropic force may cooperate with the frictional forces, which then causes substantial resistance to

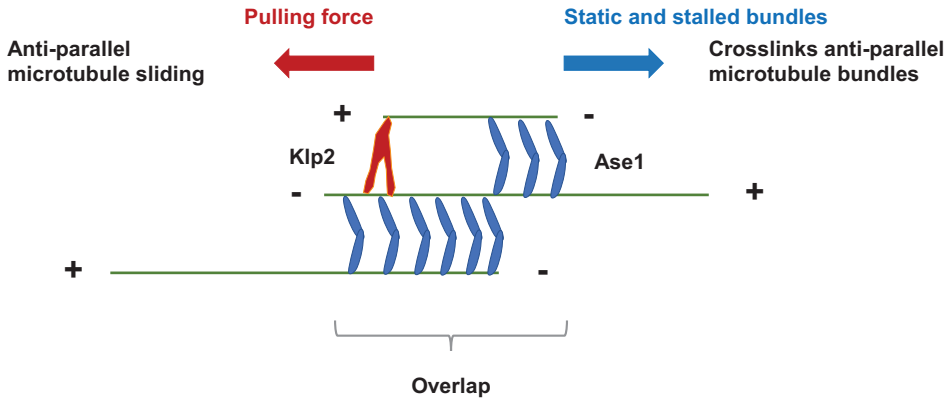

Figure 9.6. Antagonistic function of the actin bundling protein Ase1 and the minus-end-directed kinesin motor protein Klp2. Ase1 (blue) crosslinks antiparallel actin bundles and hence impairs the sliding motion of antiparallel microtubules evoked by the kinesin Klp2 (red).

microtubule sliding. How can forces generated by individual proteins on the subsecond time-scale cause micrometer-scale outputs persisting for several minutes? Micrometer-scale forces can scale with the number of motor proteins, friction can be decreased within active filament networks to move proteins and diffusible crosslinkers can cause entropic forces within bundles of microtubules. Most of these studies have been performed with combinations of proteins crosslinking microtubules to enlighten the key points of the spindle force map. However, several points need to be addressed. First, it needs to be determined how systems with high complexity, such as active and passive crosslinkers acting on the same region within a microtubule pair, can lead to diverse behaviors. Therefore, the generation of forces within microtubule pairs needs to be analyzed to reveal the crosstalk between crosslinking proteins, polymerization forces and microtubule dynamics regulators at the growing tips of microtubules. Second, are the results obtained on the microtubule extracts transferable to dividing cells? *In vivo* analysis suggests that pushing force generation that depends on the overlap length seems to need the expression of kinesin-5 mutants, which vary in length dependence and in the associated mitotic phenotype. Simple loss-of-function analyses do not seem to be suitable as they act on micrometer length-scales and not on the nanometer-sized protein length-scale. Hence the alteration of the microtubule-binding properties of distinct proteins may cause their altered localization in dividing cells and hence the clustering by frictional asymmetry affects their cellular function. In addition, several mitotic proteins are altered by post-translational modifications such as phosphorylation or dephosphorylation. How can biochemical modifications of crosslinking proteins affect their mechanical properties?

9.3 Microtubules and cell motility

A strong connection between the cytoskeleton and cell migration has been established, which finally led to the finding that actin filaments and microtubules are key regulators of cell shape and motility (Etienne-Manneville 2013, Le Clainche and Carlier 2008). In particular, the molecular interplay between actin and microtubules regulates the exertion of cell protrusions and cell adhesion to the extracellular matrix. The actin–microtubule crosstalk is precisely regulated by the small GTPases Rho, Rac1 and Cdc42, and thereby provides cell polarity, the polymerization of actin and the actomyosin-based contractility (Rodriguez *et al* 2003). Actin filaments (F-actin) are polar fibers that are assembled by polymerization of actin monomers regulated by nucleation and elongation factors (Carlier *et al* 2015) and they have the capacity to build branched scaffold networks that can push membranes outward or contract structures such as stress fibers and cortex-associated meshes. Thus, F-actin is seen as the key cytoskeletal element regulating the cell's shape and adhesion in 2D and 3D environments (Blanchoi *et al* 2014, Case and Waterman 2015, Pollard and Cooper 2009).

The microtubules are polarized tubes composed of α-tubulin and β-tubulin heterodimers (Desai and Mitchison 1997). In particular, their plus ends show frequent growth and shrinkage behavior and represent binding sites for ensembles composed of plus-end-tracking proteins (+TIPs) facilitating microtubule

polymerization, signal transduction processes and interaction with subcellular structures such as the actomyosin cytoskeleton (Akhmanova and Steinmetz 2015). In 2D cell adhesion and migration modes, the microtubules are presented as regulators of Rho GTPase signal transduction and facilitate the transport or the recycling of cell–matrix adhesion receptors such as integrins (Etienne-Manneville 2013). Moreover, these classic experiments, in which microtubules were disassembled by adding a depolymerizing drug such as nocodazole, revealed that the microtubules lead to an activation of Rac1 and impair in turn Rho activity (Krendel *et al* 2002, Waterman-Storer *et al* 1999). In 2D cell culture systems, the addition of nocodazole reduces the exertion of cell protrusions through decreased Rac1-driven actin polymerization and hence enhanced cell contractility due to Rho driven myosin II signal transduction (Krendel *et al* 2002, Waterman-Storer *et al* 1999). As the underlying molecular mechanisms are still not fully understood, the integrity of the microtubule network plays an important role in maintaining the precise balance of Rho GTPase activities, and hence the assembly of F-actin and subsequently the actomyosin contractility in 2D cell culture models (Etienne-Manneville 2013). In contrast, specific cell types such as glioblastoma cells have been demonstrated to be actin-independent, but in turn still need microtubules for their migration in 2D cell cultures (Panopoulos *et al* 2011).

The regulation of cell migration depends crucially on the interaction between dynamic microtubule remodeling and integrin-facilitated extracellular matrix adhesions such as focal adhesions (Kaverina *et al* 1998). These highly specialized cortical complexes control the interaction between cell migration and cell–matrix adhesion involving the +TIPs of microtubules (Lansbergen *et al* 2006, van der Vaart *et al* 2013, Wu *et al* 2008, Bouchet *et al* 2016a). Moreover, it has been proposed that microtubules induce the focal adhesion turnover needed for proper cell migration (Stehbens and Wittmann 2012) (figure 9.7). This microtubule function seems to be connected to the inhibition of Rho, the endocytosis of integrins and the proteolytic degradation of the extracellular microenvironment by matrix metalloproteases exocytosis and is even supposed to be critical for cell motility on stiff 2D substrates (Stehbens and Wittmann 2012, Stehbens *et al* 2014).

During embryonic development, tissue repair and immune response reaction and the malignant progression of cancer, the cellular motility operates in a 3D microenvironment and enhanced motility assays require 3D rather than 2D migration assays. Most of the current knowledge on the cytoskeleton is still based on 2D cell culture studies. In particular, the microtubule network facilitates the polarized trafficking and signaling required for the cell shape and movement in 2D. However, several studies revealed that the 2D and 3D microenvironments affect the cell morphogenesis and motility differently (Bouchet and Akhmanova 2017).

In vivo, many diverse physiological and pathological processes such as the embryonic development, tissue homeostasis, immune surveillance and cancer invasion during metastasis depend on the migration of cells through soft 3D extracellular matrices such as connective tissue or basement membranes (Even-Ram and Yamada 2005). Advances in light microscopy of thick tissue specimens and *ex vivo* 3D cell culture systems revealed insights into the cytoskeletal arrangement and structural remodeling (Fischer *et al* 2011,

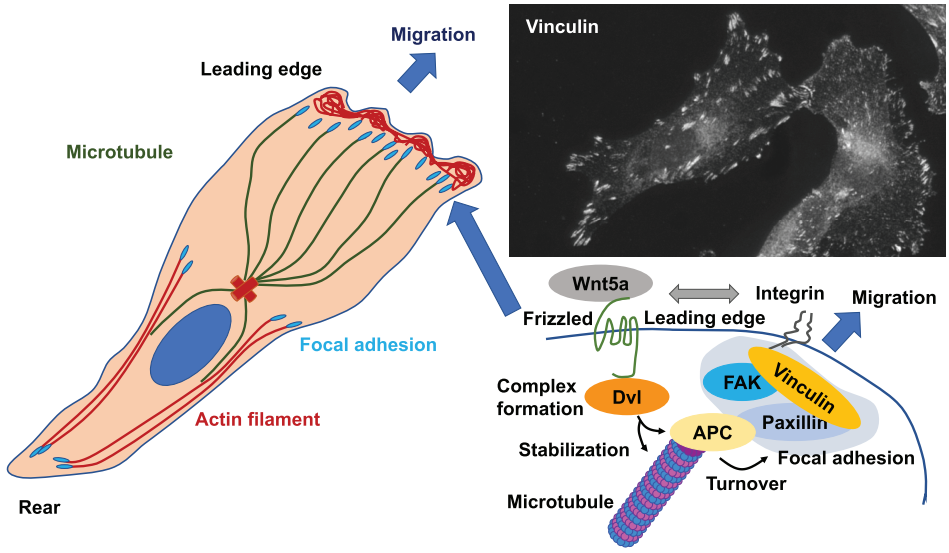

Figure 9.7. Focal adhesion turnover facilitated by microtubules. Molecular mechanism by which growth factors such as Wnt5a regulate the turnover of focal adhesions to promote cell migration. Fluorescence microscopic image shows focal adhesions of cells after staining with an Alexa Fluor 546 labelled antibody directed against vinculin.

Shamir and Ewald 2014). Without any doubt, studies of cells cultured on stiff 2D substrates provided many insights for the understanding of cellular motility in general, but the analysis of cellular motility in 3D cell culture models is highly desired for deciphering the role of microtubules in this process under more-physiological conditions. In comparison to the investigation of F-actin and its regulators in cell migration, the contribution of microtubules to 3D cell motility is rather less clearly understood (Petrie and Yamada 2012, Riching and Keely 2015). However, a clear connection between microtubules and cell motility in 3D microenvironments has been established with the focus on mostly soft 3D extracellular matrices.

9.3.1 Functional role of microtubules in cell motility in 2D and 3D

Numerous differentiated cell types and developmental precursor cells have the capacity to migrate through a 3D matrix (Friedl and Gilmour 2009, Lam and Huttenlocher 2013, Nakaya and Sheng 2008). As pathological processes require cell invasion such as metastasis of solid cancers (Chaffer and Weinberg 2011), it is essential to determine the principles of cell migration modes in 3D matrix scaffolds, which are based primarily on cellular shapes (Friedl and Gilmour 2009). In particular, a mesenchymal shape-based cell motility has been found in fibroblasts, endothelial cells, embryonic cells that perform the epithelial–mesenchymal transition (EMT), and similarly highly invasive cancer cells that exert long pseudopods in order to migrate through the 3D microenvironment (Cheung *et al* 2013, Clark and Vignjevic 2015, Friedl and Gilmour 2009, Grinnell and Petroll, 2010, Petrie and Yamada 2015). Microtubules are known to be crucial for the mesenchymal

pseudopod exertion in soft 3D matrices (Grinnell *et al* 2003, Tomasek and Hay 1984). Moreover, the remodeling or perturbation of the microtubule scaffold by microtubule-targeting agents (MTAs) impairs pseudopod-driven cell invasion (Kikuchi and Takahashi 2008, Lee *et al* 2015, Martins and Kolega 2012, Oyanagi *et al* 2012, Pourroy *et al* 2006, Rhee *et al* 2007, Tran *et al* 2009). The molecular mechanisms enabling the microtubules to foster mesenchymal protrusions formation in soft matrices are still not clear.

The mechanical involvement of the cytoskeleton in the process of cell invasion has been previously commonly connected to actin and actin regulatory proteins (Kikuchi and Takahashi 2008, Kutys and Yamada 2014, Sahai and Marshall 2003, Sanz-Moreno and Marshall 2010, Wilson *et al* 2013). In contrast, microtubules regulate the cell shape through signal transduction processes and hence indirectly provide the Rho guanosine triphosphatases (GTPases) activity, cell–matrix adhesion and cellular polarity (Etienne-Manneville 2013, Gierke and Wittmann 2012, Petrie and Yamada 2015, Rhee *et al* 2007). The loss of the plus-end tracking protein (+TIP) end-binding protein 1 (EB1) leads to impaired cell invasion of hepatocyte growth factor-treated canine epithelial cells (Gierke and Wittmann 2012). EB1 facilitates the recruitment of various +TIPs, which act in microtubule polymerization and depolymerization, interact with distinct cellular structures and fulfill roles in transport and signal transduction pathways (Akhmanova and Steinmetz 2015). However, the mechanism of EB1 function in the exertion of pseudopod protrusions is not yet revealed. Indeed, microtubules contribute to the mechanical phenotypes regulating cellular morphogenesis (Brangwynne *et al* 2006, Dennerll *et al* 1988, Fygenson *et al* 1997a, Matrone *et al* 2010, Wang *et al* 2001, Winckler and Solomon 1991). In more detail, the tensegrity model proposes that the ability of microtubules to resist compression at the cell's cortex facilitates the cellular shape in soft 3D matrices (Ingber 2003). A major restriction of such a compression resistance function is that the dynamic microtubule tips near the cell cortex undergo force-induced catastrophes (figure 9.8) (Janson *et al* 2003, Laan *et al* 2008). Are the mechanisms of catastrophe regulation related to the load-bearing function of microtubules in 3D cell morphogenesis?

In 2D cell culture systems the disassembly of the microtubule network inhibits Rac1 function and impairs hence the formation of protrusions such as lamellipodia and increases the myosin II-driven contractility through the Rho GTPase (Krendel *et al* 2002, Waterman-Storer *et al* 1999). In relatively soft 3D extracellular matrices the full disassembly of microtubules induces the retraction of the entire pseudopod, which cannot be rescued through the inhibition of myosin II and hence this effect seems to be dependent on the Rac1 inactivation (Grinnell *et al* 2003, Rhee *et al* 2007). At first glance, this result seems to be counterintuitive. The +TIP and catastrophe inhibitor SLAIN2 (van der Vaart *et al* 2011) has been found to be crucial for the mesenchymal cell invasion both *in vitro* and in *in vivo* tumor models in mice (Bouchet and Akhmanova 2017). In particular, the C-terminal part of SLAIN2 can interact with EBs such as EB1, cytoplasmic linker proteins (CLIPs) such as CLIP-170 and CLIP-associated proteins. The N-terminal part of SLAIN2 binds to ch-TOG, which is the mammalian homolog of the microtubule polymerase XMAP215 (figure 9.9). Based on multiple interactions of SLAIN2 it increases the

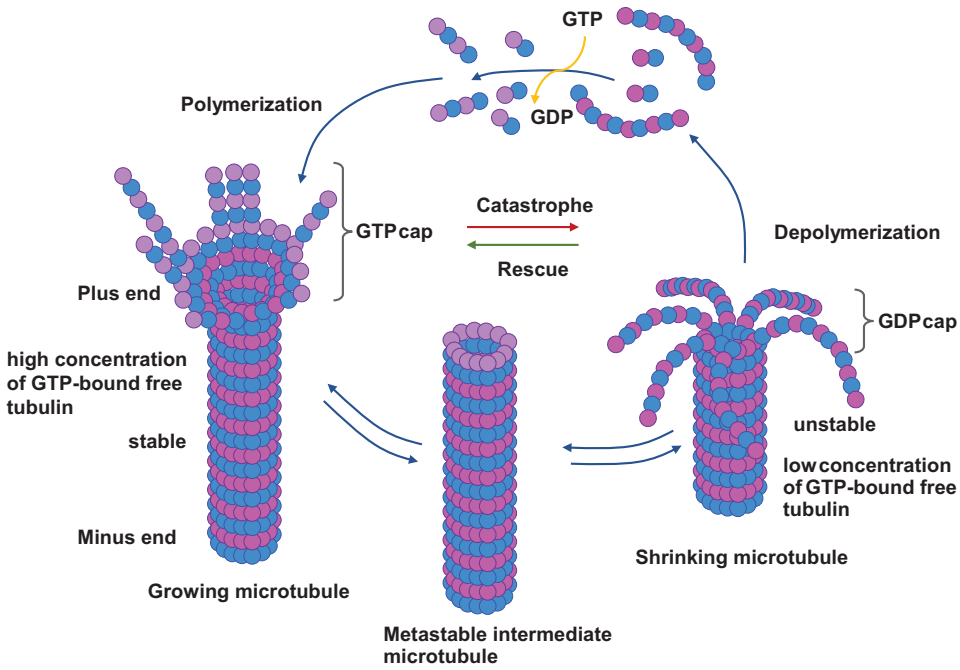

Figure 9.8. Catastrophe and rescue of microtubules. The content of free GTP–tubulin influences the balance between microtubule growth (rescue) and its rapid shortening via depolymerization (catastrophe).

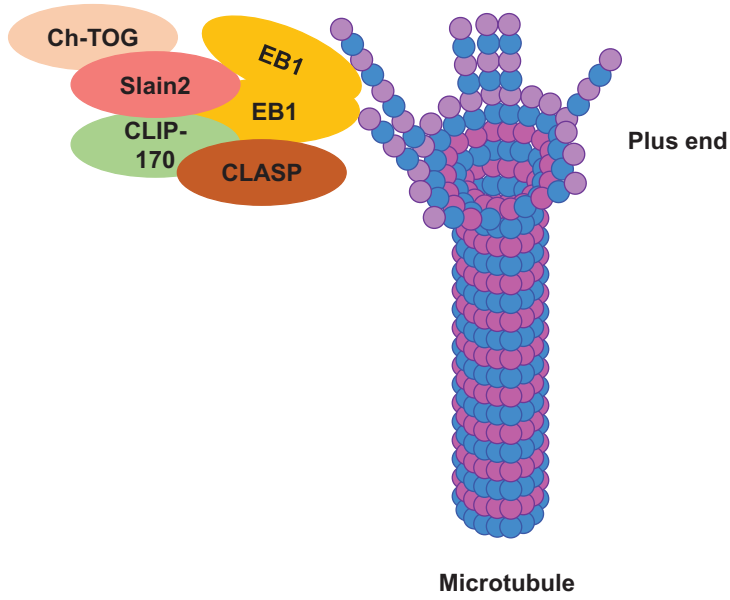

Figure 9.9. Slain2 interacts via EB1 with microtubules and other microtubule interacting proteins.

ch-TOG accumulation at microtubule plus ends and thereby it pronouncedly induces the processive microtubule polymerization in interphase cells.

In line with this, a non-destructive approach revealed that catastrophe induction by the addition of SLAIN2, the inactivation of ch-TOG or low-dose MTA treatments, creates an equally pronounced loss of invasive pseudopods. As supposed, the effect is not driven by increased Rho and myosin II activities, a downregulation of Rac1 or delocalization of these GTPases, as the microtubules are preserved in these conditions and not destroyed. Moreover, the cell adhesion and migration in 2D culture systems are critically driven by Rho GTPase activity and localization, whereas these processes are not altered by SLAIN2 inactivation. In contrast to 2D systems, a morphogenetic function of microtubules in soft 3D extracellular matrices is provided by the microtubule dynamics and thereby is mechanistically distinct from the regulation of the Rho GTPase.

Focal adhesions regulate the cell shape and are assembled in soft 3D matrices (Harunaga and Yamada 2011). Their assembly is dependent on the trafficking of integrins and on the presence of matrix proteases (Friedl and Alexander 2011, Jacquemet *et al* 2013, Stehbens and Wittmann 2012). The defects of the invasive pseudopods evoked by loss of EB1 are associated with altered myosin II activity, the presence of microtubules in pseudopods, formation of focal adhesions and trafficking of vesicles (Gierke and Wittmann 2012). In particular, this phenotype displays the perturbation of multiple different mechanisms regulated by the large numbers of +TIPs, in which association with microtubules depends on EB1 (Akhmanova and Steinmetz 2015). By inactivating SLAIN2, an increasing catastrophe frequency occurred without disturbing the +TIP localization, the density of microtubules at pseudopod tips, the trafficking of vesicles, enzymatic matrix degradation or the assembly of focal adhesions to impair the pseudopod-based cell invasion. In particular, the invasive pseudopod tips show highly persistent growth of microtubules that is associated with their buckling behavior. The buckling phenomenon appears upon microtubule compression and seems to be driven by myosin II-based actin contraction and the flow of actin (Brangwynne *et al* 2006, Gupton *et al* 2002, Wang *et al* 2001), membrane tension (Elbaum *et al* 1996, Fygenson *et al* 1997a, Fygenson *et al* 1997b), the growth of microtubules themselves (Dogterom and Yurke 1997) or even different combinations of these factors. Indeed, frequent short-scale retractions of pseudopod tips have been shown to be connected with microtubule buckling and, due to the weak matrix adhesion within soft 3D extracellular matrices (Harunaga and Yamada 2011), a compression of the entire cytoskeleton seems to be reasonable (Ingber 2003). Due to the large slenderness ratio (over 100), which is defined as the ratio of the length to the diameter of microtubules (Kurachi *et al* 1995), it is proposed that microtubules are subject to classical Eulerian buckling, which is performed under the first mode exhibiting a large wavelength under exceedingly small forces (approximately around 1 pN) (Kurachi *et al* 1995, Elbaum *et al* 1996, Fygenson *et al* 1997b, Soheilypour *et al* 2015). However, *in vivo* microtubules undergo a short-wavelength buckling regime, as they are confined by their mechanical coupling with the surrounding cytoskeleton, which leads to the suggestion that the buckling mechanism helps microtubules to withstand large

compressive forces (Brangwynne *et al* 2006). Several computer simulations have confirmed this hypothesis, in which a single microtubule is surrounded by a viscoelastic cytoplasm under compression, when the buckling amplitude, wavelength and microtubule growth rate in dependence of the properties of surrounding cytoplasm are determined (Li *et al* 2008, Brangwynne *et al* 2006). Moreover, as the microtubules are modeled as single elastic cylindrical tubes that are embedded within the viscoelastic cytoplasm, it has been concluded that the cytoskeletal resistance to compressive forces depends, on the one hand, on the mechanical properties of the microtubules and, on the other hand, on the cytoplasm, in which the rather stiff microtubules are located in the viscoelastic cytoplasm acting as an effective composite material for bearing compressive forces (Jiang and Zhang 2008).

However, this catastrophe function of the microtubules depends on the compression resistance of dynamic microtubule plus ends and seems to be independent of the Rho GTPase activity state, vesicle transport and the assembly of focal adhesions. In order to investigate the resistance of microtubules to compression, the ability of cells to exert membrane protrusions after the disassembly of the actin filamental network has been analyzed. Indeed, it has been revealed that SLAIN2/ch-TOG-facilitated growth persistence is essential, while other EB1-associated +TIPs seem to play no major role.

In contrast, in the presence of actin, additional factors are required for the prevention of catastrophes, which seems to be based on a different biochemical microenvironment of the actin-rich cell cortex or higher forces exerted at pseudopod protrusions. One factor the CLASP1, which contributes to catastrophe suppression by acting specifically at pseudopod tips, has been characterized. CLASP1 acts as a microtubule-stabilizing protein and a rescue factor (Al-Bassam and Chang 2011, Mimori-Kiyosue *et al* 2005). The reduction of CLASP1 initiates catastrophes only at the cell cortex, which is consistently with the local function of CLASPs at the cell's leading edge (Akhmanova *et al* 2001, Wittmann and Waterman-Storer 2005). Other CLASP1 interacting proteins such as LL5β or +TIPs acting in the same complexes such as spectraplakins (Drabek *et al* 2006, Lansbergen *et al* 2006) may additionally play a role in the regulation of microtubule dynamics within pseudopods. In line with this, the localization or activity differences of CLASP1 or its partners provide an explanation for the alterations in microtubule growth persistence between protruding or retracting pseudopods. The interaction between microtubules and actin can contribute to the different actin organization in 2D and 3D, as cells in 3D systems lack prominent actin-based structures such as stress fibers and large lamella. Alterations in the actomyosin-based retrograde flow, which can also modulate microtubule dynamics (Gupton *et al* 2002, Waterman-Storer and Salmon 1997), can explain the more persistent growth of microtubules at pseudopod tips in 3D than at the edges of lamella in 2D.

SLAIN2 and another +TIP, CLASP1, facilitate the exertion of long invasive pseudopods in mesenchymal cells by inducing highly persistent microtubule growth at their tips (Bouchet and Akhmanova 2017). When the persistent microtubule growth is altered, the impairment of microtubule depolymerization solely provides the maintenance of the invasive pseudopods, but not their remodeling. It has been

revealed that microtubule growth persistence at the cell cortex is required for the invasive cell shape and the 3D motility of mesenchymal cells *in vitro* and *in vivo*. An increase in microtubule catastrophes at pseudopod tips cannot hinder the pseudopod initiation, whereas it abolishes the pseudopod elongation in a 3D microenvironment that results in impaired motility, as the cells cannot assembly distant adhesion sites to pull themselves through the extracellular matrix (Bouchet and Akhmanova 2017). What are the implications of these results on the mechanical role of dynamic microtubules during cell invasion?

There exists a fundamental difference between neurons and 3D grown mesenchymal cells in how the cell shape depends on the microtubule growth. In particular, SLAIN2 inactivation induces limited defects in axon elongation (van der Vaart *et al* 2011) and low doses of paclitaxel can even promote the axon outgrowth (Witte *et al* 2008). Neurons possess many stabilizing MAPs that impair neurite retraction when the growth of microtubules is perturbed. In contrast, the dynamic remodeling of the microtubules enables mesenchymal cells to extrude long protrusions and move within 3D microenvironments. Neuronal MAPs strengthen the mesenchymal cells to be less sensitive to retraction evoked by perturbations of microtubule growth, however, basic processive microtubule growth is still required for 3D cell motility.

Catastrophe inhibition seems to support a load-bearing role for the microtubules during the cell elongation via pseudopods in soft 3D extracellular matrices. Hence a model is presented in which SLAIN2/ch-TOG and CLASP1 decrease the frequency of microtubule catastrophes at the cortex and hence enable microtubules to withstand the compression generated by cellular pre-stress in soft 3D extracellular matrices (Ingber 2003). Similarly, during mesenchymal cell invasion, microtubules withstand cell retraction, whereas the actin and cell–matrix adhesion provide the cell elongation. This special mechanism seems to be important on top of soft matrices and within them, whereas on stiff matrices, where the matrix holds itself together with strong cell–matrix adhesion, represents a mechanical element to impair cell retraction. In line with this, perturbation of the persistent growth of microtubules on top of a soft 2D gel pronouncedly reduced cell spreading and adhesion, whereas it did not impair cell motility, as the cells can move forward in a ‘roll over’ manner, although their protrusions are small. However, within a 3D gel the inability to elongate pseudopods diminishes the formation of distant adhesions and subsequently impairs movement. In summary, the microtubule dynamics is required for the exertion of cell protrusions and depends on matrix stiffness, whereas long protrusions are required for cell movement and depend on the dimensionality of the substrate. In addition to the mechanical model of microtubule-dependent regulation of the cell shape, alternative pathways involving either signal transduction processes or trafficking may play a role that couples the microtubule growth persistence to the elongation of pseudopods in soft 3D matrices.

Interphase cells rather than mitotic cells represent a major target of MTAs in cancer therapy (Komlodi-Pasztor *et al* 2011, Mitchison 2012). Hence, targeting the interphase cell migration may be a very potent anti-metastatic strategy involving the impairment of MTAs in invasive cancers (Cheung and Ewald 2014, Palmer *et al* 2011). In particular, the inactivation of SLAIN2 in cancer cells impairs tumor

invasion and metastasis in an interphase-specific manner and therefore represents a potential target of cancer therapeutics. This approach is based on the idea that the anti-metastatic action of MTAs is associated with their anti-migratory effects in interphase cells. Moreover, it hence reveals SLAIN2 as a potential target for metastatic cancer treatment.

9.3.2 Microtubules in cell morphogenesis and 3D motility

Most results on the role of microtubules in 3D cell migration such as invasion is based on *in vitro* cell culture studies, since an *in vitro* cell culture system based on collagen type I hydrogels has been established (Elsdale and Bard 1972). In the next step, the development and standardization of well-controlled 3D cell culture setups are combined with advanced live imaging and genetic engineering (Shamir and Ewald 2014) and hence these 3D culture assays are regarded to be superior to 2D cultures in modeling the behavior of motile cells under physiological conditions. After the emergence of these 3D culture systems, the morphology and behavior in 3D matrices of different motile cell types including fibroblasts (Bard and Hay 1975, Bell *et al* 1979, Bellows *et al* 1981, Elsdale and Bard 1972), developmental precursors such as neural crest cells (Davis 1980) or endocardial cushion cells (Bernanke and Markwald 1982), leukocytes (Grinnell 1982) and endothelial cells (Schor *et al* 1983) have been performed.

Cells can utilize diverse migration modes to invade through 3D matrices, such as the mesenchymal and amoeboid migration modes, which represent the most common ones. The microtubules have been identified to be important for the mesenchymal cell morphogenesis in soft 3D matrices, which has been investigated using fibroblasts cultured within 3D collagen fiber matrices (Bell *et al* 1979, Tomasek and Hay 1984). The treatment with colcemid depolymerizes the microtubules and thereby impairs the bipolar and elongated morphology of fibroblasts in 3D culture systems (Elsdale and Bard 1972) by forcing the cells to adapt a pear-like shape (Bell *et al* 1979). Both microtubule-stabilizing, such as paclitaxel (synonymously known as Taxol), and microtubule-destabilizing, such as nocodazole, reagents induce a loss of long protrusions in fibroblasts, which they usually build in 3D collagen fiber matrices (Tomasek and Hay 1984). In particular, these protrusive pseudopods are involved in the onset of 3D cell motility (Bard and Hay 1975, Schor *et al* 1980, Schor 1980) (figure 9.10). Indeed, the importance of microtubules for cell morphogenesis differs in soft 3D collagen gels from that of cell cultures on 2D plastic dishes (Unemori and Werb 1986). A colcemid-induced disassembly of microtubules impairs the spreading of fibroblasts on solid 2D substrates (Ivanova *et al* 1976, Vasiliev *et al* 1970), whereas the pseudopod-based cell elongation is not altered on soft 3D collagen matrices (Unemori and Werb 1986). This result has been reproduced by a nocodazole-induced microtubule disassembly in cells cultured in soft 3D collagen fiber matrices compared to stiff 2D substrates (Rhee *et al* 2007). Moreover, the addition of nocodazole to the fibroblasts impaired their motility in 3D matrices (Doyle *et al* 2009). In particular, the microtubule-dependent pseudopod elongation has also been identified in cancer cells with a mesenchymal morphology in 3D matrices such as MDA-MB-231 cells

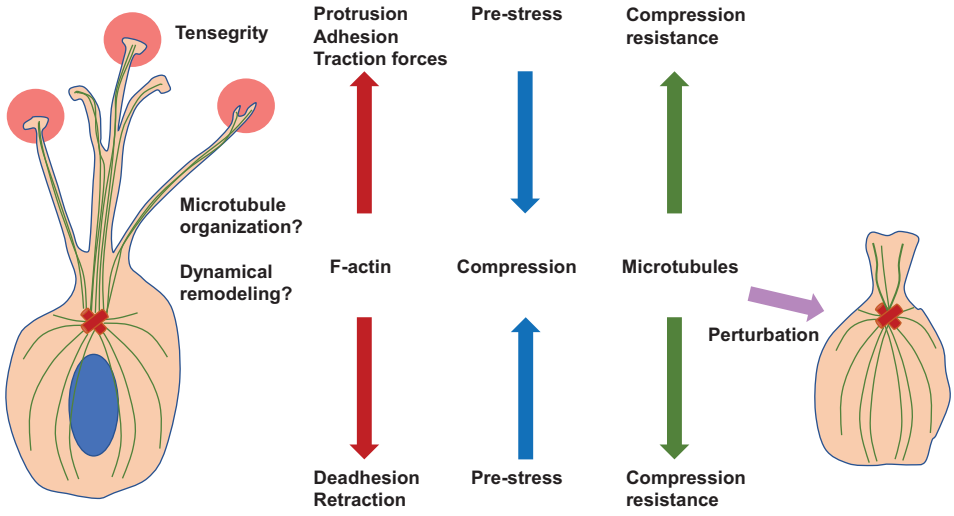

Figure 9.10. Possible role for microtubules in cell tensegrity in soft 3D extracellular matrices. In mesenchymal cells, F-actin facilitates the protrusive and adhesion-based traction forces. The cellular pre-stress is generated by contractility, which induces compression. Microtubules may help the cells to withstand compression during their elongation into the matrix. Perturbations such as mechanical forces or signal transduction processes can evoke a rounding of the entire cell. A central question is yet to be answered: how are microtubules organized and regulated at the pseudopod tip and the cell's rear end?

(Kikuchi and Takahashi 2008, Oyanagi *et al* 2012). In line with this, high doses of paclitaxel or nocodazole abolished their migration in soft 3D collagen fiber matrices (Carey *et al* 2015). However, even low doses of various MTAs that cannot inhibit cell division, are able to abolish matrix invasion by MDA-MB-231 human breast cancer cells (Tran *et al* 2009). In addition, microtubules are needed for pseudopod-based motility in 3D extracellular matrices using inhibitory MTA-based approaches in endothelial cells (Lyle *et al* 2012, Martins and Kolega 2012, Pourroy *et al* 2006). The addition of nocodazole produces defects in the elongation of invadopodia, which are small specialized protrusions observed in cancer cells involved in the malignant progression of cancer (Di Martino *et al* 2016, Schoumacher *et al* 2010). Moreover, the formation of podosomes, which share similarity with invadopodia, are specialized adhesive and invasive protrusions regulating the migration and invasion in 3D extracellular microenvironments of certain cell types such as macrophages or endothelial cells and require microtubules (Linder *et al* 2000, Maridonneau-Parini 2014, Seano and Primo 2015). Finally, a universal role for microtubules in the mesenchymal cell protrusion and elongation in soft microenvironments can be suggested.

Taken together, the loss of pseudopods causes a microtubule disassembly and hence strongly decreases cell motility in soft 3D matrices, whereas the effect is less strong on cell movement on top of soft 2D matrices (Myers *et al* 2011). Long pseudopods are necessary for mesenchymal motility within soft 3D matrices and not on top of soft 2D matrices, as 3D migration depends on the formation of distant

adhesions serving as anchoring points for contraction-driven cell body displacement (Friedl and Alexander 2011). In contrast, in 2D environments without elongated pseudopods and with maintained cell adhesion, mesenchymal cells are supposed to migrate freely (randomly) at the matrix surface, as they are not restricted by the confinement of meshwork pores (Wolf *et al* 2013). At the same time, microtubules have been shown to be necessary for amoeboid migration in 3D (Lam and Huttenlocher 2013) by using a 3D collagen fiber matrix assay for the investigation of leukocytes (Grinnell 1982). However, microtubules still only fulfill a limited role in amoeboid migration in 3D matrices, as several MTAs cannot impair the motility of leukocytes in these soft 3D collagen fiber matrices (Nikolai *et al* 1999, Ratner *et al* 1997). These results are supported by *in vivo* studies in zebrafish that revealed no impairment of the 3D migration of leukocytes, such as macrophages (Redd *et al* 2006) and neutrophils (Yoo *et al* 2012), upon the depolymerization of microtubules. However, microtubules are indeed necessary for the directionality of the migration of amoeboid cells in 3D collagen fiber matrices (Redd *et al* 2006, Yoo *et al* 2012). In particular, the uropod is a specialized structure formed at the cell's rear when these cells migrate by utilizing an amoeboid migration mode and hence is required for the directionality of cell migration (Hind *et al* 2016). In 3D microenvironments, microtubules exert this protrusion, which has been identified in leukocytes migrating in soft collagen gels (Ratner *et al* 1997), as well as *in vivo* (Yoo *et al* 2012), and hence it seems to facilitate the actin assembly and actomyosin contractility (Hind *et al* 2016). The term 'amoeboid migration' includes a broad spectrum of motility modes driven by cytoskeletal mechanisms, which vary between the submodes (Lammermann and Sixt 2009) and can then require different microtubule functions.

Single cell and collective migration during the development are examples of physiological cell motility in 3D (Aman and Piotrowski 2010, Keller 2005, Kurosaka and Kashina 2008) and hence the microtubule organization has been investigated using electron microscopy studies in migrating myogenic cells (Jacob *et al* 1978), epiblasts made invasive by performing an EMT during gastrulation (Granholm and Baker 1970) and cardiac cushion cells, which are grown inside 3D collagen fiber matrices (Bernanke and Markwald 1982). The depolymerization of microtubules plays a role in developmental processes such as the protrusion formation during epithelial tissue sealing (Eltsov *et al* 2015). Indeed, the 3D cell migration is facilitated by EMT and hence is a characteristic feature for the migration of mesoderm progenitors during the gastrulation and the movement of neural crest cells (Aman and Piotrowski 2010). The microtubule disassembly upon nocodazole treatment leads to a premature breakdown of the basement membrane during the gastrulation step (Nakaya *et al* 2008), whereas the microtubule functions during the mesoblast migration in 3D systems are not yet clear. However, paclitaxel and nocodazole affect the migration of melanoblasts, which are a subgroup of neural crest cells, *in vivo* and thus microtubules fulfill a role in developmental cell motility in 3D microenvironments (Li *et al* 2011a, Thomas and Erickson 2008). Similar to other mesenchymal cells in 3D culture systems, drug-induced microtubule network disorganization leads to a loss of long pseudopods in mouse melanoblasts and subsequently evokes an abolishment of their migration (Li *et al* 2011a). In neural

crest cells, the pseudopod loss causes the abolishment of the motility only in 3D systems upon nocodazole treatment, whereas in 2D systems these cells can migrate, but change their morphology to a round cell shape (Moore *et al* 2013). As neuron migration within a 3D microenvironment mirrors the *in vivo* situation during brain development more physiologically, the results obtained by 3D systems are commonly used to investigate this process (Gil and del Rio 2012). It has been established that both microtubule organization and microtubule-based motor proteins such as dynein and its cofactors are required for neuronal migration, and their inactivation of these components can lead to severe brain defects during development (Coles and Bradke 2015, Kapitein and Hoogenraad 2015, Moon and Wynshaw-Boris 2013).

As the microtubule dynamics and functions have been investigated largely in 2D systems, they need to be refined in more physiological 3D systems, as there exist large differences in the microtubule requirements in cell morphogenesis between stiff and soft matrices (Rhee *et al* 2007, Unemori and Werb 1986).

9.3.3 The cell–matrix adhesion and trafficking in 3D microenvironments

The microtubule function in the vesicular transport of the cell–matrix has been studied in great detail using 2D cell migration assays (Etienne-Manneville 2013, Stehbens and Wittmann 2012). In more detail, endocytosis-based integrin recycling has been shown to control the dynamic remodeling of focal adhesions, which is necessary for 2D cell migration (Paul *et al* 2015, Schiefermeier *et al* 2011). Moreover, the integrin recycling connects the microtubules with cell motility in 3D extracellular matrices in a mechanistic manner (Jacquemet *et al* 2013, Paul *et al* 2015). In particular, a well-known pathway regulating cancer cell migration in 3D microenvironments is the recycling of $\alpha 5\beta 1$ integrin. The recycling is driven by Rab25 (a protein related to Rab11), which localizes to recycling and late endosomes, and by the chloride intracellular channel 3 (CLIC3), which localizes to late endosomes and lysosomes (Caswell *et al* 2007, Christoforides *et al* 2012). Another example is the Rab4-driven recycling of $\alpha v\beta 3$-integrin that provides the invasion of cancer cells in 3D extracellular matrices with relatively low fibronectin content (Christoforides *et al* 2012).

Alternative culture methods have been established to overcome the limited working distance of the optics (of microscopic devices) in live fluorescence imaging. These cultures include cell-derived matrices such as Matrigel, which are extracellular matrix secretion products of high-density cell cultures and can be used to perform fibrillar 2D cultures for visualization of the endosomal markers Rab25 and Rab11 in pseudopods of cancer cells (Caswell *et al* 2007, Dozynkiewicz *et al* 2012, Jacquemet *et al* 2013). In particular, vesicle-associated membrane protein 3 (VAMP3) has been detected in pseudopods of canine MDCK cells under stimulation with the hepatocyte growth factor (HGF), when cultured within Matrigel/collagen I hybrid matrices (Gierke and Wittmann 2012). The functional connection between VAMP3 and 3D cell migration needs to be revealed similarly as in 2D systems (Kean *et al* 2009).

In addition, caveolae represent another type of membrane structure that seems to contribute to integrin recycling, focal adhesion turnover and directional cell

migration (Echarri and Del Pozo 2006, Grande-Garcia and Del Pozo 2008, Stehbens and Wittmann 2012). In 2D cell cultures, microtubule plus-end destabilization has been correlated with enhanced trafficking of caveolae components and their decreased delivery to the cell membrane (Wickstrom *et al* 2010). In line with this, the expression of the caveolae-associated protein caveolin-1 is associated with 3D matrix invasion in cancer cells (Hayashi *et al* 2001, Lin *et al* 2005) and endothelial cells (Parat *et al* 2003). What are the functions of caveolae in 3D cell motility and how are they associated with microtubules? In cells migrating on 2D substrates, the post-Golgi secretory vesicles are targeted in a polarized manner to the cell's leading edge, where they are exocytized (Schmoranzer *et al* 2003, Schmoranzer and Simon 2003). This function facilitates the motility of cells on 2D substrates and relies on the organization of the Golgi complex that is regulated by the +TIPs cytoplasmic-linker-associated protein 1 (CLASP1) and CLASP2 (Miller *et al* 2009, Yadav *et al* 2009). However, the contribution of the post-Golgi carriers to extracellular matrix adhesion in 3D matrices is still elusive.

In 2D cultures, it has been shown that vesicles containing matrix metalloproteases are targeted to focal adhesions and thereby promote cellular motility, as these proteases rupture the integrin connection with the extracellular matrix (Stehbens *et al* 2014, Takino *et al* 2007, Wang and McNiven 2012). The matrix metalloproteinase 14 (MMP14 or synonymously termed MT1-MMP) is necessary for the migration of endothelial cells in 3D microenvironments (Genis *et al* 2006) and is also associated with several developmental and pathogenic processes depending on 3D matrix migration (Bonnans *et al* 2014). Moreover, MT1-MMP membrane association together with the physical properties of extracellular matrix is a driving factor for the mesenchymal cell migration in 3D microenvironments (Wolf *et al* 2013). For mesenchymal migration and invasion of cancer cells, the trafficking of MT1-MMP and its delivery to the cell membrane are regulated by microtubules through the employment of late endosomes (Frittoli *et al* 2014, Macpherson *et al* 2014, Remacle *et al* 2005, Rosse *et al* 2014). The delivery of MT1-MMPs to the cell membrane is additionally controlled by F-actin and N-WASP (Yu *et al* 2012). What type of contribution is essential for the MT1-MMP trafficking in 3D migration and invasion?

9.3.4 The microtubule-related signaling in 3D microenvironments

Similar to the recycling of cell–matrix adhesion receptors, the majority of the data on signal transduction pathways downstream of the microtubules have been obtained from 2D assays. Microtubule-based signal transduction pathways include the regulation of Rho GTPases, which regulate the actin polymerization, actomyosin contractility and the assembly of focal adhesions (Etienne-Manneville 2013). The microtubule depolymerization (upon treatment with nocodazole) has been observed to downregulate Rac1, which raises the question as to what protein regulates the connection between the Rho GTPases and the microtubules to provide cell protrusions and cell migration (figure 9.11). The Rac1 activator ARHGEF4 (synonymously known as Asef) binds mutants of tumor suppressor adenomatous polyposis coli protein (APC) (a tumor suppressor and +TIP), which have been found

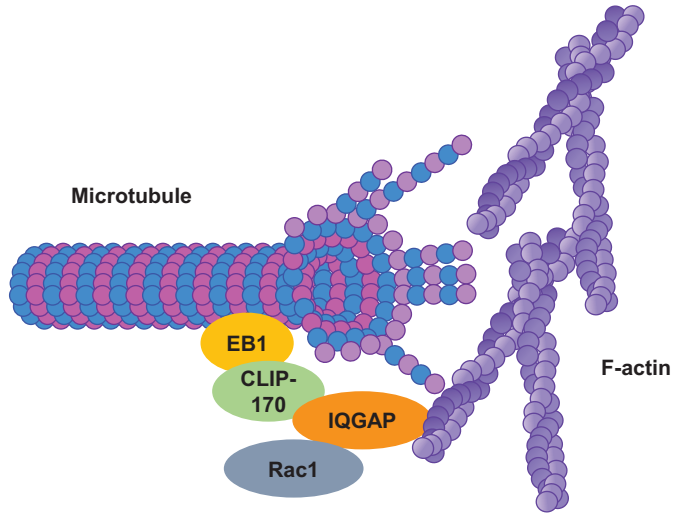

Figure 9.11. IQGAP provides the microtubule linkage to the actin cytoskeleton.

in colon cancer, and hence through this interaction Asef seems to be activated (Kawasaki *et al* 2000, 2003). As Rac1 promotes the polymerization of actin and subsequently lamellipodium extension, this mechanism seems to be associated with a pro-migratory function of microtubules in pathogenic situations, although this has solely been analyzed in 2D cell cultures. Another Rac GEF, TIAM2 (synonymously known as STEF), seems to be necessary for focal adhesion disassembly during nocodazole washout in 2D cell culture assays (Rooney *et al* 2010). Thus, it has been proposed that TIAM2 is activated by the regrowth of the microtubules. Other Rac1 activators, such as the +TIP triple functional domain protein (TRIO), which is a Rac1 and RhoG specific GEF (Blangy *et al* 2000, van Haren *et al* 2014), and TIAM1, which interacts with microtubule-associated protein 1B (MAP1B) and is a Rac1 GEF (Montenegro-Venegas *et al* 2010), seem to be additionally involved in the microtubule-driven cell protrusion exertion and migration.

Microtubules are associated with the inhibition of Rho and hence impair the contractility caused by myosin II. GEF-H1 (synonymously known as ARHGEF2) seems to be inactive when bound to microtubules, but it is activated by the depolymerization of microtubules that cause the activation of Rho and subsequently cell contraction (Chang *et al* 2008, Krendel *et al* 2002). In particular, GEF-H1 induces the activation of Rho at the cell's leading edge and locally promotes the polymerization of actin and the protrusion of the lamellipodium in 2D model systems (Nalbant *et al* 2009). However, GEF-H1 function can be more difficult, as it is possibly also a Rac1 GEF (Callow *et al* 2005, Ren *et al* 1998) and thereby acting in the process of exocytosis that also regulates cellular motility (Pathak *et al* 2012). Additionally, feedback mechanisms have been identified in which Rac1 stabilizes microtubules through the inhibition of the microtubule-destabilizing protein stathmin (STMN1) (Steinmetz 2007) facilitated by the p21-activated kinase 1 (PAK1) (Wittmann *et al* 2003, 2004). Similarly, Rho stabilizes microtubules through the

formin mDia1 (synonymously known as DIAPH1). This mechanism seems to be independent of mDia1's actin-nucleating activity, however, the binding of mDia1 to the +TIPs EB1 (synonymously known as MAPRE1) and APC within the lamella of cells grown in 2D play a role (Bartolini *et al* 2008, Palazzo *et al* 2001, Wen *et al* 2004). In addition, mDia1 builds a complex with another +TIP, CLIP-170 (synonymously known as CLIP1), which acts as a nucleator for F-actin assembly at the microtubule plus ends (Henty-Ridilla *et al* 2016). However, this mechanism represents a new function for microtubules in 3D cell migration systems. The inhibition of TRIO by Par3 and finally the inactivation of Rac1 alters the growth of microtubules at cell–cell contacts of migrating neural crest cells *in vivo* (Moore *et al* 2013). In particular, it is hypothesized that TRIO and Rac1 facilitate the stabilization of the microtubules and hence induce mesenchymal cell protrusion, whereas Par3 is able to abolish this pathway by contributing to the contact inhibition of movement (Moore *et al* 2013). However, the precise functions of TRIO and Rac1 need to be further examined in 3D migration systems.

The microtubule depolymerization did not impair the spreading of fibroblasts on stiff substrates, whereas it causes a loss of pseudopods and decreases cell elongation in soft substrates and hence may impact the microtubule-driven regulation of Rho GTPases in 3D systems (Rhee *et al* 2007, Unemori and Werb 1986). However, the inhibition of Rho effectors, which are regulating the activation of contractility such as myosin II or its activating kinase Rho-associated protein kinase (ROCK) cannot prevent the adaption of a rounded cell shape by the fibroblasts upon nocodazole-treated soft 3D cell cultures (Grinnell *et al* 2003, Rhee *et al* 2007). Thus, these results demonstrate that increased Rho activity and contractility are not involved in the loss of the pseudopod after the depolymerization of the microtubules. There is still the possibility that Rac1 inactivation and hence decreased actin-driven protrusion can explain this effect (Rhee *et al* 2007). A novel approach to investigate the regulation of Rho GTPase in 3D cell migration utilizes biosensors such as Förster resonance energy transfer (FRET)-based probes (Donnelly *et al* 2014). Examples are activity measurements of various Rho GTPases such as Rac1 and Rho in mesenchymal cancer cells *in vivo* (Hirata *et al* 2012, Timpson *et al* 2011), in 3D cell cultures (Hirata *et al* 2012, Petrie *et al* 2012) and during the amoeboid migration of germ cells during development in zebrafish embryos (Kardash *et al* 2010).

9.4 Effect of microtubules on cell mechanical properties

The effect of microtubules on cellular mechanical properties has not been investigated in great detail, however, the mechanical properties are highly important for the function of microtubules in cell morphogenesis, cell division and cell migration in 3D extracellular matrices. Indeed, a role for microtubules in contributing to cellular mechanical properties and hence providing cellular shape has been suggested in several studies (Brown *et al* 1996, Dennerll *et al* 1988, Janmey *et al* 1991, Rudolph and Woodward 1978, Tomasek and Hay 1984). In parallel, a connection between microtubules and the 3D matrix contraction by invasive mesenchymal cells has been

revealed using artificial 3D collagen fiber matrices (Bell *et al* 1979, Kolodney and Wysolmerski 1992, Kraning-Rush *et al* 2011). Indeed, microtubules are needed to generate wide-range traction forces in adhesive and elongated cells such as mesenchymal cells in 3D extracellular matrices (Kraning-Rush *et al* 2011). This behavior shows that microtubules even function mechanically, when regulating cell adhesion and contractility. The effect of microtubules on the remodeling of the 3D extracellular matrix is correlated with cell contractility (Danowski 1989) and additionally is further supported by the connection between microtubules and the activation of Rho as well as subsequently myosin II. Moreover, a direct mechanical function of the microtubules in the control of the cell shape has been suggested (Danowski 1989). Another hypothesis is favored, suggesting that microtubule-driven maintenance of the balance between Rho and Rac1 levels contributes mainly to cell elongation in soft 3D extracellular matrices (Rhee *et al* 2007). In comparison to F-actin, microtubules represent relatively stiff polymers (Hawkins *et al* 2010, Mizushima-Sugano *et al* 1983) that are able to generate through their polymerization pushing forces in the range of a few piconewtons (Dogterom and Yurke 1997). Hence, microtubules apply pushing forces to the cell membrane and can even resist cell compression (Elbaum *et al* 1996, Fygenson *et al* 1997a, 1997b, Hotani and Miyamoto 1990, Waterman-Storer *et al* 1995). In particular, the buckling of microtubules in living cells demonstrates their function act as load-bearing fibers (Brangwynne *et al* 2006, Robison *et al* 2016, Wang *et al* 2001). Based on these observations, the mechanical role for microtubules in terms of cell tensegrity needs to be further refined. Moreover, the 3D matrix stiffness alters the microtubule stability and subsequently downstream signaling such as the regulation of Rho, however, the mechanisms still remain not fully understood (Heck *et al* 2012, Myers *et al* 2011).

The tensegrity model seems to be limited by the fact that microtubule tips in close spatial proximity to the cortex are mostly dynamic and the flow of actin even promotes their destabilization (Gupton *et al* 2002, Waterman-Storer and Salmon 1997). Another limitation is that the microtubule plus ends are sensitive to compression-induced catastrophes (Janson *et al* 2003, Laan *et al* 2008), which in turn further restrict the microtubule load-bearing capacity. However, the retrograde actin flow can be reduced in pseudopods of cells cultured in 3D compared to the lamella of cells cultured in 2D, or specific regulators may decrease the sensitivity of microtubule plus ends to depolymerize at locations where the load-bearing microtubule function provides the elongation of the mesenchymal cells, such as the formation of pseudopod tips in 3D. The migration of cancer cells under a strong confinement such as in 3 μm diameter channels is more strongly altered by the inhibition of microtubule polymerization than by inhibition of actin polymerization, actomyosin contractility or integrin adhesion (Balzer *et al* 2012). In particular, tube confinement redirected the microtubule polymerization towards the cell's leading edge, which seems to suggest that growing microtubules generate pushing forces to promote the forward movement of the cell's leading edge (Balzer *et al* 2012). Hence, under distinct 3D conditions, the microtubule–cortex interactions are regulated in a manner that increases their mechanical role during cell protrusion exertion.

Cell migration is essential for tissue morphogenesis and requires alterations in cellular polarity upon changes in mechanical and chemical properties of the cellular microenvironment (Abercrombie *et al* 1971, Roubinet *et al* 2012, Schwarz and Gardel 2012). In particular, the polarization of the cell cortex for motility evokes a cortical asymmetry by the establishment of a leading edge and a cell rear within migrating cells (Akhshi *et al* 2014). Hence, the actin cytoskeleton undergoes dramatic alterations at the cell's leading edge and rear of motile cells (Ridley 2011). Lamellipodia (the ruffling of membranes) and filipodia are exerted by extensive actin polymerization at the leading edge of moving cells in order to crawl forward on a flat surface (figure 9.12). In the lamellipodium, F-actin undergoes treadmilling and is newly polymerized at the filament tips utilizing the released G-actin during the depolymerization of F-actin at the base (Bugyi and Carlier 2010, Small 2010).

The stress fibers built in migrating cells consist of stable F-actin bundles connected to active non-muscle myosin II. The stress fibers are coupled to the extracellular matrix microenvironment through focal adhesions. In turn, the extracellular matrix alters the formation and disassembly of cellular focal adhesions. In particular, the contraction of stress fibers generates rearward forces that help to push the cell forward and subsequently the cell's rear is contracted through the disassembly of focal adhesions at the rear end. There exist parallels between the cortical alterations facilitating cytokinesis and cell migration. In particular, the rear of a migrating cell seems to be similar to the equatorial plane of a mitotic cell during cytokinesis and hence possesses high cortical tension and contractility, whereas the actin network assembled at the cell's leading edge of a migrating cell is similar to the poles of a mitotic cell and displays lower tension (Roubinet *et al* 2012). Cells can migrate in response to extrinsic cues, which regulate the activity of RhoA, Rac1 and Cdc42 and their downstream effectors to adapt the actin cytoskeleton and thereby influence the

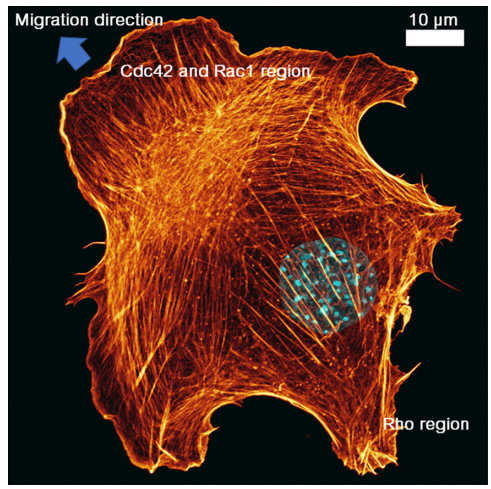

Figure 9.12. Lamellipodial protrusions of a fibroblast migrating on a flat substrate. The F-actin cytoskeleton is stained with Alexa Fluor 546 Phalloidin.

microtubule stability (Nobes and Hall 1999, Goode *et al* 2000, Rodriguez *et al* 2003, Burridge and Wennerberg 2004, Raftopoulou and Hall 2004, Takesono *et al* 2010, Ridley 2011, Hall 2012).

In the cell's rear, active RhoA controls the assembly of stress fibers by providing the polymerization of long, unbranched F-actin through formins (Watanabe *et al* 1997). In more detail, active RhoA binds to the GTPase binding domain (GBD) of formin and hence creates a conformational alteration to end its autoinhibitory conformation (via the Dia autoregulatory domain termed DAD). Thereby the FH2 dimerization domains are accessible and the FH1 domains interact with profilin–actin in order to assemble F-actin from their barbed end under ATP energy consumption (Higashida *et al* 2004, Romero *et al* 2004, Kovar 2006, Kovar *et al* 2006, Goode and Eck 2007). The activated RhoA promotes the Rho kinase (ROCK) to build active non-muscle myosin II filaments, which can generate contractility (Amano *et al* 2010a). In addition, ROCK induces LIMK activity to decreases ADF/cofilin levels and thereby impairs the disassembly of F-actin (Arber *et al* 1998, Bernstein and Bamburg 2010, Mizuno 2013). In summary, the regulation of the F-actin turnover is crucial for the assembly of lamellipodia at the cell's leading edge (Arber *et al* 1998, Yang *et al* 1998, Bernstein and Bamburg 2010, Mizuno 2013).

Active Cdc42 regulates WASP (Wiskott–Aldrich syndrome protein) and active Rac regulates Wave (WASP-family verprolin homology proteins)/Scar (suppressor of camp receptor) complexes to facilitate the activation of Arp2/3 for the initiation of the nucleation of short actin filament branches (Aspenström *et al* 1996, Machesky and Insall 1998, Castellano *et al* 1999, Eden *et al* 2002, Kunda *et al* 2003, Chesarone and Goode 2007). This also affects the stability of microtubules, as microtubules in Tiam1 (a Rac GEF) knock-out cells are found to be disorganized and unstable, all of which indicates a connection between Rac signaling and microtubule remodeling dynamics (Petgel *et al* 2007). The mechanism of this interaction is not yet clear, but microtubules in turn regulate the Rho GTPase activity to induce a polarization of migrating cells (Kaverina and Straube 2011).

In general, microtubule growth, such as the growth phase of dynamic instability, activates Rac, whereas the depolymerization of microtubules causes RhoA activation (Enomoto 1996, Waterman-Storer *et al* 1999). In nocodazole-treated cells, in which the assembly of microtubules is prevented, RhoA is activated by GEF-H1 that is released from microtubules upon their depolymerization (Krendel *et al* 2002, Chang *et al* 2008). In migrating cells, the active RhoA is located in spaces of the cell, in which microtubules are depolymerizing, and promotes the formation of mature focal adhesions and controls the contractility. In turn, the growing microtubules arriving at the cell's leading edge or growing towards mature focal adhesions elevate the levels of active Rac in order to facilitate the polarization of the cortex for cell migration or for the disassembly of focal adhesions. The growth of microtubules seems to activate Rac through the GEFs Tiam1, Sif and STEF (Tiam2) and hence causes the disassembly of focal adhesions (Ezratty *et al* 2005, Petgel *et al* 2007), which is consistent with the observation that in STEF-deficient cells the focal adhesions possess decreased disassembly rates and hence are enlarged (Rooney *et al* 2010). However, the targeting of microtubules to focal adhesions is not yet fully

understood. It has been speculated that microtubules are tracked along actin filaments to focal adhesions and thereby are guided by septins in polarizing epithelial cells (Krylyshkina *et al* 2003, Small and Kaverina 2003, Bowen *et al* 2011). There remains an open question: how can microtubules and the actin filaments interact to facilitate the polarization of cells?

Focal adhesion turnover is regulated by microtubules and its focal adhesion disassembly enables the cell to move forward and establish a focal adhesion at a new location (Petit and Thiery 2000, Parsons *et al* 2010, Wehrle-Haller 2012). The core elements of focal adhesions are the cell–matrix integrin receptors, which consist of heterodimeric α and β subunits, that interact with ligands of the extracellular matrix. Each heterodimeric integrin receptor recognizes a distinct ligand and thereby represents a mechano-sensory system translating environmental cues into cell movement (Luo *et al* 2007, Wolfenson *et al* 2013). Ligand-bound integrins are internalized to order to be recycled back to the cell membrane, which enables the formation of new focal adhesions during cell migration and invasion (Ezratty *et al* 2009). On their intracellular face of the cell membrane, the focal adhesions are coupled to the cytoskeleton via actin bundling proteins such as α-actinin, vinculin or talin, which in turn regulate the recruitment or release of other focal adhesion proteins dependent on the forces (Humphries *et al* 2007, Carisey *et al* 2013). In particular, the size of focal adhesions is controlled by the focal adhesion kinase (FAK) (Mitra *et al* 2005). FAK is activated by Src kinase-dependent phosphorylation at Tyr861 and hence associates with cytoskeletal-associated components such as paxillin and talin. The binding to paxillin or talin enables FAK to interact with the growth factor receptor-bound protein 2 (Grb2) and hence associates the dynamin GTPase to focal adhesions. In turn, dynamin induces the internalization of integrins by endocytosis in order to decrease the focal adhesion size (Burridge 2005, Ezratty *et al* 2005, 2009, Wang *et al* 2001). In more detail, the turnover of focal adhesions is necessary for cell migration and its turnover rate seems to determine the migration speed of cells (Berginski *et al* 2011, Stehbens and Wittmann 2012, Kim and Wirtz 2013). Both microtubules and stress fibers control the formation of focal adhesions and their size and turnover. In particular, focal adhesions are strengthened through their connection with stress fibers, which are assembled by active RhoA and ROCK (Chrzanowska-Wodnicka and Burridge 1996, Rottner *et al* 1999, Katoh *et al* 2007, Pellegrin and Mellor 2007, Vicente-Manzanares *et al* 2009, Amano *et al* 2010a). In line with this, monocytes treated with RhoA inhibitors such as C3 or with ROCK inhibitors such Y-27632 de-adhere and hence integrins are impaired in their localization, which suggests improper assembly of focal adhesions (Worthylake *et al* 2001). Hence, RhoA seems to stimulate ROCK for both functions, the myosin-based contractility and integrin organization for cell–matrix adhesion. Depolymerization of microtubules through treatment with nocodazole causes a stabilization of focal adhesions, as RhoA is activated and hence releases GEF-H1, which then facilitates the formation of stress fibers and large focal adhesions, which are not able to disassemble (Krendel *et al* 2002, Chang *et al* 2008, Nalbant *et al* 2009). Moreover, the disassembly of focal adhesion needs targeted microtubule growth and increased Rac activity (Kaverina *et al* 1998, 1999, Krylyshkina *et al* 2002, 2003, Ezratty *et al* 2005).

Another regulator of focal adhesion turnover the integrin-linked kinase (ILK), which interacts with integrins and hence regulates the signal transduction from the extracellular matrix to the intracellular machinery (termed outside–in signaling) (Li *et al* 1999, Legate *et al* 2006). The disassembly of focal adhesions employs targeted microtubule growth and Rac activity (Kaverina *et al* 1998, 1999, Krylyshkina *et al* 2002, 2003, Ezratty *et al* 2005).

9.5 The interactions of microtubules with actin filaments

The three main cytoskeletal polymers, actin, microtubules and intermediate filaments, are connected by coordinated protein crosslinks to create a complex 3D cytoskeletal network. The interaction of actin filaments with microtubules plays an important role in several cellular processes such as cell division, cell motility, vesicle and organelle transport, and axonal growth (Mohan and John 2015). In line with this, various proteins such as signaling molecules, motor proteins, and proteins directly or indirectly associated with microtubules and actin fulfill a coupling function of the three distinct cytoskeletal components. The MAPs belonging to the MAP1, 2, 4 family and tau proteins are reported to be crosslinkers of the actin and microtubule cytoskeletons. How can the dynamics of these coordinated networks be analyzed? Moreover, the identification of the underlying mechanisms of actin and microtubule interactions seems to be crucial for the understanding of cancer, wound healing and neuronal regeneration processes.

9.5.1 Microtubule-associated proteins (MAPs)

The interaction between actin filaments and microtubules is crucial for many cellular processes such as cell division, cell migration, vesicle or organelle transport, axonal growth and wound healing after tissue injury (Rodriguez *et al* 2003). In particular, actin filaments provide a scaffold for microtubules to guide and stabilize them specifically at the cell cortex. Indeed, the growth of microtubules has been found to be nearby and along F-actin bundles using fluorescence speckle microscopy (Waterman-Storer and Salmon 1997) and in turn the growth of microtubules is abolished in neuronal growth cones when the supporting actin scaffold is perturbed in its organization (Zhou *et al* 2002). In more detail, actin filaments and microtubules are crosslinked either by direct linkage facilitated by crosslinking proteins or indirectly via multiple protein complexes or signal transduction molecules. Interactions mediated by crosslinking proteins are usually static interactions, whereas the interactions provided by motor protein complexes or signal transduction molecules are dynamic interactions.

MAPs are key players in providing interactions between microtubules and actin filaments. There are two major different types of MAPs such as structural MAPs and the end-binding 1 TIP proteins (+TIPs). The structural MAP proteins contain members of the MAP1, MAP2, MAP4 family and tau proteins and the name structural MAPs has been chosen due to the fact that these proteins are non-enzymatic proteins, which bind along the surface of the microtubules and hence increase their assembly and the stability of the tubulin polymer (figure 9.13). In contrast, the +TIPs control the dynamic properties of the microtubules by binding

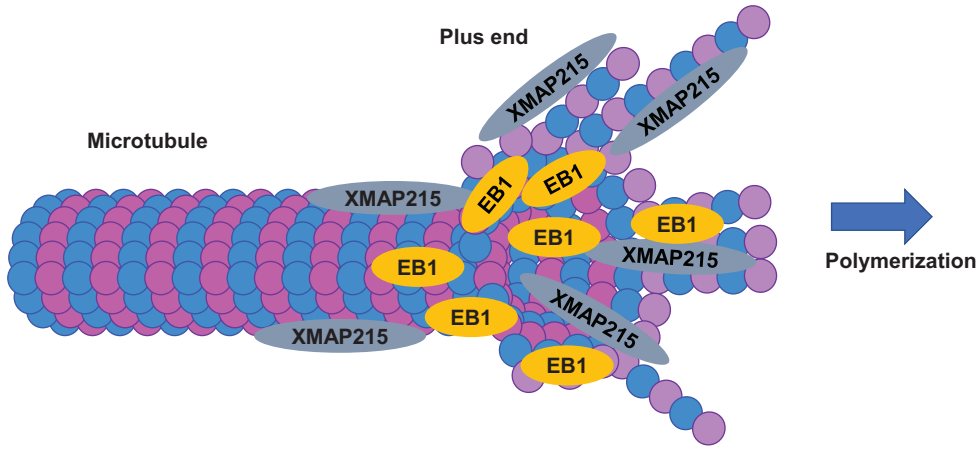

Figure 9.13. EP1 and XPMAP215 stabilize the microtubules through their binding to the plus ends and promote their elongation.

specifically to the growing ends of the microtubules, which leads then to dynamic and transient interactions with other protein complexes, through which they are indirectly engaged with actin filaments. Moreover, microtubule-associated motor proteins such as kinesins and dyneins additionally provide dynamic interactions between these two cytoskeletons. Key components, such as the spectraplakin family proteins, structural MAPs belonging to the MAP1, 2, 4 family and tau proteins, have been identified that can directly connect actin and microtubules. Spectraplakins are relatively large proteins containing multifunctional domains, which enable these proteins to associate with actin and microtubules simultaneously (Kodama *et al* 2003). MAP proteins of the MAP1 and 2 family as well as tau proteins are usually expressed in neurons, where these proteins facilitate the assembly, maintain the stability and rearrange the organization of the microtubules. Although MAPs prefer to interact with microtubules, they are able to bind actin and hence promote interactions between microtubules and actin filaments. The interaction of microtubules with actin has been analyzed extensively using biochemical assays (Griffith and Pollard 1978). The first studies of MAP-mediated microtubule–actin interaction have been simply performed with purified proteins by using different techniques such as viscometry, cosedimentation, spectroscopy, electron microscopy, light microscopy and photobleaching approaches.

9.5.2 The role of MAPs in 3D cell migration

The functions of microtubules in 3D cell motility rely on specific MAPs. An example is the protein Spastin, that is present in migrating macrophages of *Drosophila* embryos. When the expression of Spastin, which is a microtubule-severing protein is inhibited, the distribution of migrating macrophages along the ventral midline within the embryo is pronouncedly altered (Stramer *et al* 2010). Moreover, a mutation of Orbit, which is the *Drosophila* homolog of the +TIPs CLASP1 and CLASP2, is similarly responsible with the altered motility upon contact inhibition.

Moreover, the *Drosophila* CLASP seems to promote the assembly of a microtubule bundle or so-called 'arm' points towards the cell's leading edge and the depolymerization of the microtubule arm causes fast cell repolarization upon cell–cell collision and thereby alters the cell trajectory. As CLASPs are known to suppress microtubule catastrophes, promote the rescue of microtubules (Akhmanova and Steinmetz 2015) and are spatially enriched at the cell cortex, at which they facilitate the remodeling and turnover of focal adhesions (Stehbens *et al* 2014), the investigation of the CLASP function during cell migration through a 3D microenvironment is assumed to be suitable in modeling the *in vivo* situation.

Spectraplakins are another example for the involvement of +TIPs in cell migration, which can also facilitate the interaction between microtubules and actin (Suozzi *et al* 2012). In particular, the *Caenorhabditis elegans* spectraplakin VAB-10 is necessary for leader cell migration during the gonad development (Kim *et al* 2011) and the mammalian MACF1 (synonymously known as ACF7) is needed for epidermal cell migration *in vivo* (Yue *et al* 2016). ACF7 is known to target microtubules to focal adhesions by providing the crosslinking between microtubules and actin (Wu *et al* 2008), however, this function requires still detailed analysis in 3D cell migration.

EB1 represents a key regulatory protein for assembly of +TIP complexes (Akhmanova and Steinmetz 2015). Although the depletion of EB1 is negligible for the 3D matrix invasion of the mesenchymal MDA-MB-231 breast cancer cells (Morimura and Takahashi 2011), it decreases the pseudopod elongation in HGF-stimulated MDCK cells in 3D cell cultures (Gierke and Wittmann 2012). The EB1 depletion in MDCK cells reduces the phosphorylation of myosin II, causes defective microtubule arrangement into pseudopods and abolishes the assembly of focal adhesions and the trafficking of vesicles. EB1 regulates the recruitment of multiple +TIPs, which are associated with distinct cellular functions to the microtubules and hence it needs to be revealed which EB1 interacting proteins cause the phenotype of the EB1 depletion.

The tubulin-binding protein stathmin, which is a tubulin-sequestering and microtubule-destabilizing protein, represents another specific factor regulating the assembly of the microtubules and is associated with 3D cell motility (Gupta *et al* 2013, Steinmetz 2007). Stathmin inhibition through the addition of the apoptosis regulatory protein (Siva1) has been associated with microtubule stabilization, decreased 3D matrix invasion and abolishment of EMT and metastasis within a breast cancer model (Li *et al* 2011b). Moreover, the tubulin-sequestering activity of stathmin causes an alteration in the morphology of cancer cells grown within soft 3D extracellular matrix, with induces a switch from mesenchymal to amoeboid morphology and supports the migration in 3D (Belletti *et al* 2008). Cells undergoing stathmin-induced amoeboid motility in 3D are more metastatic in mice, indicating that the morphology switch enhances 3D motility *in vivo* (Belletti *et al* 2008). Consistently, during 3D extracellular matrix invasion, the amoeboid migration mode may be more efficient and hence favored than the mesenchymal migration mode (Friedl and Wolf 2010). In particular, the amoeboid cell migration facilitates the promotion of tumorigenic alterations that are associated with the destabilization

of microtubules and the increased invasion in 3D (Hager *et al* 2012). *In vivo*, the velocity of amoeboid migration cells has been revealed to be increased by nocodazole-induced microtubule destabilization (Yoo *et al* 2012). Moreover, the microtubule destabilization by stathmin seems to possess a pro-migratory effect under the amoeboid migration mode (Friedl and Wolf 2010, Lammermann and Sixt 2009, Liu *et al* 2015).

Microtubule-severing enzymes such as katanin are directly connected to the regulation of cell migration in 2D systems (Sharp and Ross 2012). The inhibition of the microtubule-severing protein fidgetin-like protein 2 (FIGNL2) have been found to induce epidermal cell migration *in vivo* (Charafeddine *et al* 2015), which implies a role for them in 3D cell migration.

In addition to specific MAPs, post-translational modifications of tubulin represent a control mechanism for multiple microtubule functions such as the binding and enrichment of +TIPs and microtubule motors (Akhmanova and Steinmetz 2008, Etienne-Manneville 2013, Janke and Bulinski 2011, Song and Brady 2015). An example is the acetylation of tubulin, which is a post-translational modification, that is facilitated by α-tubulin N-acetyltransferase 1 (ATAT1 or synonymously termed αTAT1) and can be removed by histone deacetylase 6 (HDAC6) (figure 9.14) (Song and Brady 2015). In 2D cultured cells, inhibition of HDAC6 enlarges the focal adhesion sizes (Bouchet *et al* 2011a, Tran *et al* 2007) and impairs the turnover of focal adhesions (Tran *et al* 2007). HDAC6 interacts with the focal adhesion protein paxillin, which, in turn, inhibits deacetylation (Deakin and Turner 2014). αTAT1 interacts with clathrin-coated pits and focal adhesions at the ventral side of cells in 2D culture assays, which then induces the acetylation of microtubules (Montagnac *et al* 2013). In 3D microenvironments, the acetylation of microtubules drives the transport of the matrix metalloproteinase MT1-MMP, however, its effect on 3D migration seems to be more complex as the inactivation of both HDAC6 (Rey *et al*

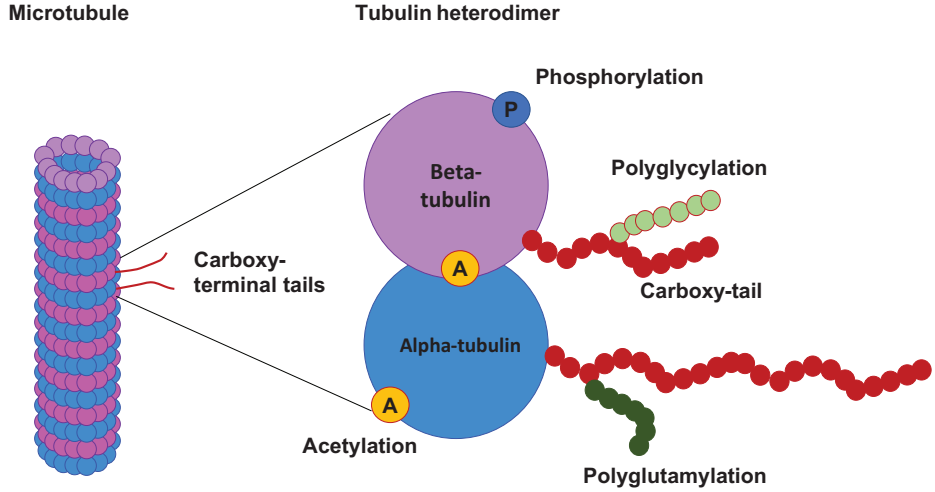

Figure 9.14. Acetylation of microtubules.

2011) and αTAT1 (Castro-Castro *et al* 2012, Montagnac *et al* 2013) impairs their 3D extracellular matrix invasion.

Multiple microtubule-associated factors such the kinesins KIF1C, KIF5B, KIF3A/B and KIF9, +TIPs EB1 and CLASP1, as well as deacetylase HDAC6 are associated with the exertion of podosomes (Bhuwania *et al* 2014, Biosse *et al* 2014, Cornfine *et al* 2011, Destaing *et al* 2005, Efimova *et al* 2014, Maridonneau-Parini 2014, Wiesner *et al* 2010, Zhu *et al* 2016). In particular podosomes, representing structures that are characterized by the specialized organization of actin and adhesion molecules and the ability to degrade the extracellular matrix, seems to rely on the microtubule regulation and microtubule-based transport for their proper function.

9.5.3 Direct interaction between microtubules and actin

The actin filaments and microtubules are highly dynamic cytoskeletal components that can fulfill a broad range of intracellular processes including the formation, maintenance and the dynamics remodeling of cell–cell and cell–substrate interactions. Moreover, the interactions between actin filaments and microtubules seem to be crucial for the assembly and maintenance of entire cell structure in 3D microenvironments. Cytoplasmic actins are differentially distributed compared to the microtubule system. Selective depletion of β- or γ-cytoplasmic actins demonstrated a selective interaction between microtubules and γ-actin, but not β-actin, through the microtubule +TIPs protein EB1. The EB1-positive comet distribution analysis and quantification revealed enhanced effective microtubule growth in the absence of β-actin. Microtubule +TIPs protein EB1 has been found to interact mainly with γ-actin in epithelial cells (Dugina *et al* 2016).

Although actin microfilaments undergo highly dynamic remodeling at the cell periphery with turnover times in the order of seconds (Amann and Pollard 2000), they can still efficiently provide the cellular shape. Indeed, microtubules are also known to be highly dynamic, as they are continuously growing or shortening, even though no visual changes can be detected in their specific localization of the cytoplasm. In particular, the ends of individual microtubules are elongating or shortening over distances of several microns (Mitchison and Kirschner 1984a, 1984b) and the entire system exchanges continuously with the pool of monomeric tubulin at turnover times of 5–20 min (Vorobjev *et al* 2001, Vorobjev *et al* 1999). The assembly of microtubules involves the enrichment of newly polymerized portions (the plus ends) of microtubules with GTP–tubulin and the specific interaction of microtubule +TIPs (Galjart 2010, Akhmanova and Steinmetz 2010). +TIPs are a large group of structurally and functionally diverse microtubule regulators including end-binding (EB) proteins, cytoplasmic linker proteins (CLIP) and CLIP-associated proteins (CLASP), which are all associated with the microtubule interaction with the cell cortex (Akhmanova and Steinmetz 2015). The mutual interaction between microtubules and the actin cytoskeleton is required for essential cellular processes such as the establishment and maintenance of cell shape, cell migration and division, intracellular vesicle transport and cell–cell interactions (Akhmanova and Steinmetz

2015, Akhmanova and Steinmetz 2008). Direct or indirect interactions between these two cytoskeletal systems are proposed. Microtubules are able to regulate the organization of the actin cytoskeleton by promoting local alterations in the actomyosin contractility at the stress-fiber ends (Small and Kaverina 2003). The interaction of microtubules and actin filaments forms the basis for the entire endothelial cell barrier function (Birukova *et al* 2004a, 2004b, Alieva *et al* 2013, Tian *et al* 2014a).

The actin cytoskeleton in non-muscle cells is built by two actin isoforms, the non-muscle β- and γ-cytoplasmic actin (β- and γ-actin), which are encoded by ACTB and ACTG1 genes, respectively. Both actins are ubiquitously expressed in cells (Vandekerckhove and Weber 1978, Rubenstein 1990) and are required for cell survival (Harborth *et al* 2001). The ratio between β- and γ-actin depends on the individual cell type (Vandekerckhove and Weber 1978, Khaitlina 2001, Khaitlina 2007, Sheterline *et al* 1995). Alterations of the actin isoform expression is associated with pathological processes (Chaponnier and Gabbiani 2004) and indeed gene transfection studies revealed that the two actin isoforms interact in opposite directions on the myoblast architecture (Schevzov *et al* 1992). Using specific monoclonal antibodies directed against β- and γ-actins and siRNA depletion of each cytoplasmic actin, it has been found that β-actin functions in contractile and adhesion structures, whereas γ-actin plays a role in the assembly of the cortical network, which is required for the flexibility of the cell shape and motile activity in normal fibroblasts and epithelial cells (Dugina *et al* 2009). Both actins are visualized at the apex of polarized epithelial cells in close association with to intercellular contacts (Dugina *et al* 2009, Dugina *et al* 2008), however, these actin isoforms regulate different junctional complexes. In particular, β-actin interacts with adhesion junctions, whereas γ-actin interacts with tight junctions (Baranwal *et al* 2012). The selective siRNA-based knockdown of γ-actin, but not β-actin, induced a epithelial to myofibroblast transition (EMyT) of various epithelial cells (Lechuga *et al* 2014). The EMyT is characterized by enhanced expression of α-smooth muscle actin and other contractile proteins and by the reduced expression of genes involved in cell proliferation. Hence all these findings show a unique role for γ-actin in regulation of the epithelial phenotype and suppression of EMyT, which subsequently reduces cell differentiation and tissue fibrosis (Lechuga *et al* 2014).

These two actin isoforms fulfill different roles in neoplastic cell transformation. The β-actin has been shown to acts as a tumor suppressor, as it alters epithelial differentiation, cell growth, the invasion of colon and lung cancer cells *in vitro* and the tumor growth *in vivo*. In contrast to β-actin, γ-actin increases the malignant phenotype of cancer cells, when their actin network is regulated through the γ-actin isoform (Dugina *et al* 2015).

9.5.4 Microtubules and actin isoforms are segregated in different compartments

Using confocal immunofluorescence microscopy, it has been revealed that the two cytoplasmic actins are segregated into dorsal and ventral zones within normally spread keratinocytes and fibroblasts (Dugina *et al* 2009). Moreover, these two actin isoforms are also segregated into different cell compartments of breast cancer cells in

in vitro cell culture assays, whereas their distribution within the cell membrane in breast cancer lesions is homogeneous (Dugina *et al* 2008). These two actin networks displaying distinct structural organization have been analyzed using improved STORM with dual-objectives (Xu *et al* 2012). Indeed, two vertically separated actin layers have been identified in the sheet-like cell protrusion despite its thinness. These two actin layers identified by STORM, in BSC-1 epithelial cells and in COS-7 fibroblast-like cells, correspond to β-actin-containing ventral bundles and γ-actin-containing dorsal cortical network. The radial microtubule system is supposed to be localized between these two vertically separated layers of actin. Although the imaging capability of 3D-SIM did not provide the distinct localization of individual cytoplasmic γ-actin within the cortical network, the difference in localization of β-actin and γ-actin compared to the microtubules has been clearly detected. Moreover, the 3D microtubule arrays are closely associated to the cytoplasmic γ-actin cortical network.

9.5.5 The epithelial cell migratory capacity is driven by an EB1–γ-actin interaction

The members of the growth-arrest-specific 2 (GAS2) family facilitate the interaction between F-actin and microtubules, as they generate an essential linkage during axon extension (Applewhite *et al* 2010, Alves-Silva *et al* 2012, Lee and Kolodziej 2002, Stroud *et al* 2011). Although all GAS2 proteins localize to actin and microtubules, only exogenously expressed G2L1 and G2L2 alters the stability of the microtubules, their dynamic remodeling and guidance behavior along actin stress fibers (Stroud *et al* 2014). This specific type of crosstalk between actin bundles and microtubules in fibroblasts is associated with cytoskeleton stability and its maturation, however, an interaction between microtubules and cytoplasmic γ-actin is correlated with cancer cell motility. In particular, the coordination between the actin cytoskeleton and microtubules seems to be facilitated by the guidance of microtubule growth through the actin bundles (Rodriguez *et al* 2003). The guidance also occurs in migrating cells, in which the growing microtubule tips are targeted to focal adhesions (Kaverina *et al* 1998, Wu *et al* 2008, Stehbens and Wittmann 2012, Preciado Lopez *et al* 2014). In particular, the plus ends of the dynamic microtubules can be connected to the cell cortex through IQGAP1 (Fukata *et al* 2002) or APC (Moseley *et al* 2007).

EB1 forms a comet-like accumulation, which is 0.5–2.0 μm long, and it turns over rapidly at the microtubule plus end (Mimori-Kiyosue *et al* 2000, Bieling *et al* 2007, Seetapun *et al* 2012). Indeed, the EB1 comet length has been found to be altered, which suggests that the microtubule dynamics are altered by the cellular actin isoform composition. In mammalian cells, EB1 enhances persistent microtubule growth and diminishes the catastrophe frequency (Komarova *et al* 2009). In *in vitro* studies EB1 has been identified as a factor reducing the maturation time of growing microtubule ends (Maurer *et al* 2014), which associates the EB1 localization and the regulation of microtubule dynamics. In more detail, the length of EB comets mirrors the size of the microtubule GTF cap (Seetapun *et al* 2012). In β-actin-depleted cells, the shortening of microtubule comets is related to the frequent switch between very short periods of growth and depolymerization. Within these cells, the microtubules

are in more parallel bundles than in untreated control cells, whereas in γ-actin-depleted cells the microtubules are more entangled (figure 9.15). The functional role of EB1 in cell motility has been revealed in melanoma cells (Schober *et al* 2009), where EB1 depletion impaired the lamellipodia formation. Moreover, the over-expression of EB1 is related to glioblastoma progression and increased migration, whereas the downregulation of EB1 using shRNA abolished cell migration and proliferation *in vitro* (Berges *et al* 2014). Alteration of the γ-actin expression causes similar functional alterations in normal (Tondeleir *et al* 2012) and neoplastic cells (Dugina *et al* 2009, Dugina *et al* 2015, Shum *et al* 2011). Using Boyden chambers assays, it has been found that actin isoform regulation causes alterations in the migratory characteristics of cancer cells. Cells with down-regulated γ-actin displayed decreased cell motility than control cells. In contrast, MCF-7 cells with loss of β-actin migrate even more effectively.

EB1 is expressed at high amounts in breast cancer cells (Dong *et al* 2010), which enables the spatial detection of endogenous EB1 and identification of the length of the comet tips. The EB1 comet length is significantly altered in β-actin-depleted cells. Moreover, EB1 comets are decreased after the treatment with low doses of microtubule-destabilizing drugs (Akhmanova and Steinmetz 2010, Alieva *et al* 2013) reducing the growth rate of microtubules. The shortening of microtubule comets preferentially in close proximity near the cell edge of β-actin-depleted cells is associated with reorganization of microtubules at the cell periphery and hence it is hypothesized that the relative levels of the actin isoforms facilitate the regulation of microtubule dynamics. In addition, microtubules become more Triton-X-100-resistant in γ-actin-depleted MCF-7 cells. Indeed, higher stability of microtubules after γ-actin siRNA treatment are observed in neuroblastoma cells (Pouha *et al* 2015). In summary, the β- and γ-actin filament networks fulfill opposite functions in the regulation of microtubule dynamics and organization.

+TIPs proteins are hypothesized to indirectly interact or facilitate the crosstalk between the microtubules and the actin cytoskeleton (Rodriguez *et al* 2003, Preciado López *et al* 2014, Gundersen *et al* 2004). The combination of biochemical and biophysical techniques revealed that EB1 binds skeletal F-actin *in vitro* and the F-actin binding site on EB1 overlaps with the EB1-microtubule binding site. Competition experiments and mutagenesis experiments revealed that EB1 can either bind exclusively to F-actin or to microtubules. This interaction may support cells to differentially regulate microtubule stability within the actin-rich cortex compared to the actin cytoskeleton of the cell interior (Alberico *et al* 2016). In contrast, in cancer

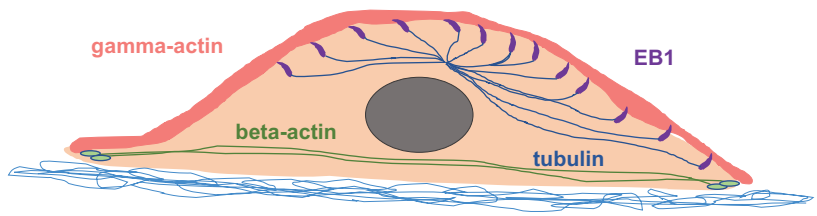

Figure 9.15. EB1 binds to γ-actin and thereby connects the microtubules to the cell membrane.

cells, a microtubule–actin cortical network interaction has been observed (Dugina *et al* 2016). In more detail, the interaction is pronounced in the absence of β-actin bundles and impaired in the presence of bundle enrichment after γ-actin depletion.

It has been hypothesized that microtubules associate directly with the γ-actin cortical network through a +TIPs protein complex. Using super-resolution microscopy with colocalization analysis, such as protein localization approaches (PLA) and Co-Immunprecipitation (CoIP) microtubules seem to be directly or indirectly bound to γ-actin through a +TIPs protein EB1-containing protein complex that contains IQGAP, APC and Rac. Multiple interaction partners of γ-actin such as ERK1/2, p34-Arc, WAVE2, cofilin1 and PP1 have been identified in lung and colon cancer cells (Dugina *et al* 2015). These proteins function in the structural and signal transduction of cellular motility and proliferation, which indicates that the EB1 interacts with a specific cytoplasmic actin isoform (Dugina *et al* 2016). In line with this, in neuroblastoma cells during mitosis (Simiczyjew *et al* 2017) the γ-actin seems to be involved in the regulation of the microtubule apparatus.

What is the biological significance of the microtubule–γ-actin interaction?
The proposed 'search-and-capture' model of microtubule dynamics (Kirschner and Mitchison 1986) hypothesizes that during the transitions of microtubules between growth and shortening of their plus ends, they rapidly sense the 3D intracellular microenvironment and search for interaction or capture target proteins. Key targets for microtubule investigation are γ-actin-containing filaments within the cell cortex. This provides a possible mechanism to couple the actin-based remodeling of the cortex with microtubule dynamics. However, the precise functional regulation of this connection is still not yet clearly understood.

9.6 Effect of the microtubule–actin interaction on the mechanical properties of cells

At the periphery of epithelial cells act two opposing cytoskeletal forces that are generated by dynamic systems, the growing end of microtubules pushing against the cell-membrane border and the actin cortex contracting the connected cell membrane. How can structural and dynamical alterations of the actin cortex impact the microtubule dynamics? Drugs target actin polymerization and cell contraction to diminish cell division and invasiveness, whereas their function on microtubule dynamics remains elusive. Human MCF-7 breast cancer cells coexpressing GFP-tagged microtubule EB1 and cytoplasmic fluorescent protein mCherry are used to identify the trajectories of growing microtubule ends in close proximity to the membrane (Ory *et al* 2017). Based on EB1 tracks and the membrane boundary, the speed, distance from the membrane and straightness of microtubule growth can be determined. Latrunculin-A-dependent actin depolymerization decreases EB1 growth speed and causes the trajectories' extension far beyond the cytoplasmic boundary. In addition, the direct myosin II inhibitor blebbistatin decreases the EB1 speed and causes less straight EB1 trajectories. The inhibition of the upstream signaling molecules of myosin II contractility through the ROCK inhibitor Y-27632

changed the EB1 dynamics in a different manner compared to blebbistatin. Indeed, these findings show that decreased actin cortex integrity causes distinct alterations in microtubule dynamics. Tumor stem cell characteristics are also enhanced by drugs which decrease actin contractility or stabilize microtubules, and it needs to be determined how cytoskeletal drugs change the interactions between these two filament types in cancer cells.

9.6.1 Interaction between microtubules and actin affects barrier function

Rapid alterations in microtubule dynamics cause regional activity of the small GTPases RhoA and Rac1, which are involved in regulation of the actin cytoskeleton and endothelial cell permeability. The role of EB1 during increased and decreased endothelial cell permeability is evoked by thrombin or the RhoA GTPase and HGF or Rac1 GTPase, respectively. The addition of thrombin to human lung endothelial cells impaired the peripheral microtubule growth. In contrast, the addition of HGF to endothelial cells induced peripheral microtubule growth and the protrusion of EB1-positive microtubule plus ends to the endothelial cell peripheral membrane region. The knockdown of EB1 using siRNAs has no effect on the partial microtubule depolymerization, activation of Rho signal transduction pathways and permeability response on thrombin stimulation, whereas it suppressed the HGF-based endothelial barrier strengthening. Moreover, the EB1 knockdown reduces the HGF-driven activation of Rac1 and Rac1 cytoskeletal effectors such as cortactin and PAK1, abolishes the HGF-induced assembly of cortical cytoskeleton regulatory complex (WAVE-p21Arc-IQGAP1) and inhibited the HGF-based strengthening of peripheral actin cytoskeleton and VE-cadherin-positive adherence junctions (figure 9.16). In summary, it has been shown that EB1 acts in coordination of microtubule-facilitated

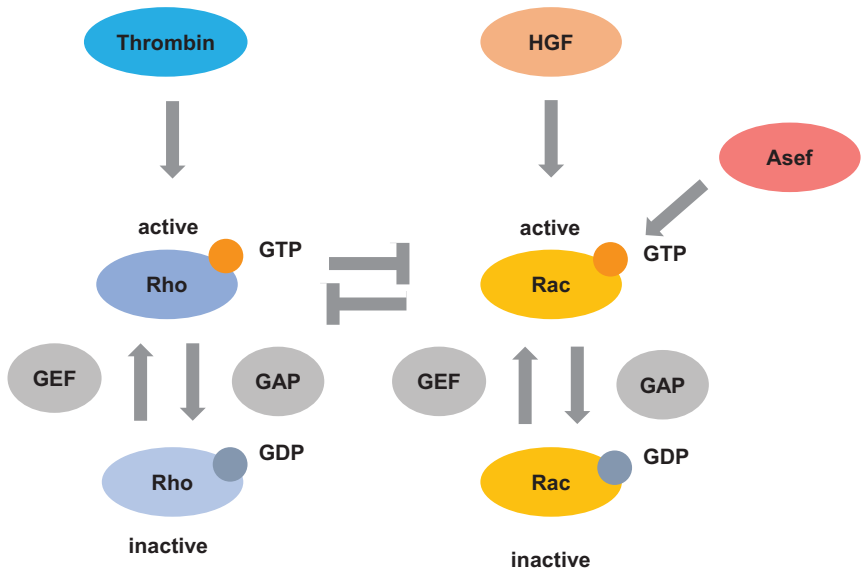

Figure 9.16. Interaction of Rho and Rac and their activation.

barrier enhancement upon HGF-stimulation, however, it is not involved in the acute increase of endothelial permeability evoked by a barrier-disruptive agonist. Increased peripheral EB1 distribution seems to be a crucial factor of the Rac1-driven pathway and the peripheral cytoskeletal remodeling, which is required for agonist-driven enhancement of the endothelial cell barrier function.

The maintenance of the endothelial cell peripheral actin cytoskeleton and cell adhesions are critical for the vascular barrier. In turn, activation of barrier-enhancing mechanisms is crucial for the impairment of vascular leakiness in the lung or other organs caused by bacterial pathogens, excessive mechanical forces or cytokines during sepsis or trauma (Frank and Matthay 2003, Tremblay *et al* 2006, Maniatis *et al* 2008, Deng and Standiford 2011). The activation of small GTPase RhoA and its downstream target ROCK can be performed by several agonists, pathological mechanical forces, or inflammatory cytokines and subsequently evokes a dysfunction of vascular endothelial cell barrier (Beckers *et al* 2010, Spindler *et al* 2010). In contrast to the RhoA-driven mechanism of endothelial cell contraction and thereby enhanced permeability, the increase of endothelial cell barrier function by stimulation with prostacyclin, sphingosine 1-phosphate or HGF causes the activation of Rac1 and Rap1 GTPases, which lead to cortical actin polymerization, peripheral cytoskeletal remodeling, assembly of endothelial VE-cadherin-based adherence junctions and subsequently the strengthening of endothelial cell–cell junctions (Pannekoek *et al* 2009, Birukov *et al* 2013).

As microtubules are not directly regulating the physical maintenance of the endothelial cell barrier, it has been proposed that dynamic alterations in the polymerization of microtubules regulate the permeability of endothelial cells. The full or partial disassembly of microtubules by plant-derived alkaloids (Verin *et al* 2001) or inflammatory cytokines (Shivanna and Srinivas 2009) causes the release of microtubule-associated Rho-specific guanine nucleotide exchange factor GEF-H1 (Ren *et al* 1998, Krendel *et al* 2002), the activation of Rho signaling, ROCK-facilitated microfilament reorganization, contraction of the actomyosin cytoskeleton and endothelial cell permeability (Krendel *et al* 2002, Birukova *et al* 2004). Subsequently this mechanism reveals a signaling crosstalk between microtubules and the actin cytoskeleton, which includes the maintenance of the endothelial cell barrier. Barrier disruption inhibits microtubule growth and hence destabilizes microtubules by targeting the regulatory proteins providing the microtubule polymerization and stability. An example represents thrombin which leads to Rho-dependent phosphorylation of tau and dephosphorylation of stathmin, promoting the disassembly of peripheral microtubule network (Birukova *et al* 2004, Song *et al* 2000).

In contrast to endothelial cell barrier-disruptive mechanisms activated by microtubule depolymerization, a new mechanism of agonist-induced endothelial cell barrier enhancement has been revealed, which needs enhanced microtubule peripheral growth. Using HGF, which increases the basal endothelial cell barrier function through the activation of the Rac1 pathway (Birukova *et al* 2007, Singleton *et al* 2007), it has been revealed that HGF promoted peripheral microtubule growth and microtubule protrusion towards the endothelial cell peripheral submembrane region (Tian *et al* 2014a). HGF-based microtubule growth causes the relocalization of the

microtubule-associated Rac1-specific GEF Asef to the periphery of the cells and thereby induces the local stimulation of Rac1 signal transduction processes (Tian *et al* 2014c).

The EB1 binds to the growing plus ends of microtubules and provides microtubule polymerization. However, EB1 cannot track microtubule plus ends processively, as it is exchanged rapidly at the microtubule tips (Akhmanova and Steinmetz 2008). EB1, together with the end-binding protein CLASP2 establishes a local connection of microtubules to actin filaments through IQGAP1, which is known to promote cell motility (Watanabe *et al* 2009). However, the function of EB1 as a general transmitter of RhoA and Rac1-driven signaling in the vascular endothelium needs more investigation. The EB1 involvement in the regulation of agonist-induced endothelial cell permeability seems to be highly important (Tian *et al* 2017). In particular, the specific role of EB1 is the regulation of the microtubule extension and the activation of Rac-specific peripheral actin and the remodeling of cell junctions, all of which strengthen the endothelial barrier function. In contrast, EB1 is not associated in the acute permeability increase driven by barrier-disruptive agonists such as thrombin or TNFα, which cause destabilization and disassembly of peripheral microtubules (Birukova *et al* 2004a, 2006, Petrache *et al* 2003) stimulating a RhoA-facilitated dysfunction of the endothelial barrier. Indeed, these barrier-disruptive effects are not altered by the knockdown of EB1.

EB1-driven interactions with growing microtubules are required for the coordination of cell shape alterations and directed migration of epithelial cells in a 3D microenvironment (Gierke and Wittmann 2012). However, the precise mechanisms of EB1-driven regulation of cell motility and functional responses of the vascular endothelium, such as induced vascular endothelial permeability, require in depth analysis. Indeed, HGF causes pronounced peripheral microtubule growth in pulmonary endothelial cells. The growing microtubule tips reached the cell cortex and are then captured by the multifunctional scaffold protein IQGAP1 (Tian *et al* 2014b). These results indicate that EB1 is indeed necessary for peripheral targeting of signal transduction molecules, which cause the activation of cytoskeletal and the remodeling of cell junctions.

An inhibitory effect of EB1 knockdown has been observed after HGF-induced increased transendothelial electrical resistance that has been connected to the suppression of HGF-induced Rac1 activation as well as Rac1-dependent cytoskeletal responses (Tian *et al* 2017). Moreover, the morphological analysis of endothelial cells using immunofluorescence staining revealed that the knockdown of EB1 affected the HGF-based remodeling of the cortical actin, the peripheral accumulation of the cytoskeletal Rac effectors cortactin and p21Arc and reduced the enhancement of VE-cadherin positive adherence junctions. However, the EB1 knockdown has no detectable effect on the basal endothelial cell barrier function or the distribution of F-actin and VE-cadherin. Additionally, HGF-induced association of IQGAP1 with actin cytoskeletal partners such as WAVE and p21Arc is facilitated by EB1. In particular, the knockdown of EB1 reduces the accumulation of p21Arc, a member of Arp2/3 complex, at the periphery of HGF-treated endothelial cell and subsequently the assembly of entire p21Arc/WAVE/

IQGAP1 complex, which is consistent with effects of the known Rac1-mechanism of Arc/WAVE complex activation and the formation of branched F-actin network (Miki *et al* 1998, Ten Klooster *et al* 2006). This mechanism seems to be crucial for the enhancement of peripheral actin cytoskeleton and a strengthened endothelial cell barrier function, which is regulated by EB1.

In contrast to HGF-stimulation, EB1 plays no role in the acute phase of thrombin-induced endothelial cell permeability that is facilitated by RhoA. The knockdown of EB1 has no effect in the acute phase of endothelial cell barrier dysfunction without affecting the thrombin-induced activation of RhoA and its downstream signaling (Tian *et al* 2017). This can be explained on the one hand by the thrombin-based inhibition of microtubule growth and the partial depolymerization of the peripheral microtubule network, all of which is not regulated by EB1. On the other hand, microtubule dynamics regulate the Rho signal transduction by binding the RhoA-specific guanine nucleotide exchange factor GEF-H1 (Ren *et al* 1998, Krendel *et al* 2002, Chang *et al* 2008). Alterations in the amount of the microtubule-bound GEF-H1 are connected to the activation of Rho and hence regulate barrier properties of epithelial (Birkenfeld *et al* 2008, Kakiashvili *et al* 2009) and endothelial cell monolayers (Birukova *et al* 2006, 2010, Tian *et al* 2014a).

The presence of EB1 is increasingly required during endothelial cell monolayer recovery after thrombin-driven disruption of its barrier function, which is associated with the activation of Rac1/Cdc42-dependent mechanisms (Kouklis *et al* 2004, Birukova *et al* 2013, Tian *et al* 2015a). As expected, the investigation of endothelial cell barrier properties after thrombin addition has been shown to be time-delayed after knockdown of EB1, which may point to an involvement of EB1 in the re-activation of microtubule growth and elongation towards the cell cortex. Moreover, it seems to be that the microtubule elongation is similar to the microtubule remodeling caused by HGF-stimulation. In particular, the growing microtubule tips entering the cell cortex become bound to linker proteins, which may unload signaling molecules such as Rac-specific GEFs that facilitate Rac-dependent mechanisms of cytoskeletal remodeling and rescue the endothelial cell barrier function.

Finally, the differential role of EB1 in maintaining the permeability responses of pulmonary endothelial cells to barrier-enhancing and barrier-disruptive agonists has been demonstrated. The EB1 involvement in HGF-induced endothelial cell barrier enhancement seems to include EB1-driven capturing of microtubules to the cell cortex through the interaction of EB1, IQGAP1 and cortactin, which seems to be required for the distinctly localized activation of the Rac signaling, enhanced interaction of Rac-dependent actin-binding proteins, which are activators of the polymerization of cortical actin with IQGAP1, and the strengthening of endothelial barrier function. Moreover, this mechanism may be additionally play a role in the rescue of the endothelial cell barrier function after thrombin stimulation, which causes the endothelial barrier disruption. Hence, the pharmacological regulation of the microtubule dynamics during the rescue of the endothelial cell barrier function seems to be crucial for future strategies rapidly improving the vascular barrier function upon vascular leakage.

9.7 The impact of microtubule alterations on diseases such as cancer metastasis

Distinct microtubule alterations observed in human diseases are related to pathogenic cell migration *in vivo*. Deregulated gene expression and mutations causing microtubule defects have been identified in metastatic cancers (Kavallaris 2010). In particular, the overexpression of stathmin is found to be connected to poor prognosis in metastatic cancers (Belletti and Baldassarre 2011). The inactivation of tumor suppressors such as p53, p21Cip1 and p27Kip1, which is frequently detected in cancers, has been correlated with alterations in microtubule dynamics (Baldassarre *et al* 2005, Bouchet *et al* 2011a, Galmarini *et al* 2003). The expression or even function of other microtubule regulators such as survivin (Chen *et al* 2016, Rosa *et al* 2006), ATIP3 (Molina *et al* 2013, Velot *et al* 2015), the +TIPs EB1 (Liu *et al* 2009, Stypula-Cyrus *et al* 2014) and APC (Etienne-Manneville 2009) is changed in various cancers, but their precise role in 3D cell migration still remains elusive. In addition, mutations in genes encoding tubulin isoforms and different microtubule regulators including +TIPs seem to play a role in neurodevelopmental diseases such as disorders of neuronal migration (Breuss and Keays 2014, Chakraborti *et al* 2016, van de Willige *et al* 2016). It needs to be determined which of their functions represent general mechanisms in 3D cell motility and which are specific mechanisms of neurons.

Microtubule perturbation is not solely a cause of human disease, it may also be a target for a therapeutic strategy. Hence, MTAs are at the forefront of anti-metastatic therapies and their discovery is still an ongoing field of research (Dumontet and Jordan 2010). Although MTAs are mainly seen as mitosis-blocking agents, there is evidence that interphase cells such as migrating cancer cells represent their major target *in vivo* (Janssen *et al* 2013, Komlodi-Pasztor *et al* 2011, Mitchison 2012). Inhibition of cell migration seems to be highly interesting as a promising direction for the targeted therapy of metastatic cancer (Cheung and Ewald 2014, Palmer *et al* 2011) that aims not only at tumors as a target, but similarly also the invasion of endothelial cells inside primary tumors. However, the characterization of the molecular mechanisms regulating the microtubule function in 3D cell motility seems to be highly important.

Most cytoskeleton studies have investigated the functions of microtubules in cells cultured on stiff 2D substrates. Hence microtubules are regarded as an essential scaffold for polarized trafficking as well as signal transduction and therefore physiological tools are necessary for the precise analysis of microtubule functions such as the easily accessible soft 3D extracellular matrix cell cultures. The analysis of cell adhesion and actin regulators in 3D cell culture models provided considerable advances in the unraveling of cytoskeletal functions during 3D cell motility and its high plasticity *in vivo* models (Paul *et al* 2015, Petrie and Yamada 2016). The mechanobiology research combines matrix properties with cytoskeletal dynamics and investigates how the feedback regulation between them adapts the cell behavior precisely in 3D microenvironments (Charras and Sahai 2014). As a next step, the function of microtubules in these processes can be defined. In particular, the

characterization of specialized MAPs with multiple functions such as +TIPs and their minus-end-associated counterparts (−TIPs) enables us the refine microtubule functions in 3D cell migration in a precise manner (Akhmanova and Hoogenraad 2005).

Taken together, the usage of light-sheet microscopy for the investigation of microtubule plus-end dynamics in 3D (Chen *et al* 2014, Wu *et al* 2013, Yamashita *et al* 2015) in combination with the development of sophisticated 3D tissue models such as organoids (Shamir and Ewald 2014) and the production of microfabricated and microfluidic devices (Paul *et al* 2016), has greatly enhanced the quality of cytoskeletal studies in living cells migrating and invading 3D microenvironments. The following questions still need to be answered: how do microtubules interact with actin filaments to modify microtubule-related signal transduction, alter cell adhesion and vesicle trafficking, and sense mechanical properties within soft 3D matrices? *In vivo* models and 3D cell culture systems with precisely defined chemical and tunable biophysical properties combined with the high-resolution microscopy enable us to address the issues and widen our knowledge on the function of microtubules.

The microtubule cytoskeleton represents a broad network of filaments filling the cell's cytoplasm. Microtubules are known to be composed of α- and β-tubulin heterodimers, which assemble to protofilaments that laterally associate with hollow tubes (Nogales 2000). These highly dynamic structures are constantly lengthening and shortening throughout the interphase, mitotic phase and rest phase of the cell cycle, which involves the addition and removal of tubulin heterodimers at both microtubule ends. The integration of the tubulin heterodimers, such as their orientation in microtubules, provides a polar molecule that displays different structures and kinetics at each end of the microtubules (Nogales 2000). In particular, the dynamics of the tubulin heterodimer addition and release are slower at the microtubule minus end, which terminates in α-tubulin proteins, compared with the microtubule plus end, which terminates with β-tubulin proteins that are still in the GTP-bound state (Bowne-Anderson *et al* 2015, Downing and Nogales 1998). Hence, the microtubules are nucleated at their minus ends, whereas their plus ends undergo more dynamic lengthening and shortening processes (Nogales 2000). The rapid transition of a microtubule from a period of lengthening to one of shortening is termed a catastrophe and the restoration of microtubule lengthening after a period of shortening is termed rescue. Each individual microtubule will dynamically switch between these phases. Moreover, interactions between microtubules and a large network of proteins facilitate the switch between these phases and hence provide their stability (Janke 2014, Valiron *et al* 2001, Akhmanova and Steinmetz 2015).

During interphase, most microtubules are nucleated at the centrosome and are located radiantly towards the cell's periphery (figure 9.17) (Downing and Nogales 1998). A major function of interphase microtubules is the maintenance of cell's shape and the trafficking of proteins and organelles (Janke 2014). Motor proteins employ the microtubule cytoskeleton for the translocating cell components such as proteins, receptors or vesicles on microtubule tracks and co-ordinate this process by protein–protein interactions with other adaptor proteins (Janke 2014).

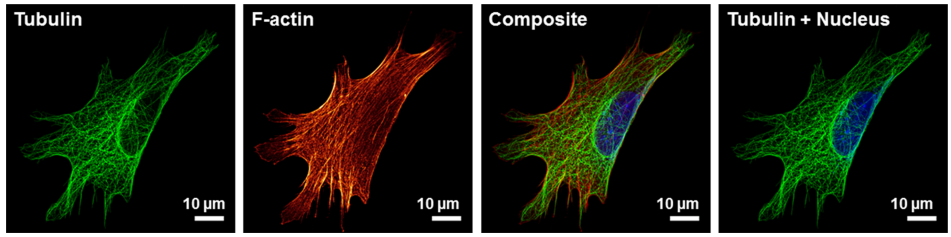

Figure 9.17. Fluorescent images of a fibroblast stained with anti β-tubulin antibody (green), with Alexa Fluor 546 Phalloidin (red), a composite image of anti β-tubulin, Alexa Fluor 546 Phalloidin and Hoechst 33342 stain (nucleus, blue) and a composite image of anti β-tubulin and Hoechst 33342 stain (nucleus).

During the mitotic phase of the cell cycle, the microtubule network undergoes remodeling to form the mitotic spindle. The dynamic nature of the microtubule filaments provides the precise segregation of the chromosomes, whereas failure in the correct attachment or the segregation of the chromosomes causes cell cycle arrest at the mitotic checkpoint and finally may cause apoptosis (Zich and Hardwick 2010). The tubulin-binding agent (TBA) class of chemotherapeutics including taxanes, vinca alkaloids and epothilones directly affect β-tubulins to impair the dynamics of spindle microtubules, which in turn abolishes the chromosomal segregation and subsequently leads to cell cycle arrest and cell death (Jordan and Wilson 2004). Particular classes of tubulin-binding agents can bind to both α- and β-tubulin proteins at the interface between the dimers (Gigant *et al* 2005).

In humans, microtubules consist of mixed combinations of eight α-tubulin isotypes and seven β-tubulin isotypes (Luduena 2013). The distinct tubulin isotypes are encoded by different genes on distinct chromosomes, are tissue specific and undergo developmental distributions (Luduena 1993, Verdier-Pinard *et al* 2009). The tubulin isotypes share high structural homology, whereas they are clearly distinguishable from each other by specific divergent sequences at their carboxy-terminal (C-terminal) tail (Sullivan and Cleveland 1986). The N-terminal and intermediate protein domains are highly conserved and form a rigid globular tubulin body that builds up to assemble protofilaments, while the C-terminal tail of the tubulins represents a highly disordered peptide of 18–24 amino acids in length. Post-translational modifications occur frequently at these C-terminal tails and facilitate interactions with a large number of proteins, which provides the unique functionality to each tubulin isotype (Janke 2014). In a coordinated manner, a cohort of chaperones and post-chaperonin cofactors assists the folding of nascent peptides into the highly rigid globular fold created by the tubulin body while they additionally facilitate the formation of tubulin heterodimers (Lewis *et al* 1997, Serna *et al* 2015). Microtubules should not accommodate deviations in the tubulin fold, as the correct tubulin fold is crucial in regulating microtubule structure and dynamics. The associations of tubulins with their post-chaperonin cofactors additionally support the dynamic exchange of tubulin heterodimers between the polymerized and soluble tubulin reservoirs, which enables this process to function far from equilibrium (Lewis *et al* 1997). However, the tubulin globular fold is highly

conserved and how the folding of tubulin may impact the regulation of the tubulin isotype composition is not yet clearly understood.

In a wide range of cancer types, the tubulin isotype distribution of cancer cells is perturbed compared to the surrounding healthy tissue microenvironment. The aberrant expression includes increased or decreased expression of one or more tubulin isotypes or the expression of tubulin isotypes that are not usually found in the healthy tissue (Vilmar *et al* 2012b). However, the aberrant expression of different tubulin isotypes is crucial for the patient outcome and the analysis of the treatment response. The knowledge of the role of tubulin isotypes can be utilized for the improvement in chemotherapy drug resistance, disease aggressiveness and tumor biology. The focus of many studies is on the importance of β-tubulin proteins, which is based on the specificity of tubulin-binding agents for β-tubulin proteins and tools for the determination of the effect of α-tubulin isotypes. Similar to β-tubulin isotypes, α-tubulin isotypes are found to be associated with cancer patient outcome, which makes it essential to understand the overall function of tubulin isotypes in tumorigenesis.

9.7.1 Role of tubulin isotypes in cancer

The β-tubulin isotype composition clearly affects the microtubule dynamics and the sensitivity of microtubules to TBAs. However, the drug resistance observed in several cancer types cannot be explained by the individual β-tubulin isotype such as the βIII-tubulin that provides resistance to various non-tubulin targeting agents such as DNA-damaging agents, antimetabolites and inhibitors of the topoisomerase (Narvi *et al* 2013, McCarroll *et al* 2010, 2015, Gan *et al* 2007). Moreover, βIII-tubulin expression determines the tumorigenic and metastatic potential of NSCLC and pancreatic cells *in vivo* (Ferrandina *et al* 2006, McCarroll *et al* 2010, 2015, Lee *et al* 2007), which supports the importance of this β-tubulin isotype in cancer. The role of βIII-tubulin in the progression of primary tumors seems to be cancer-type-specific, as suppression of βIII-tubulin cannot alter the primary tumor growth in breast cancer models (Kanojia *et al* 2015). Tumor progression facilitating intracellular signal transduction processes such as oncogenic stress response and cell death signaling also provides drug resistance, which involves the altered expression of also β-tubulin isotypes that in turn affects drug resistance and thereby increases the aggressiveness of the cancer type. Hence β-tubulin isotypes are associated with signaling processes facilitating the malignant progression of the primary tumor.

9.7.2 The role of tubulin in oncogenetic signal transduction

Although tubulin isotypes are aberrantly expressed in various cancer types, they cannot be classified as oncogenes, which are defined as a genetically altered genes driving the malignant transformation of normal cells. However, βIII-tubulin may function as downstream target of several oncogenic pathways, as its expression is elevated by mutant Ki-ras2 Kirsten rat sarcoma viral oncogene homolog KRAS expression and by the stimulation of the Ki-ras2 Kirsten rat sarcoma viral

oncogene homolog EGFR stimulation through a post-translational mechanism (Levallet *et al* 2012). The correlation between increased βIII-tubulin expression and KRAS mutant tumors in NSCLC (Levallet *et al* 2012) indicates that βIII-tubulin plays a role in Ras-driven transformation, however it needs to be refined in *in vivo* experiments. βIII-tubulin expression has been connected to the phosphatase and tensin homolog (PTEN) signal transduction pathway. In prostate cancer, PTEN deletions are associated with elevated βIII-tubulin expression, indicating that altered βIII-tubulin expression seems to be evoked by PTEN-facilitated reprogramming events during the initiation of the primary tumor formation (Tsourlakis *et al* 2014). Moreover, βIII-tubulin expression is correlated positively with ERG expression and the rearrangement of TMPRSS2:ERG in prostate cancer (Tsourlakis *et al* 2014).

In colon cancer, the inhibition of the protein kinase B (AKT) or the extracellular-signal-regulated kinase (ERK) signal transduction pathways reduces the expression of βIII-tubulin indicating that βIII-tubulin is regulated by both AKT and ERK pathways (Xiao *et al* 2016). The suppression of βIII-tubulin expression in NSCLC cells increases PTEN expression, which acts upstream of AKT to impair its phosphorylation and hence activation (McCarrol *et al* 2015a). Moreover, it is functionally associated with enhanced sensitivity to anoikis (a special type of programmed cell death) and reduced tumor growth in mice (McCarrol *et al* 2015a). The PTEN/phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is commonly dysregulated in cancer cells and is strongly associated with tumorigenesis and metastasis through the increased cancer cell survival and proliferation during the malignant cancer progression and even in response to chemotherapy agents. Finally, tubulin isotypes seem to act as mediators of oncogenic signaling promote the progression of cancer.

What role does tubulin play in the malignant progression of cancer (metastasis)?

There is some evidence that tubulin proteins play a role in the metastatic progression of cancer. Several tubulin isotypes have become potential prognostic markers whose expression levels in primary tumors are correlated positively with aggressive disease progression such as metastasis, although a main focus is on βIII-tubulin expression. In particular, high βIII-tubulin protein expression has been found to be strongly positively correlated with aggressive clinical behavior of cancer cells and poor patient outcome in various cancer types such as breast, ovarian, gastric, glioblastoma, prostate, colorectal, pancreatic and NSCLC (Reiman *et al* 2012, He *et al* 2014, McCarroll *et al* 2015, Kanojia *et al* 2015, Katsetos *et al* 2001, Katsetos *et al* 2000, 2002, 2007, Vilmar *et al* 2011, Egevad *et al* 2010, Seve and Dumontet 2008, Zhao *et al* 2016). In particular, the expression of βIII-tubulin is associated with high-grade malignancy in gliomas and gastric cancers (He *et al* 2014, Katsetos *et al* 2001, 2002, 2007, Shahabi *et al* 2013, Orsted *et al* 2014). Similarly, in NSCLC, the expression of βIII-tubulin can be correlated positively with higher histological grades, poorly differentiated cancer tissue and the malignant progression of cancer disease such as metastasis (Reiman *et al* 2012, Vilmar *et al* 2011, Katsetos *et al* 2000, Seve and Dumontet 2008).

In line with this, high βIII-tubulin expression has been detected at the invasive primary tumor edge of atypical carcinoid lung cancers (Katsetos *et al* 2000), in lymph node metastases of primary adenocarcinoma NSCLC (Katsetos *et al* 2003) and in metastatic lung cancers that are derived from colon, prostate and ovarian primary tumors, but solely when the metastases are derived from primary breast tumors (Katsetos *et al* 2000). In colorectal cancer, the expression of βIII-tubulin expression similarly correlates with high-grade malignancy, the differentiation of the primary tumor and lymphatic metastasis, which supposes a role of βIII-tubulin in the differentiation of the prostate tumor and subsequently metastasis (Zhao *et al* 2016). The overexpression of βIII-tubulin has been found in breast cancer derived brain metastases and hence its expression is significantly correlated positively with distant metastases (Kanojia *et al* 2015). Moreover, in prostate cancer, βIII-tubulin expression has been seen to be associated with the TMPRSS2:ERG gene fusion dependent rearrangement, the expression of ERG and deletions in PTEN, all three represent key oncogenetic features in metastatic prostate cancer (Tsourlakis *et al* 2014). In melanoma, βIIb-tubulin mRNA levels are significantly increased in melanoma multinucleated giant cells, which are mainly located in metastatic sites and correlate positively with poor prognosis, which indicates the importance of this tubulin isotype in the metastatic process of melanomas (Mi *et al* 2016). In colorectal cancer, the elevated expression of βIVb-tubulin has been seen in the sentinel lymph node micrometastasis (He *et al* 2010). Similarly, in lung cancer cells, βIVa-tubulin expression is even enhanced under non-adherent conditions, which resemble the conditions of circulating tumor cells in the vasculature (Atjanasuppat *et al* 2015). The process of metastasis needs the completion of a multistep process that can be subdivided into a linear propagation of steps such as the dissemination of cancer cells into distant, targeted organ sites and their subsequent adaptation to these foreign tissue microenvironments.

Altered tubulin isotype concentrations can enhance the metastatic strength of cancer cells by affecting their migration and invasion capacities. Hence, as the activation of the EMT is crucial for the regulation invasion and metastasis (Thiery *et al* 2009), the reprogramming of EMT is affected by the tubulin isotype concentrations. Moreover, the expression of βIII-tubulin correlates with Snail expression levels and hence regulates the effect of Snail overexpression during EMT transition in colon cancer cells (Sobierajska *et al* 2016). In line with this, the RNA interference-based knockdown of βIII-tubulin expression decreases the movement of colon cancer cells (Sobierajska *et al* 2016). Similarly, in breast cancer, a positive feedback regulation of ZEB1 and β-tubulin isotype classes I, III, and IVB have been revealed (Lobert *et al* 2013). The knockdown of ZEB1 in human breast cancer cells decreases β-tubulin isotype classes I, III, and IVB mRNAs, whereas an increase of ZEB1 is correlated with increases in these isotype classes. Hence βIII-tubulin can serve as a biomarker for cell survival, which is facilitated by ZEB1-induced EMT leading to the aggressive phenotype in breast cancer.

A functional linkage between βIII-tubulin and metastasis has been demonstrated in lung and pancreatic cancer mouse models (McCarroll *et al* 2010,

Feldmann *et al* 2011, McCarrol *et al* 2015b). Knockdown of βIII-tubulin reduces the anchorage-independent growth, which represents a major phenotype of the metastatic potential, in NSCLC cells (McCarroll *et al* 2010). The βIII-tubulin suppression increases the adhesion-associated tumor suppressor Maspin, which impairs the outgrowth of tumor spheroids, cell migration and increases the sensitivity of NSCLC cells to anoikis (McCarrol *et al* 2015b). Silencing the βIII-tubulin expression decreases the pancreatic cancer growth, the tumorigenic capacity and cancer metastasis (Feldmann *et al* 2011). Similarly, a role for βIII-tubulin in conferring brain metastatic potential to breast cancer cells has been detected, as several key signaling molecules involved in cell adhesion and metastasis are altered (Kanojia *et al* 2015). The suppression of βIII-tubulin expression regulates the expression of L1CAM and β3-integrin to decrease the extracellular matrix adhesion and the signal transduction through the β3-integrin/FAK/Src pathway *in vitro*. In addition, this behavior is correlated with decreased metastatic capacity *in vivo* and mirrored in the improved survival in a brain metastasis model (Kanojia *et al* 2015). In summary, these findings suggest a role for tubulin isotypes, in particular βIII-tubulin, as promotors of metastasis and may be hence be suitable predictive markers for neoplastic progression of the disease and patient's outcome. However, the mechanisms by which βIII-tubulin promotes the metastatic cascade require further investigation.

The aberrant expression of β-tubulin isotypes in a wide-range of cancer types is indeed correlated positively with aggressive and drug resistant disease that finally leads to poor clinical outcomes. As the distinct isotype composition affects the interaction of tubulin-binding agents with their targets and the microtubule dynamics, it can provide an explanation for the occurrence of drug resistance to these classes of common chemotherapeutics.

Through interactions with oncogenic signaling pathways and cell survival programs that facilitate adaptation to the strong tumor microenvironment, the altered tubulin isotype composition in various cancer types seems to contribute to drug resistance and more aggressive disease. The knowledge of the complex interplay between all factors facilitating the spatiotemporal localization and function of tubulin isotypes may significantly reveal the underlying mechanisms by which the tubulin isotype composition impacts the tumor biology and impairs the treatment resistance in distinct cancer types.

The understanding of the tubulin code in cancer may contribute to the understanding of their individual isotype-specific impact on clinical outcome of the disease. In particular, the tubulin code may vary between a cancer type and between various cancer types. The individual or collection expression of tubulin types during cancer needs to be figured out and their difference to healthy control groups. A distinct manipulation of an isotype can be performed by using gene editing technologies, in which the original endogenous regulatory framework is still present. The knowledge of the entire interaction of the microtubule systems with the intracellular network will pronouncedly contribute to the understanding of fundamental cellular processes such as cell invasion and thereby help to design and develop targeted therapeutic strategies to altered tubulin isotype expression in certain cancer types.

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Chapter 10

Nuclear deformability during migration and matrix invasion

Summary

The cell nucleus represents a key hallmark of eukaryotic evolution, where the transcription of genes is performed, the replication of the genome and gene repair mechanisms take place. In addition to these complex molecular processes, the nucleus fulfills other tasks, such as providing a physical barrier towards deformation and serving physical tasks that require distinct mechanical properties. In particular, the nuclear mechanotransduction of extracellular externally applied forces and the sensing of the extracellular stiffness of the surrounding microenvironment is regulated through the physical coupling of the extracellular microenvironment, the cell's cytoskeleton and the nucleoskeleton consisting of a matrix scaffold assembled by lamins and chromatin.

The intranuclear mechanosensor elements are able to convert the applied tension into biochemical signals activating downstream signal transduction processes. Additionally, mechanoregulatory networks keep a contractile cell state in a stable configuration, in which the cells are able to provide feedback towards the extracellular matrix, cell–matrix adhesions and cytoskeletal components. However, mechanistic insights into the force-sensing processes in the nucleus are revealed and are still under strong investigation, as the nucleus decides over the cell fate.

Besides the mechanosensing function of the nucleus, it is involved in the process of cell migration, which plays a key role in physiological processes such as embryonic development and in pathological processes such as the malignant progression of cancer, metastasis formation. Moreover, the dimensionality of the migration process has a pronounced impact on the migration of cells and the assays performed in 2D microenvironments need to be refined in the more natural 3D situation. A key difference has emerged between 2D and 3D migration assays, as the

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nucleus plays a key role during 3D migration. An example is the cell movement through narrow confinements, which requires the deformability of the entire cell body and even the entire nucleus in order to squeeze through the restrictions and move through the matrix scaffold. Moreover, the deformability of the nucleus, which is of the largest cell component with a relative high stiffness, can limit the migration speed and rate.

Hence a focus is on the role of the nuclear mechanical properties in the regulation of cell migration and invasion in 3D microenvironments. In particular, the focus is on the parameters and factors that facilitate the nuclear deformability and on the mechanisms through which cells transmit cytoskeletal forces towards the nucleus to provide nuclear rotation or translocation. The 'physical barrier' represented by the nucleus affects the cytoplasmic dynamics required for cell migration and signal transduction processes, and alterations in the nuclear structure due to mechanical forces, such as mechanical stress applied towards the nucleus during the migration through 3D confinements, may in turn challenge cellular functions.

Finally, the nucleus is a crucial component regulating the 3D cell migration of healthy normal and cancerous cells within living tissues and the unraveling of the underlying mechanisms seems to be promising for impairing cancer cell migration and subsequently metastasis.

10.1 The physical role of the nucleus in cell migration

Cell migration and invasion is required for various key functions of many eukaryotic cells, such as development, immune response to acute or chronic inflammation, wound healing during tissue repair and cancer metastasis. The nucleus emerges as an important factor for the migration mode and the efficiency of cellular motility (Fruleux and Hawkins 2016). The importance of the nucleus has long been neglected, as keratinocyte cell fragments have even been shown to crawl without a nucleus (Verkhovsky *et al* 1999). However, the nucleus plays a key role in cell motility through 3D microenvironments (figure 10.1) (Khatau *et al* 2012). Indeed, the nucleus is coupled to the cell's cytoskeleton, which is already known to play a key role in cell migration. The cytoskeletal components are connected to the external microenvironment through focal adhesions. Thus, the nucleus is directly connected to the extracellular matrix microenvironment through the cytoskeleton, which forms the basis for the mechanosensitivity and the force transduction process of cells by exploring their external microenvironments during cellular migration. Moreover, the nucleus is not only a passive object of the cell to be transported, but it is rather directly involved in the process of cell migration. The position of the nucleus is critical for the polarization of the cell and subsequently directs the migration. In particular, the nucleus is more rigid and solid-like than the cell's cytoskeleton, membrane and other organelles and subsequently less deformable. This resistance of the nucleus to deformation impairs the squeezing of the cells through narrow confinements. The nuclear role during cell migration and invasion is of special interest in metastatic cancer cells, in which the nuclei possess abnormal shapes and altered stiffnesses. These alterations of the nuclei lead to distinct nuclear positioning

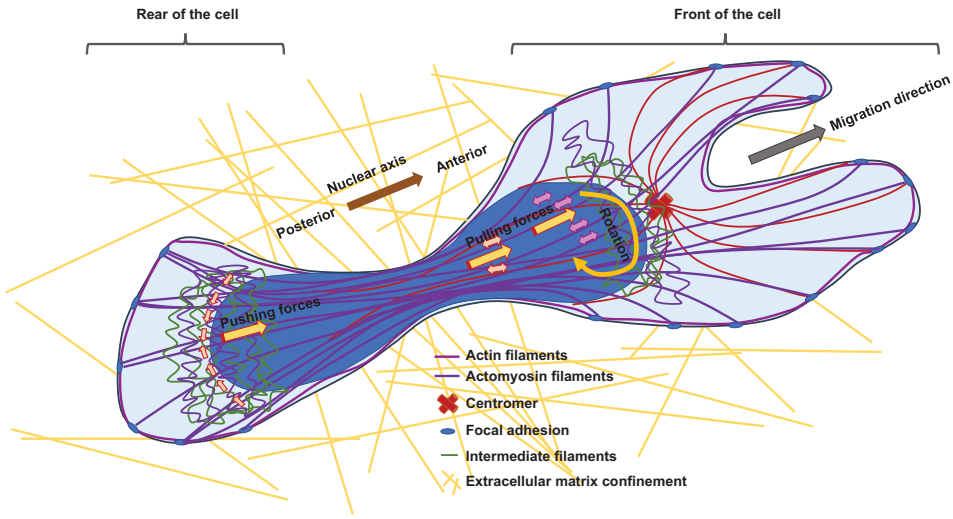

Figure 10.1. Cytoskeletal organization and dynamics during migration in confined 3D matrices. The nucleus plays a crucial role in the migration of cancer cells through narrow constrictions. When a cell squeezes through narrow pores, the nucleus separates the cell's leading edge and the rear end. The cytoskeletal network translocates the nucleus through confinements by first pushing through actomyosin contraction at the posterior of the nucleus, second, pulling through actomyosin contraction regulated by intermediate filaments, third, pulling through microtubule-associated motor proteins, such as dynein and kinesin, and fourth, nuclear rotation through microtubule-associated motor proteins.

in cell polarization, strengthened nucleus–cytoskeleton connection dependent migration and nuclear deformation during cell migration in physical constraints.

Mechanical stimuli such as alignment of extracellular fibers and stiffness are crucial for the overall cellular behavior, as they affect the architecture and organization of the cytoskeleton and the cellular contractility, which regulates biological processes such as the differentiation of stem cells, fibrosis and malignant cancer progression (Engler *et al* 2006, McBeath *et al* 2004, Baker *et al* 2015, Przybyla *et al* 2016, Duscher *et al* 2014). Active mechanical inputs such as the stretching of the cell substrate and fluid shear stresses on the cells cause similar effects on cellular behavior and pathogenesis (Cui *et al* 2015, Kurpinski *et al* 2006, Johnson *et al* 2011). The knowledge of how cells can sense the biophysical cues of their microenvironment and how they translate them into distinct biological signals is required for greater advances in the field of tumorigenesis and for the development of new clinical therapies (Szczesny and Mauck 2017).

The field of mechanobiology has enlightened the mechanotransduction mechanisms regulating these effects. Most of the studies focused on the mechanosensing at focal adhesions and their downstream signal transduction pathways (Schwartz 2010, Janmey *et al* 2013). In particular, the focal adhesions are clustered underneath cell membrane expressed integrins and other proteins that interface with the extracellular matrix and sense mechanical properties and trigger signals from the microenvironment (Zaidel-Bar *et al* 2007, Kanchanawong *et al* 2010). The force exertion at focal adhesions is necessary for focal adhesion growth, maturation and

recruitment of other 'late' focal adhesion proteins, which is facilitated by increased protein interactions that are triggered by physical unfolding and thereby the exposure of cryptic binding sites or tyrosine phosphorylation sites (Geiger *et al* 2009, Yan *et al* 2015, Harburger and Calderwood 2009). Several actin-binding partners, such as filamin, α-actinin and 14-3-3 proteins, facilitate the organization of the actin cytoskeleton and the mechanotransduction at the focal adhesions and cell–cell junctions, which affects the entire cytoplasm (Zhou *et al* 2010, Craig *et al* 2007, Sluchanko and Gusev 2010, Schlegelmilch *et al* 2011). The downstream signaling through focal adhesion maturation utilizes various pathways and is crucial for survival, proliferation, differentiation and migration of cells (Jaalouk and Lammerding 2009). Mechanical cues are able to provide the opening of stretch-activated ion channels within the cell membrane, which then changes the electrochemical potential of the cell's leading edge due to oscillations in ion concentrations, which can occur on short length-scales (locally) and on large length-scales (throughout the cytoplasm) (Martinac 2004). Ion channels such as TRAAK and TREK1 are activated by stretching the lipid bilayer and in the case of the ion channel TRPV4 through tension within the actin cytoskeleton (Anishkin *et al* 2014, Matthews *et al* 2010, Hayakawa *et al* 2008). The precise activation of the channels requires calcium ions diffusing through the mechanosensitive calcium channels, such as TRPV4 and Piezo1/2 act, which then are secondary messengers to stimulate the initiation of several signaling processes regulating cellular response to mechanical loading (Clapham 2007, O'Conor *et al* 2014, Lee *et al* 2014, Pathak *et al* 2014). In addition, enhanced calcium concentrations enable the cell to sensitize further mechanical stimuli through an increase in cellular contractility (Lierop *et al* 2002, Munevar *et al* 2004).

The mechanotransduction processes are performed at the nuclear envelope and also within the nucleoplasm (figure 10.2). In more detail, forces applied at focal adhesions are transmitted to the cytoskeleton and subsequently into the nucleus mainly through actin stress fibers and intermediate filaments (Khatau *et al* 2009, Versaevel *et al* 2012, Neelam *et al* 2015). The entire nucleus is deformed by extracellular strains and loads and in response redistributes the intranuclear structures such as the nucleoli and Cajal bodies (Maniotis *et al* 1997, Guilak 1995, Nathan *et al* 2011, Poh *et al* 2012, Booth-Gauthier *et al* 2012). Moreover, the dramatic alterations within the nuclear components cause alterations in gene expression, although the cell's spreading area is not required to be altered (Thomas *et al* 2002, Heo *et al* 2011). In line with this, isolated nuclei can remodel themselves and become stiffer due to mechanical stimulation, which indicates that the nucleus itself represents a mechanoresponsive organelle acting independent of the cytoplasm (Guilluy *et al* 2014). Whether the nuclear mechanotransduction causes changes in gene expression and cellular behavior due to biophysical stimulations, is not yet fully resolved and requires further investigations.

However, several possible purely nuclear mechanotransduction-based mechanisms have been hypothesized (Wang *et al* 2009). In particular, the forces exerted on the cell's nucleus are transmitted to the interior towards chromatin, which is then positioned at the nuclear envelope. The retranslocation of the chromatin leads to

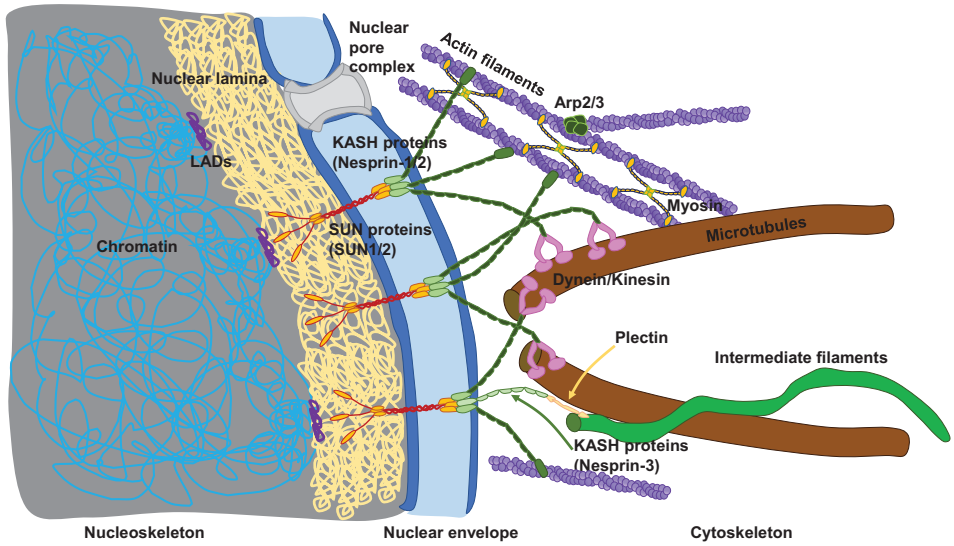

Figure 10.2. Schematic drawing of the physical connections between the nucleus and the cytoskeleton. At the nuclear periphery, chromatin interacts with lamins at lamina-associated domains (LADs). SUN protein domains (SUN1 and SUN2) are basically connected to the nuclear lamina and other components of the nuclear interior by their C-terminal end. The N-terminal luminal long end and SUN proteins of SUN1/2 form trimers that associate with KASH-domain proteins are located in the outer nuclear membrane (nesprin-1/-2/-3, cell-type specific nesprin-4 and KASH5) and build the LINC complex. The strong interaction between SUN-domain trimers and the KASH domains couple the nuclear interior with the cytoskeleton, as nesprins associate directly with actomyosin bundles or indirectly with microtubules or intermediate filaments through kinesin, dynein or plectin. The mechanical force transmission through nucleo-cytoskeletal coupling induces mechano-transduction events such as the assembling of lamins to the LINC complex that alters chromatin organization and subsequently gene expression impacting cell migration and invasion.

alterations in the gene expression that produce chromatin condensation level changes, restructuring of the topological organization or specific positioning of distinct gene loci. In an alternative manner, the nuclear loading or deformation affects the conformation and binding affinities of distinct proteins of the nuclear periphery. These changes may induce a structural reorganization of the nucleus and thereby alter the sequestration of genes and their transcription factors evoking alterations in the gene expression. Common mechanotransduction mechanisms utilize proteins, which can adapt their conformation due to the mechanical tension that subsequently challenges the post-translational modification of proteins, protein–protein interaction or their subcellular localization (Belaadi *et al* 2016). Candidates of mechanosensors are cell membrane proteins such as ion channels that sense the exerted stress directed onto the cell membrane and their activity is governed by the membrane tension (Martinac 2004, Thorpe and Lee 2017). The mechanical tension challenges not only cell surface proteins, it can also facilitate molecular processes in the cell's nucleus such as gene expression or DNA damage (Engler *et al* 2006, Bissell *et al* 1982, Denais *et al* 2016, Mammoto and Mammoto 2012, Mendez and Janmey 2012). Indeed, it has been observed that externally

exerted and internally generated mechanical forces affect the gene expression patterns pronouncedly (Denais *et al* 2016). At least two main pathways for mechanical stress transduction towards the nucleus have been hypothesized. Firstly, mechanical tension can regulate the signaling into the nucleus through a cascade of biochemical pathways facilitating the translocation of cytoplasmic proteins into the nucleus. Secondly, the mechanical stress can be transduced into the nucleus, where the mechanical force promotes the activation of signal transduction pathways affecting gene expression (Wang *et al* 2009). Numerous molecular components have been shown to shuttle from the cytoplasm to the nucleus due to mechanical stress application (Dupont *et al* 2011, Hervy *et al* 2006, Somogyi and Rorth 2004). Among these shuttling proteins are proteins containing the Lin11-Is1-Mec3 (LIM) domain (Smith *et al* 2014), Yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ) (Smith *et al* 2014), which at first glance indicates that the first transduction procedure plays a major role (figure 10.3). In summary, the load-driven remodeling and stiffening of the nucleus in turn creates changes in the surrounding cytoskeletal structural architecture and tension, which then regulates mechanotransduction processes throughout the cytoplasm as well as at the cell membrane through altered assemblies of focal adhesions. The proposed phenomenon of nuclear mechanotransduction has been broadly discussed (Shivashankar 2011, Martins *et al* 2012, Fedorchak *et al* 2014, Osmanagic-Myers *et al* 2015, Uzer *et al* 2016, Navarro *et al* 2016, Graham and Burridge 2016, Belaadi *et al* 2016), which have even been evaluated in a critical manner. In particular they figured out which of these two proposed pathways is solely or in combination with the other pathways responsible for the behavior after the biophysical stimuli action. A couple of decades ago researchers discovered the

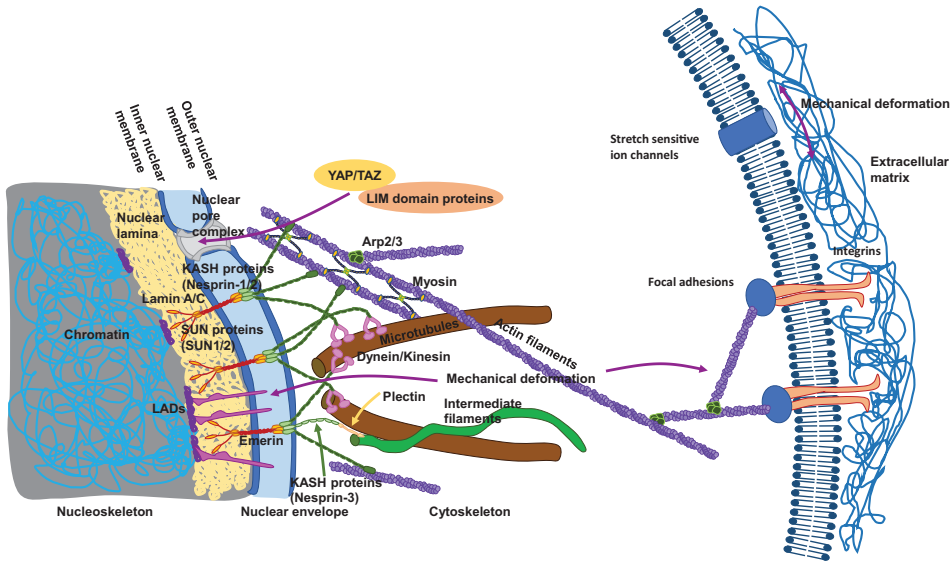

Figure 10.3. Force transmission and mechanotransduction from the extracellular matrix to the nucleus.

phenomenon that the mechanical stress can be directly transmitted from the cell's membrane surface to the nuclear compartment (Maniotis *et al* 1997). Specifically, using beads coated with integrin ligands and a simple glass micropipette, tensional forces have been exerted to the cell's membrane surface of adherent endothelial cells and as expected, the nuclear envelope displayed a distortion. Moreover, both actin stress fibers and intermediate filaments can be engaged in the process of stress transmission. These results demonstrated at first glance that cell surface integrins are directly combined with the interior of the cell's nucleus (Maniotis *et al* 1997).

What are the general cell motility mechanisms?

The initial experiments on cellular motility analyzed the crawling of cells on a glass substrate optimally suited for microscopy (Fletcher and Theriot 2004). For cell adhesion, the glass surface needs to be mostly coated with extracellular matrix proteins such as fibronectin, laminin or collagen. In these simple motility experiments, the cells polarize after cell adhesion and migrate spontaneously and in a random manner on the stiff surface. Cell processes, such as polarization and the directionality of movement, can be regulated through the implementation of chemotactant gradients to which the cells respond when they express the specific chemokine receptor on their cell surface. In addition to biochemical stimulation, cells can be directed by structural confinements such as narrow constrictions evoked by adhesion micropatterning (Maiuri *et al* 2012) or substrate stiffness gradients (durotaxis) (Lo *et al* 2000). During the migration of cells, processes such as cell adhesion (Schwarz and Gardel 2012, Humphries *et al* 2015), spreading (Wolfenson *et al* 2014), polarization (Woodham and Machesky 2014, Goehring and Grill 2013) and chemotaxis (Swaney *et al* 2010) contribute or impair cellular movement dependent on the specific cell type and microenvironment (Mierke *et al* 2011a, 2011b, Mierke 2011, 2013, 2017, Kunschmann *et al* 2017, Fischer *et al* 2017). Indeed, the networks of filamentous proteins assembling the cytoskeleton are crucial for the mechanical properties of the cells, and the shape and migration of cells. Moreover, microtubules and actin filaments have been mostly investigated, whereas the contribution of intermediate filaments and septins become the focus of mechanical properties of cells (Huber *et al* 2015; Seltmann *et al* 2013). However, the actin filaments and their interaction with myosin II are still highly important and hence the visualization of distinct proteins using fluorescence microscopy in live migrating cells and the disruption of different components through genetic impairment or drug treatments has been successful in revealing the individual roles of these proteins in cell motility.

The Brownian ratchet model of cells

The common picture of a cell migrating on a flat 2D substrate is a polarized cell extruding a wide, flat, thin protrusion at the front of the cell, which is termed the lamellipodium. Specifically, the lamellipodium consists of actin-rich protein, which is polymerized into actin filaments, assembling to fibers and bundles. The lamellipodium is an approximately 100 nm thick cellular membrane protrusion and is compared as a structural scaffold of a highly branched network formed by actin

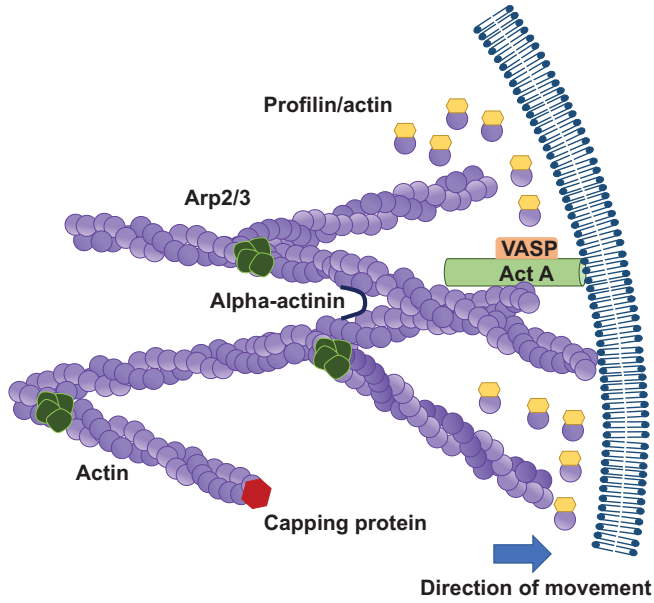

Figure 10.4. Brownian ratchet of cells model. The cell polymerizes branched actin filaments towards the membrane and hence pushes the membrane forward (actin-based forces).

filaments (Alberts *et al* 2014, Pollard and Borisy 2003). The front of the lamellipodium, termed the cell's leading edge, is hypothesized to be pushed forward by the generation of actin-based forces that are exerted by the polymerization of actin, and termed the 'Brownian ratchet' model (figure 10.4) (Peskin *et al* 1993, Mogilner 2006). In this motility system, based on protrusive cell migration, the hydrolysis of ATP provides an energy input to drive the entire system out of equilibrium, which is necessary for the binding of actin subunits to actin filaments. The actin filaments are fixed at their back by cellular adhesion to the substrate and polymerize in the direction of the cell's leading edge, thereby determining the direction of motion by pushing the cell membrane forward. At the same time, the cell adhesion to the substrate at the cell's rear end is abolished by rupturing the cell–matrix connection, which enables the cell's back to migrate forward to follow the movement of the cell's leading edge.

Although this mechanism of cell crawling can be observed without functional myosin II (Bray 2001), the contraction of the actomyosin cytoskeleton usually plays an important and even dominant key role in cellular motility. In particular, the contraction based on actomyosin is provided by clusters of myosin II molecular motors, which build mini myosin filaments that interact with the large actin filaments. The myosin molecules seem to walk along actin filaments towards the actin (plus) barbed ends. Within these actin bundles or networks, the clusters of myosin are able to connect to more than one actin filament simultaneously. However, when these two actin filaments point in different directions with their barbed ends, the myosin II motors trying to move in divergent directions will exert

stress on the filaments, which is for the actomyosin movement a contractile force exertion. Moreover, multiple models have provided an explanation of how this actomyosin contraction is generated at the microscopic length scale (Kruse and Juelicher 2000, Liverpool *et al* 2009, Hawkins and Liverpool 2014, Lenz *et al* 2012).

Can cells be treated as active gels?

At the cellular scale, continuum descriptions of the actomyosin mechanism have been successfully developed, such as the theory of active gels (Kruse *et al* 2004, 2005, 2006). These active gels are materials that work in the presence of persistent energy consumption such as ATP, which is hydrolyzed by myosin motor proteins. As cytoskeletal filaments are structurally polar, each filament defines a vector. When the filaments are aligned, the filamental structures provide the polarity of the material. On large time-scales and long length-scales, the properties of complex materials can be described as generalized hydrodynamics theory, which is based on common conservation laws and includes symmetry considerations, which have been used for the description of complex fluids such as polymers and liquid crystals. The hydrodynamics theory of active polar gels can be applied by performing several steps. The first step encompasses the identification of the relevant fields such as the conservation laws for conserved quantities and the identification of the generalized fluxes and forces in the system. In particular, the fluxes and forces define the rates of entropy production and the dissipation. The usage of the signature of forces and fluxes is with respect to time reversal.

The active matter is often out of equilibrium, which is caused by the energy input at the level of the constituent elements within the material. In particular, for the actomyosin system these elements are actin filaments and molecular motors and the input of energy input is provided by the biochemical energy from ATP binding and hydrolysis that is necessary for the function of myosin (Marchetti *et al* 2013, Ramaswamy 2010, Juelicher 2007). The unifying characteristic feature of active matter is that active matter is composed of self-driven units such as active particles that can use stored or ambient free energy for persistent movement (Schweitzer 2003). In particular, from the interaction of active particles with each other and their surrounding microenvironment emerges highly correlated collective motion and mechanical stress. The active particles are usually elongated and the direction of the self-propulsion is mainly manifested in their anisotropy and not preferentially by an external field. Orientational order is present in various active matter materials. A special feature defining active systems is the input of energy that causes the systems to drive out of equilibrium, which is mostly local at particle levels, but can be also at the cells' boundaries by the application of shear flow. In particular, each single active particle consumes and dissipates energy, which utilizes a cycle of internal alterations leading to cellular or particle motion. Moreover, active systems display a variety of intriguing nonequilibrium properties, such as emergent structures with collective behavior, which are specifically the behavior of individual particles, specific fluctuations, nonequilibrium order–disorder transitions, the formation of patterns on mesoscopic length-scales, altered mechanical properties, and propagations of waves and oscillations (Marchetti *et al* 2013).

Living systems such as cells belong to active matter and, indeed, possess extraordinary properties such as reproduction, adaptation, spontaneous motion and dynamical organization, such as force generation and force-sensing-dependent responses (mechanotransduction processes). A precise theoretical description of the general properties of living matter is still elusive as there exists a high number of parameters and largely diverse cells affecting signal transduction pathways and hence cellular behavior. However, basic universal principles, such as the conservation laws and symmetries, constrain the possible dynamical behaviors of cells and can be utilized to analyze the spontaneous dynamic organization of active matter and the motion of living systems in order to reveal the basic principles underlying these mechanisms. Indeed, for the investigation of the long-wavelength behavior of active membranes (Prost and Bruinsma 1996, Ramaswamy *et al* 2000, Manneville *et al* 2001, Ramaswamy and Rao 2001), the general theory of flocking (Toner and Tu 1995, 1998, Toner *et al* 2005) and the macroscopic mechanical properties of the cell's cytoskeleton as an active gel (Kruse *et al* 2005, Juelicher *et al* 2007) have been successfully described with these basic models and theories. Active systems can be described by the minimal agent-based models that emphasize the order and fluctuations rather than forces and mechanical properties. The phenomenon of flocking is described as a phase transition (Vicsek *et al* 1995, Gregoire and Chate 2004, Chate *et al* 2007, 2008). These models hypothesize active matter as point particles with fixed speed that move on an inert background. Due to a noisy local rule, the direction of motion is altered and hence particles need to align with their neighbors at each time point, which describes a well-defined transition of the active matter from a disordered to an ordered phase with reduced noise strength or elevated density. An example is the cell's cytoskeleton which is activated by motor proteins and can be described by compositions of semiflexible filaments on which motor protein bundles exert force dipoles (Mogilner and Oster 1996, Nedelec *et al* 1997, Pinot *et al* 2009, Head *et al* 2011).

What is the effect of the dimensionality of migration modes?
There are distinct differences between cells migrating on a flat stiff substrate and cells migrating *in vivo* or embedded in gels polymerized from extracellular matrix proteins such as collagen type I (Petrie and Yamada 2015). Several factors such as substrate rigidity, adhesion and confinement alter the migration type in cellular motility (Engler *et al* 2006, Lämmermann *et al* 2008, Lautenschlaeger *et al* 2009, Malboubi *et al* 2015). In confined microenvironments, cells can switch to a different motility type such as the amoeboid type of migration, in which actomyosin contraction at the rear of the cell facilitates a flow of actin cortex towards the leading edge of the cell. Due to the friction within the confined microenvironment the retrograde flow of actin pushes the cell forward (Poincloux *et al* 2011, Hawkins *et al* 2011, Callan-Jones and Voituriez 2013, Whitfield *et al* 2014, Maiuri *et al* 2015). In particular, highly contractile cells can form blebs on their cell surface, which originate from the cell membrane and are free of actin, since the membrane is not connected to the underlying actin cortex. When cells migrate in confinements such as matrices with pronounced contractions, the cells bleb to squeeze into the available

free space and, when the blebs retract due to the surrounding matrix fibers, a polarized and persistent motion can emerge (Charras and Paluch 2008).

Under conditions where the cells are migrating through small constrictions within extracellular matrix scaffolds or through narrow microchannels, the nucleus can get stuck and the movement is impaired. As the nucleus is less deformable than the cytoplasm of a cell, the nucleus represents the rate limiting step for cells to squeeze through constrictions such as channels or matrices that are smaller than the unconfined nucleus size (Friedl *et al* 2011, Davidson *et al* 2014, Lee *et al* 2012). Indeed, the motor protein myosin seems to be required for generating a force to push the nucleus through these small spaces (Thomas *et al* 2015). How is the nucleus of a living cell deformed? What is the connection between nuclear deformability and cellular motility? How is the nucleus connected to the cytoskeleton and subsequently to the extracellular matrix microenvironment? How do the mechanical properties of the nucleus contribute to nuclear function?

10.2 Mechanical properties of the nucleus

The structural architecture of the nucleus and its connection to cytoskeletal structures
The nuclear envelop consists of an inner and outer membrane that separates the nucleus physically from the surrounding cytoplasm (figure 10.5). On the internal and

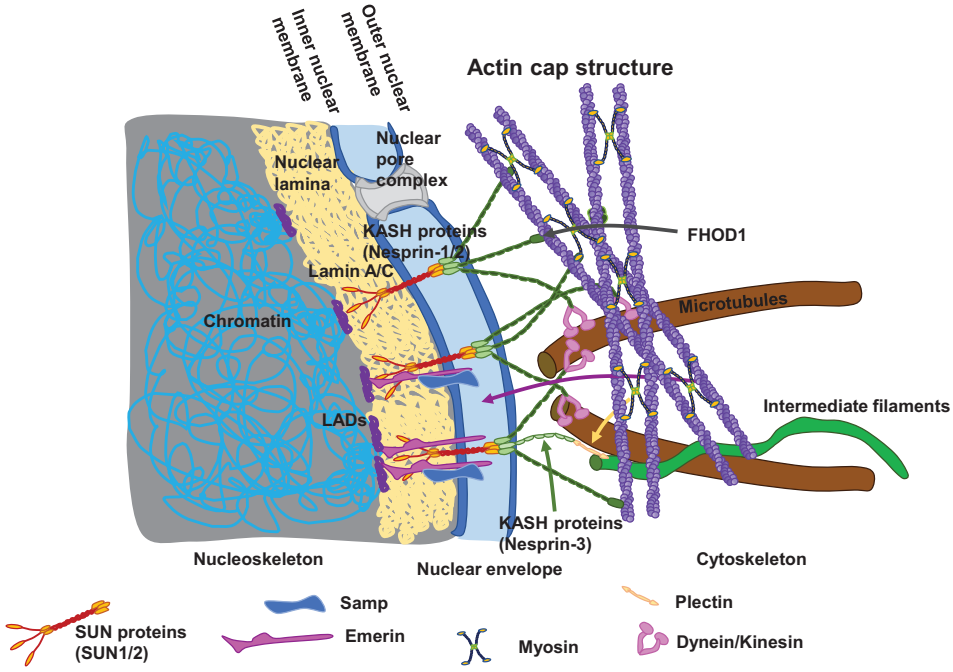

Figure 10.5. Schematic drawing of the structure of the nuclear envelope. Underneath the inner nuclear membrane is the nuclear lamina consisting of an intermediate filament network such as lamin A/C that structurally supports the nuclear shape. Heterochromatic lamina-associated domains bind to the lamina and nuclear envelope associated proteins such as emerin. The chromatin is anchored to the nuclear lamina by lamina associated domains (LADs).

nuclear surface of the nuclear envelope is the nuclear lamina, which is a thin meshwork of intermediate filaments such as lamins A/C, B1 and B2 and supports maintenance of the nuclear shape and structural morphology (Gruenbaum and Foisner 2015, Davidson and Lammerding 2014, Shimi *et al* 2008, Broers *et al* 2004). In particular, lamin A/C (two isoforms of the same LMNA gene) are in dynamic equilibrium as soluble dimers within the nuclear interior, termed the nucleoplasm, and they represent insoluble network assemblies of the peripheral lamina network (Osmanagic-Myers *et al* 2015, Broers *et al* 2006). Mechanical load alterations can impact the conformation of lamin A/C, which then change the accessibility to binding and phosphorylation sites regulating the assembly, disassembly and the degradation of lamin A/C (Swift *et al* 2013, Buxboim *et al* 2014, Ihalainen *et al* 2015, Kim and Wirtz 2015, Machowska *et al* 2015). These remodeling processes of the nuclear lamina represent the most important mechanism by which the cell can challenge the nuclear stiffness due to alterations of the mechanical properties of the microenvironment, such as stiffness and mechanical loading (Swift *et al* 2013, Lammerding *et al* 2006). In addition to the structural role of lamins, they can connect to chromatin and multiple other nuclear proteins, which includes the association with transcription factors (Taniura *et al* 1995, Wilson and Foisner 2010). Due to the various functions of lamin A/C, the nuclear lamina fulfills an important role in the nuclear mechanotransduction process (Lammerding *et al* 2004, Cupesi *et al* 2010), the differentiation of stem cells (Uzer *et al* 2016, Swift *et al* 2013, Akter *et al* 2009, Mao *et al* 2015) and the pathology of diseases such as cancer (Capell and Collins 2006, Burke and Stewart 2006).

Chromatin is the structural ensemble of DNA and associated proteins such as histones filling the entire volume of the nucleus. Specifically, the linear DNA molecules are wrapped around core histone complexes and thereby assemble to nucleosomes (McGinty and Tan 2015). Moreover, these nucleosomes provide compaction via internucleosomal interactions into 30 nm chromatin fibers that are even more condensed into topologically associating domains (TADs) and chromosome territories (Cremer and Cremer 2001, Woodcock and Ghosh 2010, Philipps-Cremins 2014). The condensation of chromatin, and its organization and positioning are precisely coordinated and also strongly related to gene density and gene expression levels (Parada *et al* 2004, Bolzer *et al* 2005, Huebner and Spector 2010). In particular, chromatin-containing actively transcribed genes are present in a less condensed state, which is termed euchromatin, whereas the silent genes are present in a more compact and condensed state, termed heterochromatin. The lamina-associated domains (LADs) within the DNA sequence connecting to the nuclear lamina are heterochromatic (Guelen *et al* 2008). For gene activation, these loci become decondensed and relocated to specific intranuclear regions, which are termed transcription sites, consisting of multiple genes, RNA polymerase and multiple transcription factors (Peric-Hupkes *et al* 2010, Lund *et al* 2013, Demmerle *et al* 2013, Misteli 2007). The reorganization of the genome represents a characteristic feature of stem cell differentiation and seems to be additionally connected to nuclear mechanotransduction processes (Martins *et al* 2012).

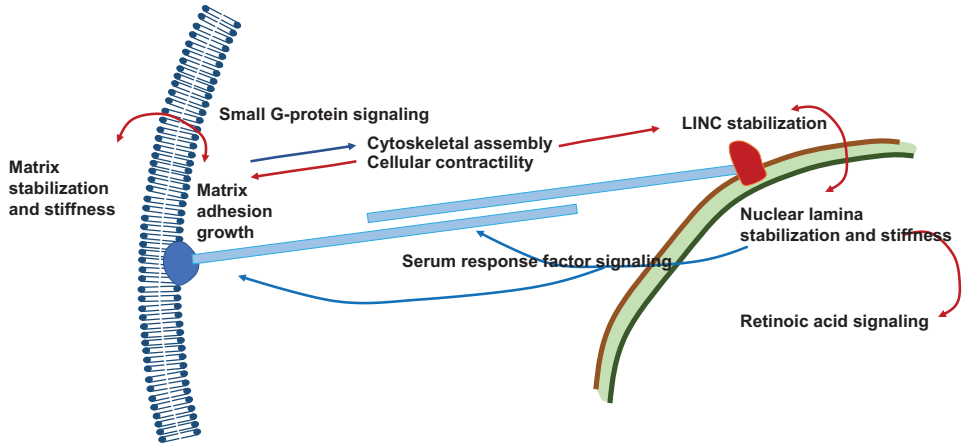

Figure 10.6. The mechanobiochemical feedback mechanisms between the extracellular matrix, the cytoskeleton and the nucleus provide the maintenance of the cell's contractile state. The stabilization and growth of cell–matrix and cell–nucleus attachments drive the propagation of forces inside–out and outside–in. These forces and the associated signal transduction pathways induce the assembly of the matrix, actomyosin and nuclear lamin filaments and subsequently increase cell contractility. Cytoskeletal-based contractile forces evoke matrix and nuclear deformations that in turn cause elastic restoring forces.

The nucleus is physically linked to the cell's cytoskeleton through a linkage complex of nucleoskeleton and cytoskeleton (LINC) complexes that is located in the nuclear envelope (Crisp *et al* 2006, Chang *et al* 2015, Rothballer and Kutay 2013) (figure 10.6). SUN proteins are embedded in the inner nuclear membrane, which is attached to the nuclear lamina and other nuclear membrane-linked proteins. Within the perinuclear space between the outer and inner nuclear membranes are the SUN molecules associated with the KASH domain of proteins, which are coupled to the outer nuclear membrane. These KASH proteins such as nesprin-1 giant, nesprin-2 giant and nesprin-3 are needed to assemble the full LINC complex through the connection of actin filaments, intermediate filaments and microtubules to the cytoplasm. Several other proteins have been revealed to fulfill major functions in the maintenance and reinforcement of these linkages (Guilluy *et al* 2014, Borrego-Pinto *et al* 2012, Kutscheidt *et al* 2014, Bone *et al* 2014), which leads to the hypothesis that the LINC complex is the analog of focal adhesions underneath the cell membrane in the nuclear membrane (the nuclear envelope) (Antoku *et al* 2015). The specific combinations of subcomponents facilitating the assembly of the LINC complexes determine the distinct role of this complex in numerous cell functions such as nuclear positioning, cell migration, morphology, organization of the cytoskeleton and transmission of intracellular forces (Rothballer and Kutay 2013). For cellular mechanotransduction the coupling between the nucleus and the contractile actomyosin fibers in the cytoplasm is crucial. The precise interaction between the LINC complex interface and the actin cytoskeleton is not yet fully understood in detail. However, specific actin stress fibers are detected that envelope the nucleus and build a caged network that is termed the perinuclear actin cap (Khatau *et al* 2009). These stress fibers end in focal adhesions underneath the cell

membrane at each end (Khatau *et al* 2009, Kim *et al* 2012) and simultaneously they are physically linked to the apical surface of the nucleus through nesprins and form transmembrane actin-associated nuclear (TAN) lines that are composed of linear arrays of LINC complex proteins at the surface of the nucleus (Luxton *et al* 2010, Nagayama *et al* 2011, Versaevel *et al* 2014). These TAN lines are termed synonymously actin cap stress fibers and transmit forces to the apical nuclear surface that are able to deform and even concentrate intranuclear DNA (Ihalainen *et al* 2015, Kim *et al* 2015, Versaevel *et al* 2014, Arsenovic *et al* 2016, Nagayama *et al* 2013). Hence, they are proposed to fulfill a critical role in the transition of load towards the nucleus, affecting nuclear shape and driving possibly nuclear mechanotransduction pathways (Khatau *et al* 2009, Versaevel *et al* 2012, Kim *et al* 2012, Khatau *et al* 2012). Indeed, sufficient nuclear stiffness is needed for migration in 3D microenvironments (Khatau *et al* 2012) and the nuclear deformability is essential for the squeezing of cells through narrow constrictions (Friedl *et al* 2011). Why are distinct mechanical properties of the nucleus required for cellular movement? In eukaryotic cells, the nucleus encompasses the chromatin-containing DNA and associated proteins and represents the largest organelle within the cell and thereby fills a large fraction of the total cell volume. The nucleus is enclosed by the nuclear envelope. The nuclear membrane possesses nuclear pores for the active transport of distinct molecules in and out of the nucleus and a wide range of transmembrane proteins such as the LINC complexes mechanically linking the cytoskeleton to the nucleoskeleton. In the interior of the nucleus the nuclear membrane is supported by the lamina, a thin (approximately 100 nm) network of intermediate filaments building the nucleoskeleton (Alberts *et al* 2014). This network is composed of intermediate filaments such as lamins type A, B and C. Moreover, lamins are supposed to alter the organization of the chromatin and are considered to provide the increased mechanical stiffness of the entire nucleus (Gruenbaum and Foisner 2015).

In normal healthy cells, the nucleus has been reported to be usually an order of magnitude stiffer than the cell's cytoskeleton (Friedl *et al* 2011) and on time-scales important to cell migration such as minutes (Maiuri *et al* 2012), the nucleus displays fully elastic behavior, when it relaxes to its original shape after deformation within seconds after force removal (Neelam *et al* 2015). The stiffness of the nuclear lamina has been simply measured by micropipette aspiration of isolated *Xenopus* oocyte nuclei containing chromatin or without chromatin, when it has been removed using a swelling procedure (Dahl *et al* 2004). However, swollen and unswollen nuclei exhibit nearly similar values for their elastic moduli of approximately 25 mN m^{-1}. The nuclear lamina seems to break beyond a specific deformation threshold confinement (Le Berre *et al* 2012). In particular, lamin A and C provide the rigidity of the nucleus, as cells deficient of lamins A and C possess more deformable nuclei, whereas lamin B has no pronounced effect on the nuclear stiffness (Ho and Lammerding 2012, Lammerding *et al* 2006). This result is in agreement with the finding that the relaxation time increases with the lamin A to B ratio, such as the increasingly elastic behavior for a constant measurement time (Swift *et al* 2013). Consistent with the finding that lamin A provides nuclear stiffness, elevated levels of

lamin A lead to less deformable nuclei and impaired migration of cells through small pores (Harada *et al* 2014). Instead, cells with reduced levels of lamin A possess highly fragile nuclei, which are frequently ruptured (Davidson and Lammerding 2014). Distinct mutations in lamins cause many diseases, such as the collectively known laminopathies such as muscular dystrophy (Davidson and Lammerding 2014). Thus, lamins fulfill a prominent role in providing the nuclear mechanical properties directly through the lamina mechanical properties and indirectly through its linkage to chromatin (Ho and Lammerding 2012). In particular, mechanical stress exerted on embryonic stem cells can initiate the decondensation of chromatin that causes a nuclear auxetic behavior (a negative Poisson ratio) (Pagliara *et al* 2014), which means that the nucleus expands on stretching in cross-sectional area perpendicular to the direction of the stretch. This phenomenon is termed auxeticity. When an interaction between lamins and chromatin is assumed to regulate the nuclear mechanical properties, this lamin–chromatin connection may be crucial in mechanotransduction by altering the gene expression in response to mechanical cues (Ho and Lammerding 2012). Dissimilar to the active cytoskeleton, the nucleus is mostly assumed to be mechanically passive. Instead, the nuclear lamina itself can signal biochemically in response to the mechanical cues of the microenvironment through the induction of an upregulation of lamin A, when adhered on stiff substrates, and through the phosphorylation of lamins that induces their disassembly, when adhered on soft substrates (Swift and Discher 2014).

Moreover, the content of the nucleus additionally contributes to the nuclear mechanical properties. In more detail, isolated chromosomes respond elastically towards force application, whereas when they are located inside the nucleus, they can even flow, which depends on how strongly they are tethered to the nuclear periphery (Schreiner *et al* 2015). In particular, isolated nuclei of the fission yeast *S. pombe* lacking lamins have been used to discriminate between the effect of the lamins and the chromatin (Schreiner *et al* 2015). In order to probe the mechanical effect of isolated chromatin, optical tweezers have been utilized for determining that these nuclei are mostly elastic with a minor viscous component, whereas nuclei with untethered chromosomes are highly deformable and exhibit shorter relaxation times, lower stiffness and viscosity. An example is stem cells, which do not express lamin A/C, and that are much less stiff than differentiated cells and show an irreversible nuclear deformation feature, which is characteristic for cellular plasticity (Pajerowski *et al* 2007). The chromatin exhibits enhanced mechanical stiffness when condensed by cations, whereas it flows in the absence of cations (Pajerowski *et al* 2007). Using micropipette-based mechanical probing of GFP labeled epithelial cells, it has been found that the lamina is stretched within these cells, whereas the chromatin flows in a viscous manner (Davidson and Lammerding 2014, Isermann and Lammerding 2013, Denais and Lammerding 2014). A broad range of cells have revealed that their interphase nucleus is significantly stiffer than the surrounding cytoskeleton. Mostly the values of the nuclear stiffness such as the elastic modulus are between 1–10 kPa (1 kPa = 1000 N m^{-2}), which depends on the examined cell type and the biophysical method employed to assess the stiffness (Caille *et al* 2002, Guilak 1995, Kha *et al* 2014, Vaziri and Mofrad 2007).

However, the overall stiffness measurements consistently revealed a two-fold to more than ten-fold higher stiffness of the nucleus compared to the cytoskeleton. In order to determine the nuclear and cytoskeletal deformations in endothelial cells, these cells are compressed between two parallel plates and revealed an effective elasticity of the nucleus of 8 kPa compared to the stiffness of 0.5 kPa for the cytoplasm (Caille *et al* 2002). In line with these results, the nuclei from lamin A/C deficient mouse embryo fibroblast and myoblast cells possess significantly softer nuclei than the nuclei of wild-type control cells (Lammerding *et al* 2004, 2006). Nuclear strain experiments on a variety of mouse embryo fibroblast cell lines lacking distinct lamin isoforms or overexpressing specific lamins isoforms, revealed that lamin C and in particular lamin A are the main contributors to the stiffness of the nucleus (Lammerding *et al* 2006). In contrast, lamin B1 deficient cells display normal nuclear mechanical properties, whereas nuclei in cells lacking lamin A and C can be deformed more pronouncedly under strain application. In particular, cells lacking lamin A, but still possessing lamin C, exhibit only modestly altered nuclear mechanical properties. In contrast, cells expressing only lamin A, but not lamin C, have even pronouncedly stiffer nuclei than cells from wild-type littermates (Lammerding 2011). These findings are in agreement with results on A and B-type lamins that have been reported to assemble in separate networks at the nuclear lamina (Shimi *et al* 2008) with a distinct structural organization (Delbarre *et al* 2006, Goldberg *et al* 2008b). When human B-type lamins are ectopically expressed in *Xenopus* oocytes, they display the formation of a thin network of 8–10 nm fibers that are located underneath the inner nuclear membrane, whereas ectopically expressed A-type lamins form thicker structures on top of the thin B-type lamin network and thereby increase dramatically the stiffness of the oocyte nucleus (Goldberg *et al* 2008b, Schaepe *et al* 2009). Since the nuclear lamina is involved in nuclear–cytoskeletal linkage, mutations in lamins or other nuclear envelope proteins can affect the transmission of forces between the nucleus and the cytoskeleton and hence cause a further alteration of the nuclear deformation under externally applied mechanical stress (load). In addition to the lamin, the nuclear interior can act as a compressible, aqueous, sponge-like and viscoelastic material, which increases in stiffness when it is compressed (Dahl *et al* 2005, Rowat *et al* 2008). As the majority of the nuclear interior is filled with chromatin, modifications of chromatin structure directly impact the physical properties of nuclei using micropipette aspiration techniques (Pajerowski *et al* 2007). In line with this, alterations in the architecture of chromatin through the addition of divalent salts (Dahl *et al* 2005) or the upregulation of heterochromatin proteins (Meshorer *et al* 2006, Pajerowski *et al* 2007) decrease the movements of chromatin within the nucleus and subsequently stiffen the chromatin. Apart from chromatin, distinct nucleoskeletal structures may also provide the mechanical phenotype of the interior of the nucleus. Nuclear lamins such as lamins A and C assemble stable structures in the interior of the nucleus and constantly exchange with the nuclear lamina (Broers *et al* 2005). The presence of these internal nuclear lamins and lamin-binding proteins such as LAP2α organizes the entire nucleoplasm in a structural architecture. Moreover, the interaction of various constituents of the interior and the periphery of the nucleus seems to be

critical for the maintenance of the mechanical phenotype of the nucleus. In particular, the loss of lamins A and C or mutations of lamin A associated with the Hutchinson–Gilford progeria syndrome evoke significant changes in the organization of the chromatin, which can be identified by a loss of peripheral heterochromatin altering the nuclear mechanical properties. In a similar manner, the nucleoplasmic protein LAP2α affects the ratio between nuclear periphery and nuclear interior localization of lamin A/C, which in turn alters the entire nuclear organization and the overall cellular function (Gotic *et al* 2010, Naetar *et al* 2008).

Differentiation of cells during embryogenesis employs extensive alterations in gene expression through the structural reorganization of the nucleus, such as the condensation of chromatin and the immobilization of the nucleoprotein. Hence it can be hypothesized that the nuclei of naive stem cells can be used to analyze their physically plastic behavior in order to confirm their undifferentiated stage, as their nuclei are more pliable than nuclei in differentiated cells.

Plasticity in developmental processes generally means the ability of the cell to adapt its gene expression (Blau *et al* 1985) and mostly reflects alterations in the structural organization of the chromatin (Slack and Tosh 2001). Stem cell nuclei are usually exhibiting more plastic behavior compared to the nuclei of fully differentiated cells (Szutorisz and Dillon 2005). Indeed, major differences are located in the chromatin conformation (Labrador and Corces 2002, West and Fraser 2005), the nuclear protein expression (Worman and Courvalin 2005, Constantinescu *et al* 2006, McKittrick *et al* 2004) and the modification of DNA and associated histones (Li 2002). The differentiation of the cells is associated with the immobilization of distinct nucleoplasmic proteins and hence provides the 'rigidification' the entire genome and subsequently of the nucleus through the establishment of a specific setting of a relatively permanent preference for individual expression profiles (Meshorer *et al* 2006, Kosak and Groudine 2004). Indeed, micromanipulation methods confirmed that the nuclei in human embryonic stem cells (ESCs) are strongly deformable and even stiffen six-fold by their terminal differentiation, however, the nuclei of human adult stem cells exhibit an intermediate stiffness and can deform irreversibly. As the nucleoskeletal component lamin A/C is not expressed in these stem cells, lamin A/C is knocked down in human epithelial cells and indeed their deformability values are similar to values of adult hematopoietic stem cells. Rheologically measurements revealed that lamin-deficient states are mostly fluid-like, especially during the first ten seconds of deformation. When nuclear distortions persist longer than ten seconds, they are irreversible. Using fluorescence-imaged microdeformation with photobleaching it can be confirmed that chromatin flows, distends and reorganizes, whereas the lamina stretches. Indeed, the rheological phenotype (viscous part) of the nucleus is thus manifested largely by the nucleoplasmatic chromatin, whereas the extent of deformation (elastic part) is facilitated by the lamina. In particular, the ESC nuclei are highly deformable and stiffen over several culture days, in which they exhibit a six-fold higher relative stiffness in the typical range of differentiated cells such as embryonic fibroblasts (Maniotis *et al* 1997). Moreover, an exponential fit yields an effective time constant of nearly three culture days for the differentiation-associated stiffening of the

nucleus compared to the to the entire cell body (Pajerowski *et al* 2007). In addition, the timescale seems to be roughly consistent with global alterations in the nucleoprotein expression such as lamins in ESCs (Constantinescu *et al* 2006, Ozolek *et al* 2007).

Hematopoietic stem cells (HSCs) isolated from the bone marrow possess a less multipotent potential than ESCs. In addition, HSCs have still the capacity to differentiate into all types of the mature blood cells (Kondo *et al* 2003) and a few solid tissue cells such as epithelial cells (Krause *et al* 2001). Micropipette aspiration measurements of human HSC nuclei are used to apply a step increase in pressure towards the nuclei. These experiments revealed a progressive flow of the nuclei into the pipette and these stem cell nuclei exhibit also greater deformation than fibroblast nuclei under the same stress. Taken together, after 200 s of aspiration, HSC nuclei can even deform more than twice as much as their cytoskeleton, which is equivalent to the ratio of nuclear to cytoskeletal deformation of ESCs after a culture of three days.

10.3 Nucleus–cytoskeleton–extracellular matrix connections

10.3.1 Nucleus–cytoskeleton connections

The role of the nucleus in regulating the migratory capacity depends strongly on the coupling between the nucleus and the motility providing components of the cytoskeleton. These interactions between the nucleus and the cytoskeleton are provided by LINC (linker of nucleoskeleton and cytoskeleton) complexes (Starr and Fridolfsson 2010). The LINC complexes transmit mechanical stresses from the cytoskeleton to the nucleocytoskeleton of the nucleoplasm during cell processes such as migration and invasion (Martins *et al* 2012, Simon and Wilson 2011, Jaalouk and Lammerding 2009). These LINC complexes are assembled by two transmembrane protein families, the SUN-domain proteins such as Sad1 and Unc-83 at the inner nuclear membrane and the nuclear envelope spectrin repeat proteins (nesprins), which are terminated by KASH domains such as Klarsicht, Anc-1 and Syne homology at the outer nuclear membrane (Lombardi *et al* 2011, Zhang *et al* 2001). In more detail, SUN-domain proteins connect to the nuclear lamina (Folker *et al* 2011) and associate with nuclear pore proteins and other nuclear envelope proteins such as Samp1 (Borrego-Pinto *et al* 2012).

Nesprins are associated to all major cytoskeletal filament networks such as actin filaments, intermediate filaments and microtubules (Gundersen and Worman 2013). Nuclear deformations evoked by a microneedle pulling on the cytoskeleton are pronouncedly reduced in cells containing disrupted LINC complexes (Lombardi *et al* 2011). The LINC complexes couple the cytoskeleton to the lamina, which is further demonstrated by the mechanical stiffness in fibroblasts being abolished by the lack of functional LINC complexes, similar to the stiffness decrease observed in lamin A depleted cells (Stewart-Hutchinson *et al* 2008).

The actin network is bound to the nucleus by interaction with the actin-binding domain of the giant isoforms of nesprin-1 and nesprin-2. These two nesprin proteins, together with their interactions with intermediate filaments through nesprin-3, may

assemble a scaffold around the nucleus restricting the size of the nucleus (Luxton and Starr 2014). A cell is able to move around its nuclei by using KASH proteins that couple the nuclei to moving actin filaments. Similar to actin filaments, microtubules are attached to the nucleus through the kinesin and dynein motor proteins connecting to various KASH proteins (Fridolfsson and Starr 2010). In more detail, these molecular motors are able to move nuclei along microtubules in different directions, such as kinesin that transports its cargo towards the plus end of microtubules, whereas dynein transports the cargo towards the minus end (Fridolfsson and Starr 2010, Tapley and Starr 2013).

10.3.2 Impact on nucleus–cytoskeletal interactions on cell adhesion

The bidirectional interactions between cells and their external microenvironment strongly affect the migration and invasion of cells, which alters not only the cellular speed, the direction of polarization and the persistence of motion, but also the type of mechanism utilized for migration. An example is the specific adhesion of cells to the substrate, which is required for cell migration on a 2D substrate, whereas it seems to be of minor importance for the migration through *in vivo* tissues, matrices or channels (Lämmermann *et al* 2008). The role of the nucleus in cell migration is modulated by interactions with the microenvironment. When cells migrate in soft 3D matrices, the migration depends on lamin A/C and LINC complexes, whereas the migration on stiff 2D substrates is independent of nuclear components (Khatau *et al* 2012). In particular, the adhesion of cells plays an important role in cell migration (Schwarz and Gardel 2012, Humphries *et al* 2015) and also the physical extracellular microenvironmental properties (Charras and Sahai 2014).

Cell adhesion is facilitated by a wide range of adhesion molecules and encompasses transmembrane proteins such as integrins, cadherins and selectins (Gumbiner 1996). Cadherins are cell–cell adherence junction proteins and regulate the intercellular adhesion of neighboring cells. The cell adhesion to the surrounding extracellular matrix is usually driven by activation by integrins and the interaction of cells for the transmigration through cell monolayers, such as endothelial monolayers lining the luminal face of blood or lymph vessels, is usually at first glance driven by the interaction of selectins with carbohydrates or sugars of two neighboring cells.

Migrating cells can perform persistent and directed motion towards gradients in substrate stiffness, which is termed durotaxis. Moreover, this mechanosensitive response is facilitated by dot-like adhesion sites termed focal adhesions coupling the cytoskeleton to the extracellular matrix microenvironment (Trichet *et al* 2012). For adherent cells on a 2D substrate, activated integrins undergo a conformational change and arrange as microclusters to facilitate the interaction with the extracellular matrix at the basal surface of the protrusive structures, such as lamellipodia or filopodia, at the cell's periphery (Ridley 2011, Choi *et al* 2008). Over a certain initial adhesion time, so-called adapter proteins such as talin and paxillin are additionally recruited towards integrin clusters to build nascent adhesions, of which some mature into focal adhesions, which provide a more stable connection of the

cytoskeleton with the extracellular matrix. In line with this, the maturation of focal adhesions occurs concomitantly with the assembly of contractile actin bundles (termed stress fibers). In particular, focal adhesions enlarge their size and alter the shape of their adhesion towards a elongated oval form with the largest diameter in the stress fibers (Choi *et al* 2008, Alexandrova *et al* 2008). Radial stress fibers, which are perpendicular to the cell's leading edge, are anchored at one end to a focal adhesion and the other end is connected to transverse arcs of actin bundles, which are located parallel to the cell's leading edge, but do not need to be connected at their ends to focal adhesions (figure 10.7) (Gardel *et al* 2010, Burnette *et al* 2011). During the crawling of a cell on a 2D substrate, the actin polymerization at the cell's leading edge extrudes the lamellipodium in the direction of motion and generates an actin retrograde flow (Renkawitz *et al* 2009). Moreover, from the effect of cell adhesion on the balance between the forward protrusion of lamellipodia or filopodia and the retrograde flow of actin has emerged the concept that cell–matrix adhesion proteins act as a 'molecular clutch' (Giannone *et al* 2009, Case and Waterman 2015).

In cells cultured on a soft substrate or in confinement, such as channels or embedded in soft 3D matrices, focal adhesions are rare or not observed (Fraley *et al* 2010). However, adhesion proteins are still able to modify cell migration, cellular protrusion and matrix deformation (Fraley *et al* 2010) through the formation of smaller dynamic podosomes or invadopodia (Linder 2007). Amoeboidal modes of cell migration in confined microenvironments are independent of integrin cell–matrix adhesion proteins and rely on non-specific friction of the cells with their micro-environment, which is sufficient for their movement (Lämmermann *et al* 2008).

The interplay between cell adhesion, mechanosensing processes and cytoskeleton–nucleus linkage is key in cellular movement in 3D microenvironments (Kim *et al* 2014a, Meehan and Nain 2014). The coupling between the nucleus and the process of cell migration is supposed to be facilitated by the perinuclear actin cap structure,

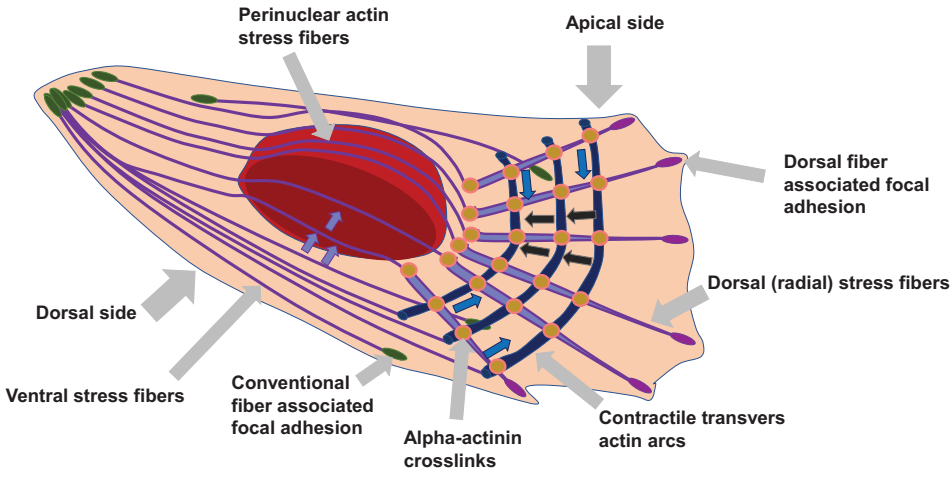

Figure 10.7. Types of stress fibers in migrating cells. Schematic drawing of the four main types of stress fibers: dorsal stress fibers, transverse arcs, ventral stress fibers and perinuclear actin caps.

which specifically arranges the actin coupling with the nucleus (Kim *et al* 2014a). This actin cap consists of actin stress fibers building structures similar to cables that cover the entire nucleus and terminate in a special subset of mechanosensitive focal adhesions (Luxton and Starr 2014, Kim *et al* 2013). Moreover, the elongated shape of nuclei in migrating cells, such as many cancer cells, seems to be regulated by the actin cap (Kim *et al* 2014, Khatau *et al* 2009). The actin cap cables utilize specific LINC complexes such as nesprin-3 and nesprin-2G and require the focal adhesion protein zyxin and hence seem to provide the transmission of external mechanical signals towards the interior of the nucleus (Kim *et al* 2012, Chambliss *et al* 2013). The perinuclear actin cap, which links the nucleus with the extracellular matrix through focal adhesion driven cell–matrix adhesion, hence fulfills a key function in the transmission of the forces of external microenvironmental cues towards the nucleus.

10.4 Mechanosensitivity and mechanotransduction

Cells are can sense and transduce mechanical cues of the microenvironment and even respond to mechanical forces, which is needed for the migration mode termed durotaxism (figure 10.8) (Lo *et al* 2000, Trichet *et al* 2012). Within cells, mechanosensitive proteins sense mechanical alterations and provide a response to these alterations. When the adhesion protein talin is stretched, it exposes a hidden vinculin binding site, which subsequently causes the binding of vinculin (del Rio *et al* 2009). Several adhesion and cytoskeletal proteins such as integrins and myosins gain enhanced affinity to their ligands under mechanical stress (Veigel *et al* 2003, Schwarz and Gardel 2012). In addition to cell–matrix adhesion proteins, various ion channels

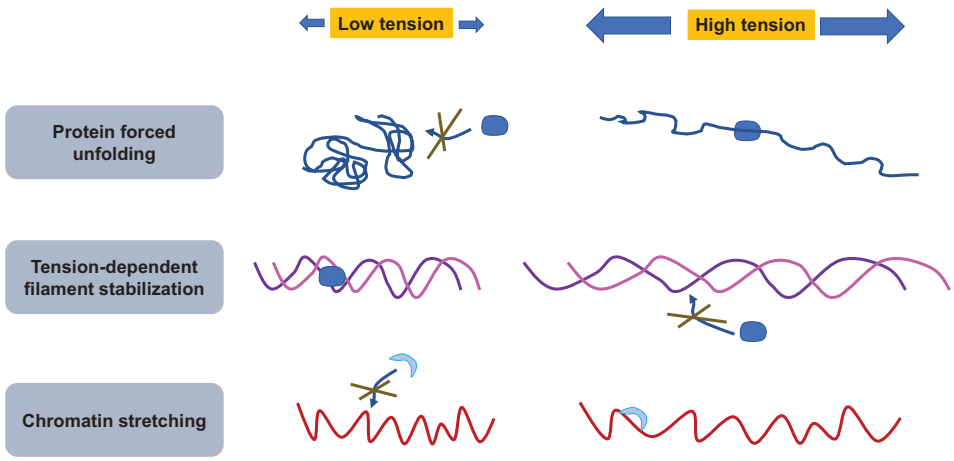

Figure 10.8. Conversion of mechanical forces into biochemical forces. The forces of unfolding linker and adapter proteins can expose cryptic binding sites that are available for binding and activation factors (upper images). Tension applied to rope-like supercoiled filaments impairs the accessibility of signaling molecules, which hence confers protection from induced disassembly, protein turnover and proteolytic degradation (middle images). The propagation of forces over the LINC complex attachments and transmission into the nucleus pulls directly on chromatin segments and hence enhances their binding affinity to RNA-polymerases and transcription factors (lower images).

(transmembrane proteins creating pores regulating the transport of ions) within the cell membrane and nuclear membrane are both mechanosensitive. These stretch-activated ion channels are able to open due to membrane tension and hence transport ions such as calcium across the cell or nuclear membrane (Ingber 2006). Mechanotransduction is a process that facilitates the conversion of the external mechanical force signal to an internal biochemical response signal and altered gene expression. The decondensation of chromatin and the nuclear entry of the transcription factor MKL is provided upon a response to force application through magnetic beads that have been bound to distinct cell membrane receptors (Venkatesan Iyer *et al* 2012). The nuclear lamina reacts through the transmission of biochemical signals to the mechanical microenvironment with increased levels of lamin A on stiff substrates and phosphorylated lamin A, which induces the disassembly as it naturally occurs at the onset of cell division on soft substrates (Swift and Discher 2014). It seems to be clear that the nucleus–cytoskeleton connections combined with cell adhesion to the external microenvironment facilitate the transduction of forces towards the nucleus. This mechanotransduction fulfills a key role in enabling the nucleus to perform its role in many cellular processes such as cell migration. How do the mechanical properties of the nucleus and the nucleus–cytoskeleton–extracellular microenvironment connections affect the migration and invasion of cells?

10.4.1 Mechanotransduction and nuclear function

Multiple signal transduction pathways accumulate in the nucleus to provide to facilitation of crucial nuclear events such as gene transcription, DNA replication and the progression of the cell cycle including cell division. In addition to the vast majority of signals transduced towards the nucleus upon stimulation with hormones or other soluble factors, the nucleus is able to react upon exertion of mechanical forces. It has been revealed how mechanical force can affect the transcription, growth and differentiation of cells. Moreover, it is even known how the force is transmitted through the cytoskeleton towards the nucleus, across the nuclear envelope to the nuclear lamina and chromatin. What are the key players in the transmission of the mechanical signals to the inside of the nucleus?

Cells can sense and respond to mechanical cues of their surrounding microenvironment (Discher *et al* 2005, Janmey *et al* 2013, Graham and Burridge 2016). The mechanical forces affect the cell-cycle-dependent cell division, cell differentiation during developmental processes and cell migration, which covers a broad range of basic processes from morphogenesis to tissue repair. The process of mechanotransduction utilizes the transformation of mechanical stimuli into cellular signaling steps, has been recognized in all eukaryotic cells and is in part based on the structural properties of the cytoskeleton acting as a conductive and viscoelastic material. Hence, the cytoskeleton transmits forces and propagates stress within a cell and also between neighboring cells, when these cells are coupled through cell–cell adherence junctions, tight junctions or gap junctions. The characterization of the components that sense, transduce and respond to physical forces has produced an

integrated network of adhesion receptors, cytoskeletal elements, and organelles (Hoffman *et al* 2011, Wang *et al* 2009). Since the morphological aberrancies of the nucleus evoked by force exertion were observed, a long time ago (Chambers and Fell 1931, Sauer 1935), it has been revealed in more detail that the forces exerted through integrins can provide a rapid (a few seconds) force transmission into the nucleus (Maniotis *et al* 1997), which alters the position and morphology of the entire nucleus. The impact of mechanical force on nuclear positioning (Starr 2009, Lombardi *et al* 2011), nuclear morphology (Guilak *et al* 2000, Pajerowski *et al* 2007), and gene activity, such as c-fos, egr-1, iex-1 and c-myc (Lombardi *et al* 2011, Gieni and Hendzel 2008, Lammerding *et al* 2005), has also been observed in different conditions. The immediate nuclear responses to force (within 30 min) such as the induction of physical alterations to the nuclear lamina (Pajerowski *et al* 2007), the repositioning of intranuclear markers (Booth-Gauthier *et al* 2012) and nuclear positioning of mechanical response factors (Dupont *et al* 2011, Driscoll *et al* 2015), have lead to the suggestion that a cell's internal mechanotransduction pathway facilitates the coordination and communication with the cell's nucleus.

On longer time-scales (of several hours or even days), the nucleus is able to change its stiffness to mirror the stiffness of the surrounding extracellular microenvironment (Swift *et al* 2013). Alterations in matrix stiffness leads to the induction of genetic programs to guide the development (Mammoto *et al* 2013), tumorigenesis (Levental *et al* 2009) and stem cell fate (Engler *et al* 2006). Finally, the nucleus seems to be a crucial component for the cellular mechanoresponse and facilitates a long-term adaptation of the cell to forces through the regulation of the transcription of genes. How can the mechanical force affect the nuclear function of the regulation of gene expression?

10.4.2 The nucleus: linking structural form to function

The nucleus possesses several stratified and interconnecting components that couple the two lipid bilayers of the nuclear envelope to the interior nucleoskeleton and the chromatin. The connection of the inner and outer nuclear membranes is facilitated through nuclear pores providing the communication between the cytoplasmic and nucleoplasmic compartments. In particular, the inner nuclear membrane is mechanically stabilized by the nuclear lamina, which is composed of filamentous lamin proteins such as lamins A, B and C and several integral membrane proteins such as the including LEM-domain containing proteins LAP2, emerin and MAN1 (Barton *et al* 2015). The nuclear lamina represents a dynamic structure, which interacts with chromatin domains and hence controls the global organization of chromatin and subsequently the expression of genes (Zullo *et al* 2012, Solovei *et al* 2013). Multiple severe pathologies such as laminopathies (Isermann and Lammerding 2013) are dependent on defects of proteins located within nuclear lamina, which seems to underestimate the structural importance of the nuclear lamina.

Early biochemistry and electron microscopy experiments revealed that the cytoskeleton interacts with the interior of the nucleus, such as the nuclear lamina (Capco *et al* 1982, Fey *et al* 1984, Lehto 1978, Berezney and Coffey 1974), whereas

currently the molecular contribution to the nuclear migration has become a focus (Dupin and Etienne-Manneville 2011). The identification of two distinct families of proteins colocalizing in the nuclear envelope such, as the SYNE/nesprin-family (Apel *et al* 2000, Zhang *et al* 2001) and SUN-family (Malone *et al* 1999, Lee *et al* 2002), have been demonstrated in the linkage of the cytoskeleton and nuclear lamina. In *Caenorhabditis elegans* mutants of ANC-1 and UNC-84 it has been demonstrated that ANC-1 (a homolog of nesprin-2) can interact with actin at its N-terminus, whereas UNC-84 (a homolog of SUN1/2) binds to actin with a domain in its C-terminus (Starr and Han 2002). In addition, UNC-84 can localize in the nuclear envelope similarly to lamins (Lee *et al* 2002). Hence, a molecular bridge connecting the nuclear lamina and the cytoskeleton has been detected and demonstrated to be crucial for the movement of the entire nucleus. Finally, the term LINC complex has been associated with these structures (Crisp *et al* 2006).

10.4.3 LINC complexes and nuclear mechanotransduction

Isolated nuclei are able to react to physical forces (Guilluy *et al* 2014, Rowat *et al* 2006), which indicates that the sensory, transducing and responding functions are nuclear properties. Similar to whole cells, which couple the extracellular micro-environment to their actin cytoskeleton through adhesion receptors, the LINC complex couples the cytoskeleton to the nucleoskeleton (figure 10.9). The structural similarity between the whole cells and nuclei raises the question whether the force-

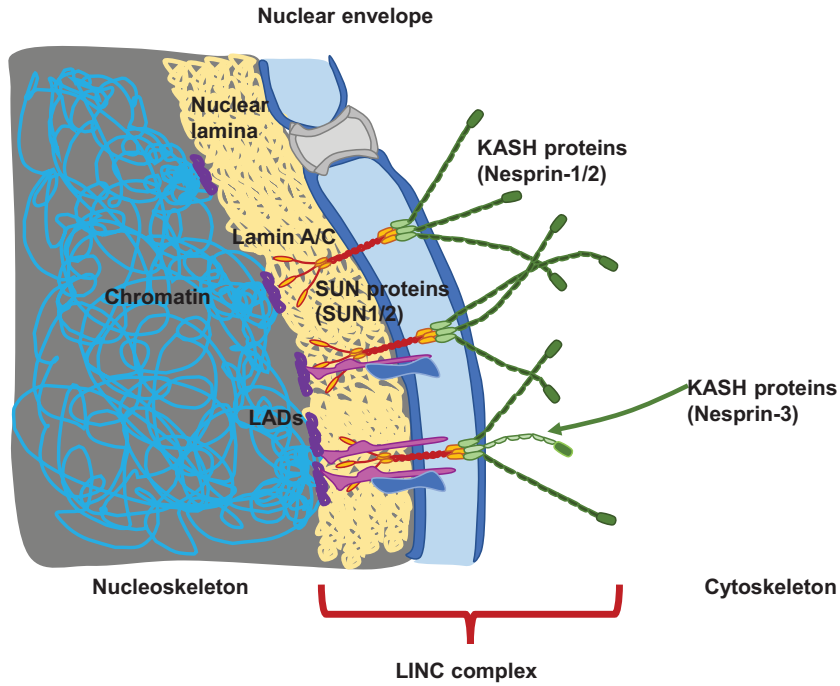

Figure 10.9. Composition of the LINC complex.

sensitive signal amplification that is performed in focal adhesions underneath the cell membrane by the focal adhesion proteins talin and vinculin occurs similarly at the nuclear membrane. The spectrin-repeats of nesprins are hypothesized to cooperatively unfold under tension, thereby expose binding sites driving the dimerization of nesprins and recruit of additional factors for providing the stability and rigidity of the entire complex. Thus, the LINC complexes seem to behave as force-sensitive signaling focal points for cytoplasmic proteins and fine-tune the nuclear responses due to various mechanosensory loads. A nesprin-dependent force-sensing mechanism can be locally amplified by the structural alterations that localize in the nuclear lamina (Guilluy *et al* 2014).

LINC complexes seem to be the primary structure providing the nuclear mechanotransduction, but how can the nuclear mechanotransduction compromise cellular functions? Indeed, the LINC complex is involved in providing the pre-stress state of the cell in mesenchymal stem cells (Driscoll *et al* 2015). In particular, the pre-stress evolves from stresses generated within and sensed by the cells in their local microenvironment. Hence, the LINC complexes seems to facilitate cell-wide tension regulation and the transfer of strain towards the nucleus. Moreover, this maintenance of the internal cellular tension balance is a key factor of the proposed intriguing cellular tensegrity model (Wang *et al* 2009, Ingber 1997). The major factors involved in the regulation of the pre-stress state of the cell, such as cell–matrix or cell–cell adhesion receptors, actomyosin contractility, and the cytoskeleton, are additionally contributing to the force transmission towards the nucleus (Maniotis *et al* 1997, Hu *et al* 2005, Wu *et al* 2014). In addition to the LINC complex, cell-generated tension can affect and regulate nuclear mechanics. The actomyosin contractility alters the stability of the nuclear lamina through providing rapid and long-term alterations to lamin A (Buxboim *et al* 2014, Swift *et al* 2013). The tension on the LINC complex, which mimics the actomyosin tension, vastly elevates the nuclear stiffness (Guilluy and Burridge 2015), which may have a broader and longer impact on the cellular pre-stress state, as has been shown for the differentiation of stem cells on substrates with different rigidity (Engler *et al* 2006).

10.4.4 Nesprins

In mammalian cells five different nesprin genes can be detected, SYNE-1–4 and KASH5, that possess a conserved C-terminal KASH (Klarsicht/ANC-1/Syne-1 homology) domain for the interaction with SUN proteins. Cytoskeletal forces are transduced to LINC complexes through specific nesprins connected to various cytoskeletal systems. In particular, nesprin-1 and -2 interact with actin (figure 10.10) (Starr and Han 2002) and link to microtubules through dynein/dynactin (Zhang *et al* 2009) and kinesins (Zhang *et al* 2009, Fan and Beck 2014). Nesprin-3 interacts with intermediate filaments binding to plectin (Wilhelmsen *et al* 2005). Nesprin-4 connects to microtubules through kinesin (Roux *et al* 2009). KASH5 associates to microtubules through dynein/dynactin (Morimoto *et al* 2012). Nesprins are highly complex as they have been found in multiple splice variants, which increase their complex functional regulation; these adaptable expression patterns provide another

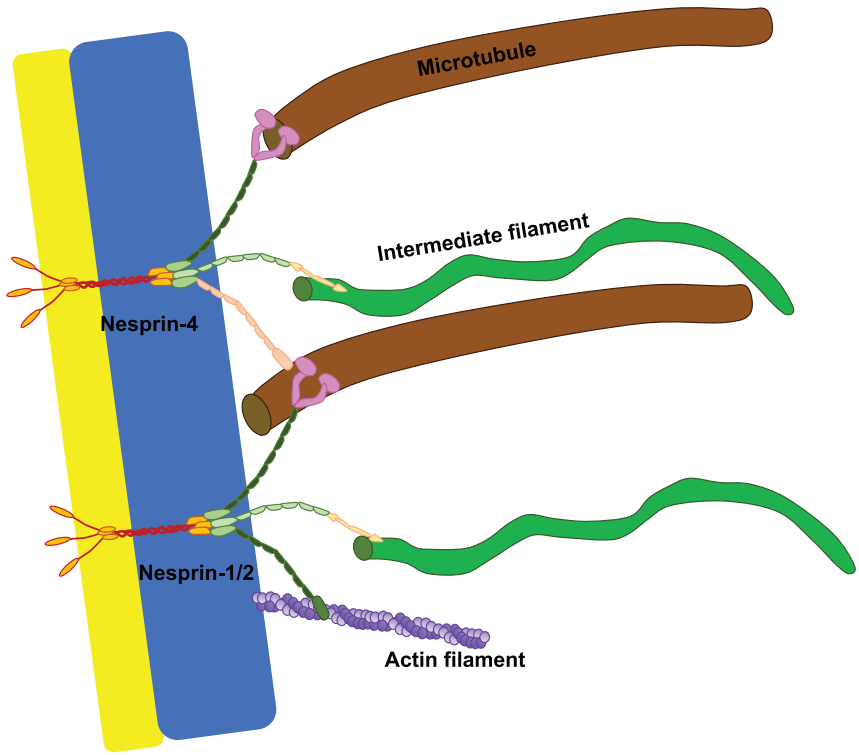

Figure 10.10. Structure of nesprins and their connection to cytoskeletal filaments.

problem for simple depletion studies and are suggested to interact both physically and functionally with one another (Zhang *et al* 2001, Lu *et al* 2012, Rajgor *et al* 2012, Duong *et al* 2014). Nesprins 1–3 seem to predominantly facilitate the force transduction towards the nucleus in most cells, these three nesprins are widely expressed compared to nesprin-4 and KASH5. The expression of nesprin-4 seems to be restricted to distinct tissues and cell types such as the sensory hairs and secretory epithelial cells of mammary, salivary and pancreatic tissues (Roux *et al* 2009, Banerjee *et al* 2014), whereas the expression of KASH5 is restricted to reproductive organs (Morimoto *et al* 2012).

The application of mechanical tension on isolated nuclei through nesprin-1 leads to stiffening of the nucleus (Guilluy *et al* 2014). In particular, the nuclear adaptation to force through nesprin-1 relies on several nuclear lamina proteins such as SUN1, SUN2, emerin and lamin A/C. Moreover, nesprins function in providing cellular responses to force in other cell systems. In mouse cardiomyocytes, the loss of nesprin-1 and -2 impairs the expression of mechanical response genes after a biaxial cell stretching (Chancellor *et al* 2010). In endothelial cells, the depletion of nesprin-1 imposes a failure to the alignment of cells due to uniaxial stretch (Morgan *et al* 2011), whereas the depletion of nesprin-3 induces a failure in reorientation of the centrosome upon fluid shear (Brosig *et al* 2010). The usage of dominant-negative approaches in order to investigate the effect of the loss of nesprin–SUN complexes

shows that the transmission of forces from the cytoskeleton towards the nucleus is abolished (Lombardi *et al* 2011). Moreover, the nuclear localization of the mechanically responsive transcriptional cofactor YAP depends on the response of nesprin-1G to stretch (Driscoll *et al* 2015). Additionally, the LINC complex is involved in the NFkB activity regulation due to cell stretching (Avvisato *et al* 2007). Finally, these results indicate that LINC complexes seem to activate other mechanoresponsive transactivators such as β-catenin (Wei *et al* 2015) and Twist (Neumann *et al* 2010). Indeed, nesprin-2 has been shown to regulate the Wnt-ligand-induced nuclear translocation of β-catenin (Friedl *et al* 2011).

The maintenance or the change of the nuclear position requires the transmission of forces from the cytoskeleton to the nucleus (Gundersen and Worman 2013, Zhang *et al* 2007b) and a loss of nesprin causes defects to this crucial nuclear positioning process in many systems (Starr and Han 2002, Tsujikawa *et al* 2007, Mosley-Bishop *et al* 1999, Postel *et al* 2011, Khatau *et al* 2012, Zhang *et al* 2009). However, it is difficult to decipher the nuclear migration defects, which also affect the entire cell polarity, migration and other processes, from defects based on the two mechano-transduction processes. However, the deregulation of these processes may be affected by the following processes. An example process is the dorsal actin stress fiber structures that traverse the apical side of the nucleus and are associated in the force transmission through their connection with the LINC complex to the nucleus (Luxton *et al* 2010, Nagayama *et al* 2013). The photoablation of nesprin-positive stress fibers over the nucleus evoked a local deformation of the underlying nucleus and finally a nuclear displacement (Nagayama *et al* 2014, Petrie *et al* 2014) indicating that the LINC complex arranges the nuclear positioning by maintaining the tension between the cytoskeleton and nucleus. The dynamic mechanical connection between the nucleus and the cytoskeleton has been most pronouncedly demonstrated in 3D cell migration. A hydrostatic pressure differential has been found to be existent between the cell's leading edge and its rear, which has been established through the force exerted on the nucleus during the cell migration in 3D microenvironments (Zhang *et al* 2007a). In particular, the actomyosin contractile force is transmitted towards the nucleus through vimentin and nesprin-3. In line with this, the depletion of nesprin-3 evokes a concomitant loss of the nuclear positioning and abolishes the intracellular pressure asymmetry during the 3D cell migration.

Moreover, the relevance of the nesprin function in mechanotransduction can be found in several human diseases. Patients with Emery–Dreifuss muscular dystrophy (EDMD) display a late-onset of neuromuscular disorders, which are associated with mutations in emerin (X-linked form), lamin A/C (autosomal dominant form) or nesprin-1 and -2 (Horn *et al* 2013). EDMD subsequently causes elevated nuclear fragility and aberrant mechanosensitive gene responses in highly contractile skeletal and cardiac muscle tissues. In mice, the deletion of nesprin-1 and -2 causes cardiomyopathy and impaired gene expression due to mechanical probing (Chancellor *et al* 2010). In addition, mutations in nesprin-4 have been detected in families displaying hereditary hearing-loss (Banerjee *et al* 2014). In line with this nesprin-$4^{-/-}$ mice displayed a gradual degradation of the highly mechanosensory

outer-hair cells of the cochlear organ, which may be caused by simultaneously occurring defects in the nuclear positioning.

10.4.5 SUNs

In mammals five SUN-domain containing proteins, SUNs 1–5, have been identified. SUN1 and 2 are widely expressed in different organs and tissues, while SUN3–5 seem to be testis specific (Goeb *et al* 2010, Yassine *et al* 2015, Huttlin *et al* 2010). The oligomerization of SUN proteins is necessary for binding with the KASH domains of nesprins (Cain *et al* 2014). Moreover, SUN proteins assemble as a trimer which binds in a 1:1 ratio of SUNs with the KASH domain of nesprins (Sosa *et al* 2012). Hence, it has been suggested that a covalent connection between the SUN–KASH domains is formed, which is then strong enough to keep the connection and provide the high-level force transmission that seem to be governed by TorsinA (a member of the AAA+ superfamily of ATPases) that fulfills roles in the endoplasmatic reticulum and a specific function in the nucleus (Sosa *et al* 2013, Chase *et al* 2017). In line with this, the TorsinA-dependent ATP-hydrolysis activity is controlled by LAP1 and LULL1 (Zhao *et al* 2013). In more detail, TorsinA dislocates SUN2, nesprin-2G and nesprin-3 from the nuclear envelope, whereas SUN1 is not altered (Vander Heyden *et al* 2009).

In addition to roles in the endoplasmatic reticulum, Torsins fulfill a specific role at the nuclear envelop. In fibroblasts, both TorsinA and its cofactor LAP1 are required for the proper assembly of TAN lines that are connected to the nuclear envelope (figure 10.11) (Luxton *et al* 2011), which consists of the LINC complexes, and is associated with retrograde flow of actin. In particular, TorsinA alters the backward motion of the nuclei during the positioning of the centrosome and is involved in the maintenance of cellular polarity in migrating cells (Saunders *et al* 2017). Another role for Torsins at the periphery of the nucleus is their contribution to the regulation of the nuclear envelope architecture. Deletions of Torsins in a wide variety of species such as human, mouse, worm and fly cells causes the development of omega-shaped 'bleb' compartments within the nuclear envelope, which seems to be similar to the bleb formation of the cell membrane (Goodchild and Dauer 2005, Jokhi *et al* 2013, Liang *et al* 2014, VanGompel *et al* 2015, Laudermilch *et al* 2016, Tanabe *et al* 2016). Moreover, the perinuclear blebs contain ubiquitinated proteins (Liang *et al* 2014, Laudermilch *et al* 2016) and components of the nuclear pore complex (Laudermilch and Schlieker 2016). In summary, Torsins fulfill a broad variety of functions at the nuclear envelope (and also at the endoplasmatic reticulum), among them some are even critical for the early developmental steps in neuronal cells (Tanabe *et al* 2016).

As cells are able to sense and respond to low magnitude vibrations, it has been found that the nucleus is a crucial component in detecting this vibrational type of mechanical probing (Uzer *et al* 2015). Using mesenchymal stem cells, it has been shown that the activation of FAK and Akt pathways by mechanical vibration causes the activation of RhoA-dependent signal transduction processes, the remodeling of F-actin and impairs the expression of adipogenic gene expression. The codepletion of SUN1/2 and the expression of the DN–KASH domain evoked a disruption of

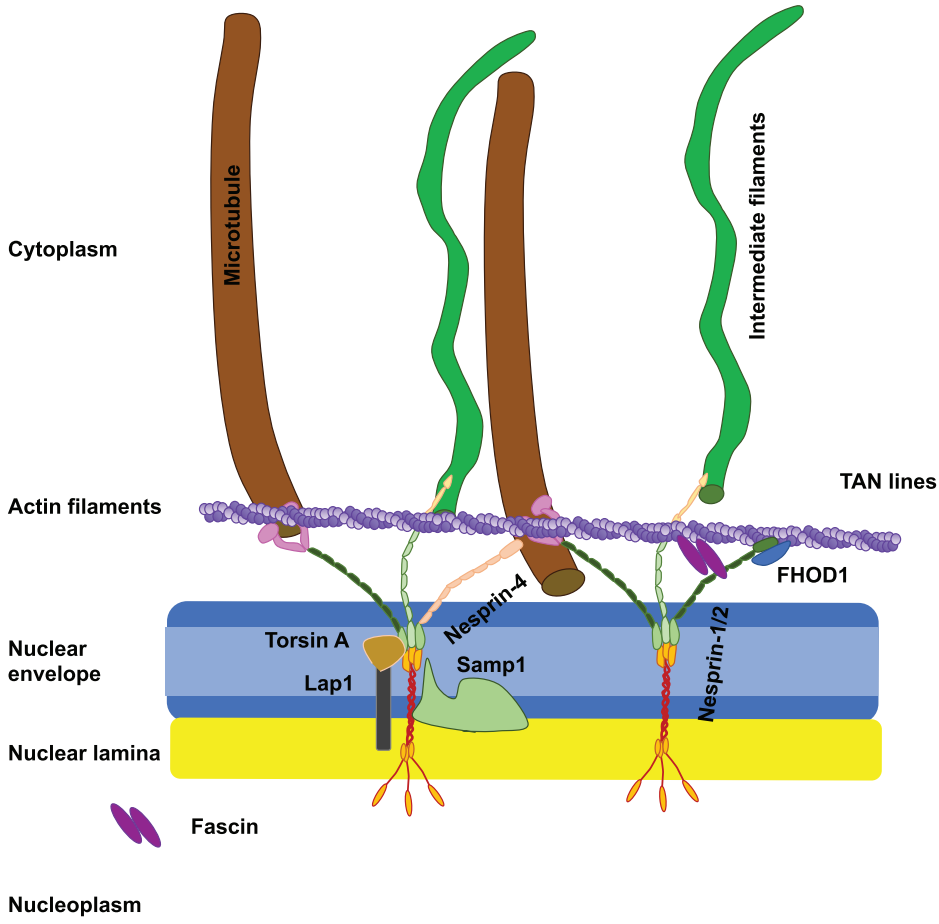

Figure 10.11. TAN lines and their connection to the nucleus.

vibration-dependent cell responses (Uzer *et al* 2015). In *C. elegans*, UNC-84 (a SUN1/2 homolog) binds to lamin in order to transmit cytoplasmic forces towards the nucleus during nuclear migration (Bone *et al* 2014). The codepletion of SUN1/2 similarly impairs nuclear stiffening due to force application to isolated nuclei through nesprin-1 (Guilluy *et al* 2014), which leads to the hypothesis that SUN1 and SUN2 operate in a separate manner and hence they seem to be functionally redundant. This finding is consistent with the results in SUN1/2 null mice, which displayed a disrupted nesprin-1 localization, whereas the localization is unaffected when just one of the two SUN proteins is expressed, such as SUN1 or SUN2 expressing cells (Lei *et al* 2009). Conversely, functional differences have also been suggested for these two proteins. Although both SUN proteins possess similar binding affinities to the KASH domain of the mini-nesprin-2G, SUN1 has been reported to be dispensable for the connection of the cytoskeleton to nesprin-2, whereas SUN2 seems to be required (Ostlund *et al* 2009). In *C. elegans*, the UNC-84 protein seems to recruit UNC-83, which is a KASH-domain containing protein, to

the nuclear envelope where they facilitate the transmission of forces during nuclear migration (Starr *et al* 2001). Moreover, UNC-84 is essential for the maintenance of the nuclear envelope architecture in high force-bearing cells (Cain *et al* 2014), which correlates with its functional role as a force transducer within the LINC complex. In lamin-deficient cells, SUN1 protein levels are elevated, which is a result of decreased protein turnover, whereas the SUN2 protein levels are not altered (Chen *et al* 2012a). Based on these results, it can be seen that different protein degradation pathways are utilized and compensatory mechanisms seem to regulate SUN1 and 2 protein levels, which should enlighten how SUN1 causes lamin pathologies.

10.4.6 Emerin

Emerin is a ubiquitous integral nuclear membrane protein that localizes to the inner nuclear membrane and interacts with nesprin-1/2, SUN1/2, lamin A–C (figure 10.5) (Haque *et al* 2010, Wheeler *et al* 2007, Mislow *et al* 2002) and other proteins (Berk *et al* 2013). Emerin mutations in their EMD domain (figure 10.12) (Bione *et al* 1995) and emerin knockout fibroblasts exhibit an impaired mechanotransduction pathway (Lammerding *et al* 2005, Rowat *et al* 2006). In particular, the Src kinase phosphorylates Emerin at its tyrosines upon the exertion of tension to isolated nuclei through nesprin-1 (Guilluy *et al* 2014). The rapid phosphorylation correlates with the accumulation of lamin A/C and the nuclear reinforcement events. Emerin facilitates the polymerization of actin (Holaska *et al* 2004), which then causes possibly an elevated nuclear rigidity, as a result of polymerization of actin at the

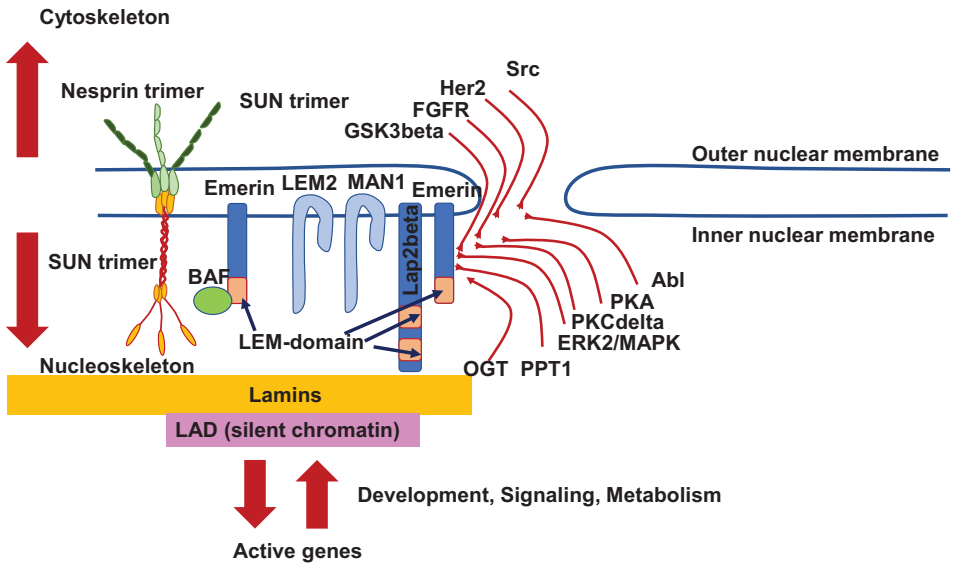

Figure 10.12. Regulation and interaction partner of emerin at the nuclear envelope. Emerin and other LEM-domain proteins (Lem2, Man1 and Lap2β) are located at the inner nuclear membrane. Emerin fulfills roles in signaling, mechanotransduction, nuclear architecture, chromatin tethering and gene regulation.

nuclear lamina under certain circumstances. However, the polymerization of actin does not seem to evoke a nuclear stiffening due to force exertion (Guilluy *et al* 2014). The phosphorylation of emerin is enhanced on substrates with increasing stiffness (Guilluy *et al* 2014) and is impaired through the inhibition of myosin II, as the entire actomyosin-based contractility is dramatically reduced. This result leads to the hypothesis that the cellular pre-stress is able to facilitate the phosphorylation of emerin and subsequently the nuclear signaling. Moreover, Emerin promotes the recruitment of the mechanoresponsive transcription factors such as Lmo7 and MKL1 (Ho *et al* 2013) and thereby provides a long-term adaption to the mechanical signals. In more detail, MKL1 dislocates from G-actin and localizes in the nucleus upon mitogen and mechanical stimulation (Vartiainen *et al* 2007). In lamin knock-out and mutant cells, the aberrant MKL1-SRF signaling phenotype can be rescued by the addition of emerin (Ho *et al* 2013). In summary, these findings show that emerin can regulate the fast stiffening of the nucleus, the actin cytoskeletal polymerization and gene activation, whereas how the function of emerin is regulated during these processes is not yet fully clear.

However, the nucleus has been demonstrated to be integral to the mechano-transductive processes in cells. Aberrancies in the LINC complex affect nuclear functions and even cause wide-ranging effects on cellular structural architecture and the behavior of cells. However, how can mutations in the ubiquitously expressed LINC complex proteins cause distinct disease states in certain cell types? Can LINC complex alterations predominantly be based on alterations in the pre-stress state of the entire cell? Indeed, the pre-stress states vary between the cell types and can even continually adapt to the mechanical cues of the specific microenvironment. Disruptions of the LINC complex have been most identified in cells that are subject to high mechanical strain such as cardiac and skeletal myocytes. Since the LINC complex facilitates the regulation of the pre-stress state in many ways, these highly stressed cell types seem to be particularly susceptible to defects within the LINC complex. In UNC-84 mutants, cells that are exposed to high mechanical strain displayed an irregular nuclear envelope architecture (Cain *et al* 2014). Hence the dynamic cellular adaptation to mechanical stress seems to be crucial for the cellular homeostasis and is extremely well defined for bone and soft tissue (Wolff's law and Davis' law, respectively) and has also been observed in various other cell types (DuFort *et al* 2011). In order to explore the role of nuclear mechanotransduction, the individual contributions of the LINC complex and nuclear lamina in regulating the pre-stress state of cells need to be revealed and how these alterations then provide the regulation of the overall cell behavior.

10.4.7 Mechanotransduction via the nuclear envelope: a distant reflection of the cell surface

As the nucleus represents the largest and stiffest organelle, it seems to be subjected to significant forces generated by the cytoskeleton in order to adapt its shape, alter its position and facilitate the cellular processes required for cell migration, differentiation or division. Indeed, mechanosensitive mechanisms are present within the

nucleus and hence control the regulation of the nuclear structure and function due to mechanical force application (Aureille *et al* 2017). While the molecular mechanisms that facilitate this response are not yet known, the nuclear envelope seems be crucial in this regulatory process. Do the nuclear mechanosensitive mechanisms possess a functional homology to the components at the cell membrane? How do these nuclear envelope mechanisms function during the adhesion and migration of cells? How can the nuclear envelope function in the cytoskeletal organization through direct physical interaction or the regulation of signaling processes?

Whenever cells adhere, divide or undergo differentiation, they need to adapt their architecture to accommodate the cellular signal transduction machinery to the requirements of these tasks. The constant restructuring and reorganization of the cell's interior is based on alterations of its cytoskeleton and the molecular motor-based forces, whose coordinated actions depend on the mechanosensitive signal transduction pathways (Hoffman *et al* 2011, Parsons *et al* 2010). Moreover, the force-dependent mechanisms, which can reinforce, repair or disassemble subcellular load-bearing structures, help to maintain the cellular integrity, while they provide the reorganization of cells (Parsons *et al* 2010). Since the nucleus is the largest and stiffest compartment within the cell, it represents a major hindrance for the cell reorganization and hence pronounced mechanical stress need to be applied to facilitate alterations in nuclear shape and position.

Mechanosensitive mechanisms in the nucleus have been identified that adapt the nuclear structure and function to the mechanical tension (Wang *et al* 2009). While the molecular mechanisms providing a response to mechanical cues are at the onset of being revealed, there is a lot of evidence that strongly proposed a key role for the nuclear envelop in this process. The nuclear envelope is built by a lipid double membrane, which is folded together and interacts with a large network of more than 80 proteins (Schirmer *et al* 2003) and subsequently assembles subnuclear structures such as the LINC complex, the nuclear pore complex and the underlying scaffold, termed the lamina. Moreover, the nuclear envelope anchors LEM-domain proteins such as lamina-associated polypeptide 2, Emerin and MAN1 and among them are proteins such as emerin initiating the interaction with chromatin (Simon and Wilson 2011). These elements have been demonstrated to establish a membrane-bound network, where the assembly and the dynamics are facilitated by the mechanical tension (Luxton *et al* 2010, Kutscheidt *et al* 2014, Guilluy *et al* 2014, Swift *et al* 2013) and this nuclear network possesses a functional homology with the cell surface mechanosensitive proteins at the cell membrane. What is the impact of the cell architecture on the force-dependent mechanisms at the nuclear envelope? Why are nuclear processes required for the cell adhesion through integrins or for the cell migration?

10.4.8 The nuclear envelope and the cell membrane share similarities in their mechanosensitivity

As the nuclear envelope is located deep inside the cells interior mass, it can be questioned whether it experiences the mechanical stresses evoked by the surrounding

cellular microenvironment. However, the nuclear envelope, like the cell membrane, can explore pronounced tensile and compressive forces that arise from the cell's cytoskeleton and/or from the cell's microenvironment (Wang *et al* 2009, Maniotis *et al* 1997). Despite the major differences in the structure and trafficking between the cell membrane and the nuclear membrane, both membranes contain protein complexes that can alter their activity and/or interaction by external mechanical stress. Based on the coevolution of the nuclear envelope and the endomembrane systems of the endoplasmatic reticulum, the mechanosensitivity may also be highly similar (Baum and Baum 2014) and is best exemplified by the LINC complex altering due to tension (Kutscheidt *et al* 2014, Guilluy *et al* 2014) and highly similar to alterations of the cell surface adhesion. However, the LINC complex is not the only candidate for a force-sensitive structure at the nuclear envelope. Hence, the tension within the nuclear membrane or through the lamina can evoke molecular responses similarly to that at the cell's surface. Although these mechanisms are mostly addressed separately, they are interdependent and function in a coordinated manner.

10.4.9 LINC complex reinforcement

While spanning the nuclear envelope, the LINC complex is built by the association of nesprin and SUN (Sad1-UNC-84) proteins and provides a physical connection between the cytoplasm and the nucleoplasm. As nesprins are associated with the outer nuclear membrane, they bind directly or indirectly with all three types of cytoskeletal filaments, i.e. actin filaments, microtubules and intermediate filaments, and can even interact with SUN proteins in the perinuclear space through their KASH domain. In particular, SUN proteins are located in the inner nuclear membrane and bind to lamins and nucleoplasmic components (Zhen *et al* 2002, Padmakumar *et al* 2005, Wilhelmsen *et al* 2005, Crisp *et al* 2006, Haque *et al* 2006, Schmitt *et al* 2007). In several aspects, the LINC complex is similar to integrin-based adhesions. In both cases, transmembrane proteins in the lipid membranes, such as nuclear nesprin–SUN, or cell surfaced located integrins enable the transmission of forces between two compartments: on the one hand through the interaction with the cytoskeleton on one side and with a fibrous extracellular matrix protein network on the other side, and on the other hand with the cytoskeleton on one side and the nuclear matrix on the other side (Berezney and Coffey 1974). Moreover, these complexes are able to form aggregates and colocalize with actomyosin filaments. The clustering of integrin-based adhesions was revealed decades ago, and the identification of LINC complex clusters was achieved more recently (Luxton *et al* 2010) and these have been termed TAN lines. Beyond the structural homology of the LINC complex with focal adhesions, it has been shown that the LINC complex behaves similarly to cell surface adhesions and can adapt its response to tensional application (Kutscheidt *et al* 2014, Guilluy *et al* 2014) and hence evoke a strengthening of the connection between the cytoskeleton and the lamina.

In analogy to integrin-based adhesions, the LINC complex is able to undergo reinforcement due to tension caused by distinct but complementary modalities. On the one hand, increased connection with the actin cytoskeleton is critical for the

reinforcement. In more detail, nesprin-2G undergoes actomyosin-generated tension that has been reported using a fluorescence resonance energy transfer-based tension biosensor (Arsenovic *et al* 2016). The formin FHOD1 has been demonstrated to interact with nesprin-2G, which enhances the avidity for the binding of actin by exposing an additional binding site (ABS) and potentially by gathering nesprin-2G molecules through the FHOD1 dimer formation (Kutscheidt *et al* 2014). Moreover, this strengthening response is suggested to provide the resistance towards the substantial force that is required to mobilize the nucleus (Kutscheidt *et al* 2014). On the other hand, the formin mDia has been shown to first alter the integrin-based adhesion maturation due to tension, although here the FH2-driven actin elongation is responsible, rather than the actin binding (Riveline *et al* 2001, Schiller *et al* 2013). In addition, tension can promote the emerin-dependent recruitment of myosin and the polymerization of actin filaments (Huy *et al* 2016) at the outer nuclear membrane, which then increases the interaction of the LINC complex with actin. Second, the transmembrane protein association due to tension is modified. In particular, the actomyosin filaments have revealed to be colocalized with clustered LINC complex components, which create the TAN lines (Luxton *et al* 2010). Moreover, the inhibition of myosin impaired the formation of nesprin-2G linear arrays, indicating that, similarly to integrins (Chrzanowska-Wodnicka and Burridge 1996), nesprin aggregates due to actomyosin contractility (Nagayama *et al* 2014). The structure of the SUN–KASH complex has been identified (Sosa *et al* 2012) and involves the binding of three KASH domains to trimeric SUN proteins. In particular, SUN proteins possess coiled-coil domains that provide the regulation of the accessibility of the SUN-domain and the SUN protein multimerization (Nie *et al* 2016). However, it needs to be known whether mechanical tension can induce intramolecular interaction between coiled-coil domains and the SUN domain and hence favor the 'active' SUN conformation, in a similar manner as has been described for integrins (Schwartz 2009). Third, there exists an increased connection with lamina. Using isolated nuclei, the application of tensional forces to nesprin-1 has been shown to trigger an enhanced association of lamin A/C with the LINC complex (Guilluy *et al* 2014), which in turn reduces the strain and elevates the resistance to the applied tension. Whereas it has been found that the force-dependent emerin phosphorylation is necessary, the molecular mechanisms for the response to tension are not fully clear, but they seem to be associated with increased interaction between SUN proteins and lamin A/C. Cells lacking lamin A have been revealed to assemble less stable TAN line slipping around the apical surface of the nucleus (Folker *et al* 2011), which shows that the composition of the lamina can affect the response of the LINC complex to tension. However, the lamina is able to restructure due to cell-generated contractility through the lamin A/C stabilization or its recruitment to the nuclear envelope (Buxboim *et al* 2014), which leads to the hypothesis that the arrangement of the lamina even promotes the reinforcement of the LINC complex. The tension-driven LINC complex reinforcement may provide an adaptive mechanism to withstand the force applied by cytoskeletal filaments, thereby providing a reliable nuclear anchor. In addition, the mechanical tension is

transmitted through the LINC complex to the nuclear membrane, where it is transferred into biochemical signal transduction pathways.

10.4.10 Nuclear membrane tension

The membrane tension within the cell membrane is a key factor in the process of mechanotransduction (Diz-Munoz *et al* 2013), where the membrane tension is transmitted to lipid-anchored protein complexes inducing the signal transduction pathways. Similar to the cell membrane, the nuclear membrane can be subjected to tension, which arises from the surrounding cytoskeleton or directly from the external microenvironment. Hence, the nuclear membrane can deform to a certain extent that relies on the amount of membrane reservoir available and the mechanical properties of the surrounding cytoskeletal network scaffold (Diz-Munoz *et al* 2013). The nuclear membrane storage such as the endoplasmic reticulum, which is continuously wrapped around the outer nuclear membrane, can accommodate rapid alterations in nuclear membrane tension and hence restrict the consequences evoked by tensional stress such as nuclear rupture. Hence, intranuclear invaginations of the nuclear envelope, termed the nucleoplasmic reticulum, have been identified in various cell types (Malhas *et al* 2011) and may represent a membrane reservoir in cells, in which the nuclear envelope is subject to frequent or strong mechanical stress. Using micropipette aspiration for the investigation of swollen and unswollen nuclei, the extent of the deformation of the nuclear envelope revealed that the lamina provides an extensible network that is able to damp the mechanical stress (Dahl *et al* 2004, 2005). Indeed, chromatin contributes to the deformability of the nuclear membrane based on its interaction with the nuclear lamina (Dahl *et al* 2004, 2005).

At the cell membrane, tension can be transferred into biochemical pathways through many different mechanisms, such as curvature sensing proteins, the translocation of proteins to the membrane and mechanosensitive channels (Diz-Munoz *et al* 2013). All these structures may possess nuclear counterparts that can transfer the membrane tension into nuclear signal transduction pathways. Firstly one option is curvature sensing proteins and the translocation of proteins. Lipid composition between the nuclear membrane and the cell membrane (van Meer *et al* 2008) can alter the processes for protein recruitment, which prefers hydrophobic rather than electrostatic interactions (Bigay *et al* 2012). In particular, the ALPS domain, which represents a well-known membrane curvature sensor, has been recognized in the NPC component Nup133 and facilitates the membrane anchoring and assembly of the NPC (Kim *et al* 2014). In line with this, the phospholipase A2 (PLA2) is able to translocate from the nucleoplasm to the inner nuclear membrane due to excessive membrane tension, which followed nuclear swelling (Enyedi *et al* 2016). In *in vivo* experiments it has been demonstrated that this mechanotransduction mechanism triggers the eicosanoid synthesis and the process of inflammation as a response to tissue damage (Enyedi *et al* 2016). Moreover, excessive nuclear membrane tension can lead to damage to the nuclear integrity and may thereby affect cell survival, as has been seen when cells migrate in confined microenvironments (Raab *et al* 2016,

Denais *et al* 2016). When substantial physical stress is exerted on the nuclear envelope during the migration of cells through narrow confined interstitial spaces, the nuclear envelope can be ruptured and subsequently a repair mechanism can be triggered through the recruitment of the endosomal sorting complexes required for transport III (ESCRT III) machinery. Secondly, another option is the mechano-sensitive channels. Nuclear pore complexes are large protein assemblies that build aqueous channels within the nuclear envelope, where the two membranes, the outer nuclear membrane and inner nuclear membrane, are fused. The nuclear pore complexes enable the nuclear import and export of molecules through a passive or active diffusion and are closely connected to the chromatin organization and the translation of mRNAs, as they provide the mRNA export (Kabachinski and Schwartz 2015, Knockenhauer and Schwartz 2016). Moreover, the transition of the mechanical stress to the nuclear envelope has been hypothesized to alter the nuclear pore complex structure, which may be regulated by adapting the size of the nuclear pore (Wang *et al* 2009). If this turns out to be true, both the nuclear membrane tension and cytoskeletal tension function coordinately to perform nuclear pore complex deformation. However, SUN1, but not SUN2, can bind to the nuclear pore complexes (Liu *et al* 2007) and connect the nuclear pore complexes with the lamina or the LINC-associated cytoskeletal filaments. Nuclear pore complexes are connected to a filamentous network, the so-called pore-linked-filaments (PLF) (Cordes *et al* 1993, Kiseleva *et al* 2004), which may link it directly to other nucleoplasmic components. Alterations in the dimension of the transport channel have been observed in cells grown at different confluence levels (Feldherr and Akin 1990). However, apart from the apparent flexibility of the constituents of the nuclear pore complex (Knockenhauer and Schwartz 2016), whether the transport through these complexes is sensitive to mechanical stress transmitted at the nuclear envelope still remains elusive and needs to be determined. In addition, mechanosensitive calcium channels have been detected in the outer nuclear membrane at a similar surface density as at the cell membrane of cardiac myocytes (Itano *et al* 2003). Moreover, nuclear calcium levels are found to be elevated in cells adhering to rigid surfaces (whereas when the cells adhere to soft substrates, the calcium levels are not altered) and the enhanced calcium levels are related with alterations in the nuclear shape (Itano *et al* 2003). The alterations in the calcium levels within the nucleoplasm may cause various types of nuclear signaling, such as the induction of gene expression through the activation of the calmodulin-dependent kinase IV, increased apoptosis or the import of proteins (Malviya and Rogue 1998).

10.4.11 Lamina remodeling

The A-type and B-type lamins belong to the intermediate filaments that assemble the lamina scaffold underneath the inner nuclear membrane (figure 10.13) (Osmanagic-Myers *et al* 2015) and therefore they provide the mechanical properties of the nuclear envelope (Dahl *et al* 2004, Osmanagic-Myers *et al* 2015). In particular, lamins play a role in numerous different nuclear processes, regulate the structure of chromatin and control the gene expression (Osmanagic-Myers *et al* 2015) through

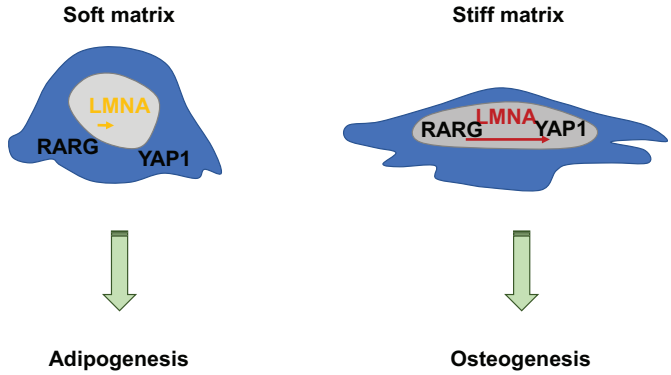

Figure 10.13. Lamin type A affects the cell fate.

both lamina-dependent or -independent pathways (Dechat *et al* 2010). Indeed, dynamic rearrangements of lamin A/C has been detected due to mechanical stress stimulation.

It has been shown that lamin A/C levels are increased with elevated tissue elasticity (Swift *et al* 2013) *in vivo*. In line with this, the myosin-generated contractility causes the dephosphorylation of lamin A/C at Ser22, which provides its stabilization (Buxboim *et al* 2014), and indeed results in enhanced stiffness of the nuclear envelope and elevated expression of the serum response factor target genes. The lamin A/C enrichment at the nuclear envelope has been found to occur due to shear stress (Philip and Dahl 2008). The mechanical tension can directly alter the conformation of lamin A/C (Swift *et al* 2013, Ihalainen *et al* 2015). The exertion of shear stress to isolated nuclei induced a partial unfolding of the lamin A immunoglobulin (Ig)-domain (Swift *et al* 2013), which seems to alter its phosphorylation state and subsequently its stability. Using antibodies targeting the conformational epitopes of C-terminal Ig-domain and the N-terminus of A-type lamins, it has been revealed that these two epitopes are not accessible at the basal nuclear envelope in cells, in which the nuclei are confined and hence compressed by an actin cap. The tension-driven reversible unfolding of Ig-domain or the structurally related fibronectin 3 domain have been found in other proteins that are associated with the mechanotransduction pathways (Ihalainen *et al* 2015). However, it needs to be investigated whether the force-dependent lamin A/C conformation exposes a cryptic site that impacts the interaction between lamin molecules. Indeed, this specific mechanism has been detected in the extracellular matrix, where the tension induces the fibronectin fibril formation (Zhong *et al* 1998) that is driven through the exposure of new (formerly hidden) intermolecular binding sites. In addition, it has been shown that the heterochromatin connection to the lamina is altered by tension through an emerin-dependent regulation of the nuclear actin levels (Huy *et al* 2016), which finally causes the regulation of gene expression. However, whether the chromatin interaction with the lamina is involved in the dynamic alterations in the mechanical properties of the nuclear envelope remains not fully understood.

10.4.11.1 Effect of nuclear remodeling on cell adhesion to extracellular matrix

What are the force-dependent nuclear envelope mechanisms during cell adhesion and migration? During cell adhesion and migration, the force-dependent mechanisms exerted on the nuclear envelope are not exclusively altering the structure and function of the nucleus, but also affect the neoplasmic architecture through physical contact with load-bearing components or through the controlling of signaling processes, thus orchestrating an autonomous response that is not yet integrated.

When cells adhere to flat 2D surfaces, the actomyosin filaments exert mechanical tension towards the nucleus, which alters the morphology of the nucleus (Sims *et al* 1992). A dome-like actin cap, which consists of contractile actin filament bundles that wrap the apical side of the nucleus, can provide the nuclear flattening (Khatau *et al* 2009) and thereby compresses to the nuclear envelope and exerts tension to the LINC complex (Nagayama *et al* 2014, Arsenovic *et al* 2016, Ihalainen *et al* 2015). While cells spread in the early phase of adhesion (up to 20 minutes), the nuclear flattening seems to be facilitated by additional contractility-independent mechanisms. Indeed, it has been demonstrated that in fibroblasts the nucleus flattens without myosin activity (Li *et al* 2015). Hence, the nuclear flattening may be a consequence of the altered cellular shape, as the intervening cytoskeletal network needs to withstand cellular expansion or compression (Li *et al* 2015). A plectin-associated dense network of intermediate filaments can control the nuclear shape in keratinocytes, which is independent of the LINC complex (Almeida *et al* 2015). Taken together, two successive mechanisms are proposed to alter the nuclear shape during cell adhesion. The first mechanism seems to be a LINC-independent and contractility-independent flattening of the cell, in which all cytoskeletal filaments are involved (up to 20 minutes). The second mechanism seems to be the generation of actomyosin contractility which is exerted on the LINC complexes. In terms of the nuclear envelope, these two phases cause diverse consequences. At the onset of cell spreading, the LINC-independent alterations of the nuclear shape prefer the nuclear deformation by restricting the remodeling of the lamina and LINC complexes. Subsequently, the cell-generated tension causes the LINC reinforcement (Guilluy *et al* 2014) and the polarization and hence stabilization of the lamina (Swift *et al* 2013, Buxboim *et al* 2014, Ihalainen *et al* 2015) to withstand the larger cytoskeletal tension evolving in the late adhesion of the cell. An adhesion maturation has been detected upon the application of pulling forces to the nucleus (Riveline *et al* 2001). This result suggests that reinforcement of the LINC complex causes the maturation of the adhesion by providing the attachment of contractile filaments. Moreover, force-induced LINC remodeling may alter gene expression during the later stages of cell adhesion. The mechanical tension leads to an emerin-dependent reduction of the nuclear actin levels that results in impaired transcription and accumulation of H3K27me3 histone methylation at facultative heterochromatin (Huy *et al* 2016), which is in line with other reports showing that the LINC complex controls the organization of chromatin (Hernandez *et al* 2016, Kim and Wirtz 2015).

Moreover, other nuclear envelope force-dependent mechanisms can affect the gene expression due to cell contractility, such as the stabilization of the lamina (Buxboim *et al* 2014) or nuclear mechanosensitive calcium channels leading to

elevated levels of the nucleoplasmic calcium levels during the cell spreading, which subsequently increases the myocyte enhancer factor 2 transcription (Itano *et al* 2003). Cell-generated contractility exerted on the nuclear envelope can induce proliferation (Versaevel *et al* 2012, Nagayama *et al* 2015), although the involvement of force-dependent mechanisms has not been ruled out and requires further investigation.

10.4.11.2 The effect of nuclear remodeling on cell migration

The mechanisms regulating the nuclear positioning during individual single cell migration can be based on all three types of cytoskeletal elements (Gundersen and Worman 2013) and depend on many other factors such as the type of migration, the mechanical properties of the microenvironment and the specific cell type. Microtubules and their associated molecular motors are able to transmit shear forces towards the nuclear envelope that induce the rotation and translocation of the nucleus. Actomyosin-driven nuclear movement exerts a variety of forces (Alam *et al* 2015), which either pull or push the nucleus (Alam *et al* 2015, Wu *et al* 2014) and facilitate the centrosome orientation through the TAN lines (Luxton *et al* 2010, Kutscheidt *et al* 2014). The migration of isolated fibroblasts has shown that the actomyosin contraction causes the nuclear translocation (Alam *et al* 2015). In line with this, the actin cap has been observed to drive the nuclear translocation and favors the persistent motion of cells, whereas the dynein-facilitated nuclear rotation induces alterations in the migration direction (Kim *et al* 2014). Independent of TAN lines or actin cap structures, the actomyosin tension causes the LINC rearrangements that fulfill a central role to withstand tension (Kutscheidt *et al* 2014). Beyond affecting the nuclear positioning, the altered LINC connection affects the entire cellular architecture, which leads to trailing edge defects in cellular de-adhesion (Alam *et al* 2015), changing the cell speed and impairing persistence (Kim *et al* 2014). In addition, the actin cap-associated adhesions are highly sensitive to alterations in the matrix rigidity (Kim *et al* 2012), which indicates that the LINC complex and its associated cytoskeleton play a role in durotaxis. The LINC complex also fulfills a key role in the organization of 3D cell migration and invasion. Defective LINC complexes lead to impaired actin-based protrusions and reduced matrix tractions (Khatau *et al* 2012). The actomyosin-coupled vimentin filaments can pull on the nucleus, which behaves similarly to a piston and can generate a forward compartment of high hydrostatic pressure that can provide lamellipodia-independent cellular displacements (Petrie *et al* 2014). As the forward compartment in turn exerts compressive forces to the anterior face of the nuclear envelope, it seems to be crucial to determine the lamina polarity and the exposure of the usually hidden 'conformation' epitope. In particular, lamina stiffness is critical for 3D migration efficiency (Harada *et al* 2014). However, the migration through constrictions such as narrow pores induces the formation of a perinuclear Arp2/3-dependent branched actin network, which causes the disruption of the lamina within dendritic cells (Thiam *et al* 2016) and hence induces the deformation of the nuclear envelope. The inhibition of myosin has no further effect on this response, which leads to the hypothesis that this perinuclear actin structure does not display contractile behavior.

Does the actin network alter the tension of the nuclear membrane or the successive cell membrane, or the friction between the two membranes?

The mechanosensitive structures of the nuclear envelope transduce mechanical signals and thereby dynamically control the cellular organization during cell adhesion, migration and many other functions associated with alterations in nuclear position or shape. The key role of the cell architecture seems to be manifested by the myriad of cellular defects that are correlated with genetic diseases involving nuclear envelope proteins (Gundersen and Worman 2013). An inside–out signaling model has been provided to explain the origin of the cell's nucleus (Baum and Baum 2014). In this model, the outer nuclear membrane, the cell membrane and the cytoplasm are derived from extracellular protrusions and preceded the cell membrane fusion. Based on this model, the LINC complex components seem to originate from archaeal S-layer glycoproteins, proposing that the development of mechanosensitive structures at the nuclear membrane may have occurred prior to the emergence of those at the cell membrane, which means that the cell surface reflects the nuclear envelope.

10.5 Nuclear positioning and cell polarization in cell migration

The direction and persistence of cell migration is provided by the cellular polarization and the positioning of the nucleus inside the cytoplasm of the cell, which is predominately located towards the rear during the process of cell migration (Kim *et al* 2012). The cell polarization is a morphological asymmetry of cells that promotes the cell motility function. For cells migrating on 2D substrates the nucleus is usually behind the centrosome (synonymously termed the microtubule organizing center (MTOC)). In geometric confinement and *in vivo* some migrating cells localize the centrosome behind the nucleus. Cells migrate on 2D substrates with the centrosome in front of the nucleus, whereas cells migrating on thin fibronectin adhesive lines localize the centrosome behind the nucleus (Pouthas *et al* 2008). Similar to the migration under confinement, fibroblasts migrating in 1D and 3D microenvironments possess the centrosome at the back and hence behind the nucleus (Doyle *et al* 2009). Using micropatterned substrates, it has been revealed that the anisotropy of the adhesive microenvironment directs the cellular polarity (Théry *et al* 2006). Moreover, the centrosome is usually located near the cell centroid and the nucleus is located mainly off center towards the rear end of the cell, where lower cell adhesion and contractile cell edges are present. Moreover, the polarized microtubule network supports the maintenance of the cellular polarization and hence promotes the persistent migration. The polarized trafficking of migration associated molecules along microtubules seems to be crucial for the persistent migration (Etienne-Manneville 2013). Indeed, the mechanisms determining the orientation of the centrosome in relation to the nucleus are important for the direction and persistence of cellular motion.

The positioning of the nucleus is based on the interactions with actin, microtubules and intermediate filaments (Kim *et al* 2012, Starr 2009, Tsai *et al* 2007, Wu *et al* 2011). In particular, these interactions are facilitated by nesprins linking the

nucleus to actin filaments, motor proteins and intermediate filaments. Molecular motors such as dynein, which move along microtubules can exert active forces on the nucleus (Wu *et al* 2011). In migrating neuronal cells, the microtubules fulfill an important role in the positioning of the nucleus and its translocation (Kim *et al* 2012). Lamins are similarly important for cellular polarization, as lamin A/C deficient cells cannot correctly polarize (Lee *et al* 2007). The nuclear position is not independent of the centrosomal positioning, as they are physically connected through kinesin and dyneins (Fridolfsson *et al* 2010). In particular, dynein molecular motors at the cell periphery pull on the microtubules, which represents a key feature for the positioning of the centrosome (Elric and Etienne-Manneville 2014).

The actin filaments fulfill a key role in the nuclear positioning process, as the movement of the nucleus to the cell's rear end in migrating fibroblasts relies on actin, nesprin and SUN proteins that are assembling the TAN lines (Luxton *et al* 2010). As the nucleus is linked to actin, it is transported to the cell's rear, when an actin retrograde flow is present. Moreover, the actomyosin contraction at the cell's rear end seems to push the nucleus in the direction of the leading edge and additionally, the actomyosin contraction at the cell's front pulls the nucleus in the direction of motion (Wu *et al* 2014).

Alterations in migration direction can utilize the nuclear rotation to reorient the polarization axis, which keeps the nucleus behind the centrosome. The nuclear rotation can be detected under various conditions and seems to be dynein-activity-dependent (Wu *et al* 2011, Gerashchenko *et al* 2009, Levy and Holzbaur 2008), which may be based on the tension between the centrosome and the nucleus that is evoked by the dynein moving along the microtubules (Wu *et al* 2011). However, multiple cell types perform alternating phases of fast persistent migration and slow low persistent migration. The fast and persistent migration depends on an intact actin cap, whereas alterations in the direction of cell migration seem to be based on dynein-dependent nuclear rotation, while the actin cap transiently vanishes (Kim *et al* 2014, Razafsky *et al* 2014). Alternatively, the actomyosin contractility can provide the nuclear rotation, when the nucleus is seen as a simple passive inclusion (Marchetti *et al* 2013, Kumar *et al* 2014, Gomes *et al* 2005). It has been found that micropatterns of adhesive stripes induce persistent migration, actin cap formation and elongated nuclei, while circular micropatterns impair the movement and cause a dynein-based rotation of the rounded cell nuclei (Kim *et al* 2014). In line with this, cells migrating along synthetic nanofibres display nuclear elongation, a specific cellular velocity and persistence of motion (Meehan and Nain 2014). Indeed, the actin bundles in elongated cells seem to compress the nucleus leading to nuclear elongation (Kim *et al* 2014, Khatau *et al* 2009, Versaevel *et al* 2012). As stress fibers are coupled to the extracellular matrix, the elongation of the nucleus and cellular polarization are both affected by the microenvironmental cues. Using micropatterns, it has been found that alterations in the local adhesion impairs the orientational ordering of stress fibers across the entire cell, which indicates that cells react to local mechanical cues at the whole cell level (Khatau *et al* 2009, Kim *et al* 2013, Mandel *et al* 2014).

10.6 Nucleus–cytoskeleton connection dependent cell migration

As the position of the nucleus is crucial for cell polarization, it also sets the direction of the motion. Moreover, the nucleus plays a key role in the migration and invasion of cells (Khatau *et al* 2012). In particular, the nucleus–cytoskeleton linkages are necessary for the migration in soft 3D extracellular matrices mimicking tissue microenvironments. Cells lacking lamin A/C can still migrate on a collagen coated 2D glass substrate, but when they are embedded within a 3D collagen fiber matrix the migration speed is pronouncedly decreased compared to the speed of wild-type counterparts. The same effect can be seen when either the LINC complexes or the nesprins are ruptured. Hence, the rigidity of the nucleus and nucleus–cytoskeleton linkages are crucial for the migration within 3D collagen fiber matrices, whereas they are less important for the crawling of cells on flat 2D glass substrates. However, it remains elusive as to what extent this effect depends on the difference in the rigidity of the two microenvironments or on the difference in the confinement geometry. Moreover, the integrity of the nucleus and nucleus–cytoskeleton linkages affect the cell migration on 2D substrates. In wound healing assays, lamin A/C deficient cells exhibit decreased migration speeds (Lee *et al* 2007). In a similar manner, nesprin-1 lacking endothelial cells display slower migration speeds (Chancellor *et al* 2010). The depletion of nesprin-1 evokes enhanced focal adhesion assembly, increased cellular traction generation and elevated nuclear height, whereas the migration speed is reduced. Thus, these results lead to the hypothesis that nesprin-1 facilitated forces on the nucleus cause a flattening of the entire nucleus and subsequently changes the morphology of the nucleus to a disk-like shape in endothelial cells. What effect may the inhibition of actomyosin facilitated forces have on the nuclear shape? The inhibition of myosin causes a comparable elevation of the nuclear height. The nucleus has hence been assumed to balance the tension based on an actomyosin cytoskeleton, but not that of the nesprin-1 evoked tension, which is balanced by the substrate (Chancellor *et al* 2010).

10.7 Cell squeezing through constrictions

Many investigations on the nuclear impact on cellular migration have been performed using various confinements, such as adhesive micropatterning (Théry 2010), 1D migration along synthetic nanofibres (Meehan and Nain 2014), 2D confinement (Le Berre *et al* 2012), microfabricated microchannels (Heuzé *et al* 2011, Lautenschlaeger and Piel 2013), micropillars (Saez *et al* 2007, Isermann *et al* 2012), invasion into 3D collagen fiber matrices (Mierke *et al* 2011a, 2011b, Koch *et al* 2012, Mierke *et al* 2017, Kunschmann *et al* 2017, Fischer *et al* 2017), embedding in matrices such as collagen (Petrie and Yamada 2015, Lämmermann *et al* 2008, Wolf *et al* 2009) and *in vivo* studies (Wolf *et al* 2009). Which of these techniques are optimally suited to reveal the special function of the nucleus? This question cannot yet be fully answered, as these different methods have not been compared in combined studies of the phenomenons of the nucleus during cell migration.

Using microfabrication techniques, it has been demonstrated that the cells moving between micropillars, in channels and through constrictions of controlled

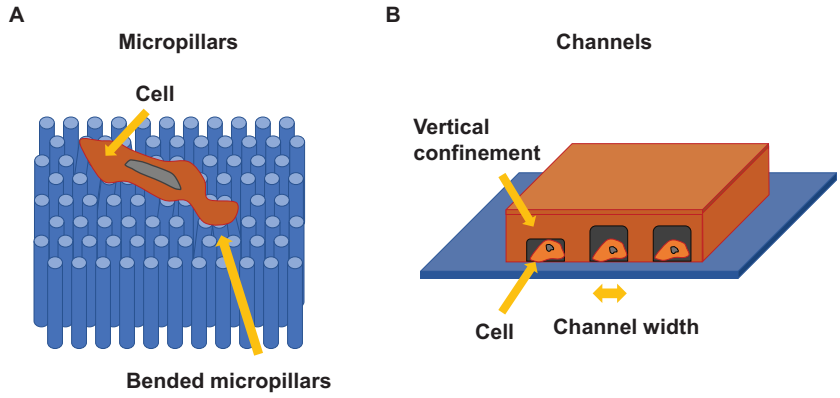

Figure 10.14. Mechanical confinements for cell migration such as micropillars (A) and channels (B) produced with microfabrication techniques.

geometry are not driven by flow (figure 10.14) (Diz-Munoz *et al* 2013, Isermann *et al* 2012). In addition, microfluidics methods, which indeed impose flow (the so-called deformability cytometry) are employed to investigate cell deformations and stiffness (Otto *et al* 2015). As expected, the dynamical remodeling of the nucleus represents the rate limiting factor for the cells squeezing through narrow constrictions (Raab *et al* 2016, Denais *et al* 2016). Cells need to deform their nuclei to pass through narrow constrictions smaller than the size of the nucleus. In normal cells, it is more difficult to deform the nucleus compared to the rest of the cell, such as the cytoplasm, however, lamin A/C deficient cells contain more deformable nuclei (Lammerding *et al* 2004). Cancer cells frequently possess lamin mutations and contain softer nuclei, and thus can pass more easily through smaller structural confinements (Raab *et al* 2016). How the cell can generate these forces to deform the nucleus is not yet clearly understood and the mechanisms are still elusive. Moreover, it is not fully revealed whether the nucleus is pushed or even pulled through these constrictions. It seems to be possible that more adherent cells pull the nucleus and less adherent cells push the nucleus (Schwarz 2009). The mechanisms responsible for the movement of the nucleus through a narrow constriction seem to involve an actomyosin contraction at the cell's leading edge or rear (Raab *et al* 2016, Kabachinski and Schwartz 2015) and the polymerization of actin (Thiam *et al* 2016). Additionally, the nuclear lamina may be broken when the cells migrate through small constrictions (Feldherr and Akin 1990, Thiam *et al* 2016).

10.8 Models of the nucleus during cell migration

In the past, the nucleus has often been ignored in models of cell motility, as it is assumed to be purely passive and hence not the driving force in cell migration and invasion. However, the nucleus seems to perform an important role in cell migration. The nucleus is, in some studies, not modeled explicitly and hence is simply included as a passive load with a corresponding frictional drag (van Meer *et al* 2008). In order to model the nucleus explicitly, the simplest model that can be used, treats the entire

nucleus as a deformable linearly elastic sphere (such as a linear elastomer) and the maximum deformation of the nucleus at a bottleneck is calculated in a quasi 1D model (Le Berre *et al* 2013). The energy necessary to deform the nucleus from an initial spherical shape to an ellipse or rod shape fitted into a cylindrical channel has been performed (Giverso *et al* 2014). In particular, two models for the nucleus have been tested: the first one assumes the nucleus as a liquid droplet that is surrounded by an elastic shell and the second one treats the nucleus as an incompressible elastic solid. Moreover, another continuum model has been applied to the nucleus that assumes it as an encapsulated liquid drop (Leong *et al* 2011). Both, the nucleus and entire cell are modeled as Newtonian fluids, which are enveloped by elastic interfaces that can be simulated using the immersed boundary method.

A more sophisticated engineering approach has been employed by using a finite element method to model the cytoplasm and nucleus in a 2D simulation (Aubry *et al* 2015). The nucleus is modeled as a viscoelastic material that can be described with the Maxwell model, which is enveloped by an elastic circle mimicking the lamina. Moreover, agent-based models such as the cellular Potts model have also been employed (Scianna and Preziosi 2013), which uses a Metropolis Monte Carlo method to stochastically simulate cells and nuclei with a lattice-based model, and the deformation of their shape costs energy. These two different models have emerged as a combined hybrid agent-based finite element model for cellular motility (Tozluoğlu *et al* 2013). Future analytical and numerical studies including the nucleus would be useful to provide experimental data analysis and help to unravel the role of the nucleus in the process of cell migration (Estabrook *et al* 2016, Fruleux and Hawkins 2016).

In addition to the knowledge of diverse cellular motility mechanisms and the mechanical properties of the nucleus, such as its coupling to the cytoskeleton and indirectly to the extracellular matrix microenvironment, the organization of the actin cytoskeleton and its coupling to the nucleus and the extracellular matrix has also been addressed. Despite this knowledge, the role of the microtubules in cell migration is less well understood and remains to be investigated. Although the coupling of microtubules to the nucleus is known, the coupling of microtubules to the extracellular matrix and the coupling between the centrosome and the nucleus remain elusive. Indeed, the role of microtubules during cellular migration and invasion has not yet been studied extensively despite its emerging important role in cellular mechanical properties and cell migration (Balzer *et al* 2012). As the role of microtubules seems to have a major impact on providing cellular polarization, it is unclear whether the position of the centrosome is the cause or an effect of cellular polarization.

It is clear that the nucleus needs to deform in cells migrating through small constrictions. What are the mechanisms by which cells push or pull the nucleus through these small constrictions? This is not yet clearly understood. In the field of cellular mechanotransduction there are many unanswered questions: How is the expression of genes altered by mechanical cues? How does the mechanotransduction of forces from the external microenvironment towards the nucleus challenge gene expression? There is still a long way to go before we understand the process of mechanotransduction in detail and refine the biophysical methods needed to analyze the mechanical properties of the microenvironment and their response in cells in 3D confinements.

Despite the great knowledge of many details on the microscopic length scale, there remain still questions in terms of integrating this knowledge at the cellular length scale. From a material physicist's point-of-view, how does the presence of the nucleus regulate the migration of the cell? When the nucleus is modeled as a passive inclusion with an excluded volume, how can the cytoskeleton flow around it? How does the nucleus constrain the cytoskeletal remodeling dynamics?

In summary, there is still a lot to be learnt about the effects of physical properties of nuclei in diseases and how the mechanical properties are correlated with cellular migration such as the motility of metastatic cancer cells during the malignant progression of cancer. Moreover, this will lead to the discovery of new approaches for diagnosis of cancer and the development of therapies in the future. However, there is still much to be discovered to fully understand the physical roles of the nucleus during migration and invasion of cells in 3D constrictions.

10.9 Nucleoskeleton

In multicellular organisms, the migration of cells is required for the developmental processes of tissues and organs, the maintenance and repair of various tissues (Munjal and Lecuit 2014). Cell migration enables immune cells to travel through tissues and respond to local challenges such as acute or chronic inflammations (Weninger *et al* 2014). Under pathological conditions, cell migration supports the invasion of cancer cells through tumor surrounding stroma and targeted tissues as well as the subsequent metastasis, which is the cause of the vast majority of cancer deaths (Chaffer and Weinberg 2011, Athirasala *et al* 2017). As most of the current knowledge regarding the molecular and biophysical principles of the migration of cells, is still based on studies involving cell migration on stiff 2D substrates (Gardel *et al* 2010), it is necessary to compare these results with the behavior of cells migrating in 3D microenvironments encountering distinct physical loadings. During the *in vivo* migration and invasion of cells, they need to navigate through multiple microstructural obstacles such as dense extracellular matrix networks and neighboring embedded cells. In particular, the pore sizes in the interstitial space range from 0.1 to 30 μm in diameter, which are comparable to the migrating cell's size or even significantly smaller (Doerschuk *et al* 1993, Stoitzner *et al* 2002, Weigelin *et al* 2012). Cells can employ at least two distinct strategies to migrate through such a confinement. First, the cells can expand the openings of the matrix scaffold through a physical remodeling and/or the proteolytic degradation of the extracellular matrix (Stetler-Stevenson *et al* 1993) or, second, the cells can convert their cell shape to squeeze through the available space (Wolf *et al* 2003). The cell membrane and cytoplasm are able to undergo a fast deformation and remodeling process to penetrate through the narrow openings of less than 1 μm in diameter (Wolf *et al* 2013). In contrast, the deformation of the nucleus represents a more tremendous challenge (McGregor *et al* 2016). However, the following main question is still left unanswered. How is the nucleus translocated by the intracellular biomechanical properties?

10.9.1 The size and rigidity of the nucleus: a physical barrier for cell migration

As the nucleus is the largest organelle in the cell with a diameter between 3 and 15 μm (Lammerding 2011, Martins *et al* 2012), it is hence even larger than many

pores that it faces during the migration through physiological tissues. In addition, biophysical measurements of isolated nuclei and intact cells have shown that the nucleus is generally 2–10 times stiffer than the surrounding cytoplasm (Lammerding 2011). Since the nucleus is large and stiff, it has been hypothesized that it pronouncedly affects the cell's capacity to migrate and invade in extracellular matrices (Friedl *et al* 2011). This hypothesis has been confirmed by cancer cells squeezing and migrating through microfabricated artificial channels with defined microconstrictions (Tong *et al* 2012, Fu *et al* 2012, Balzer *et al* 2012). A moderate confinement leads to increased cell migration speed which allows cells to utilize faster migration modes such as the amoeboid migration and chimneying compared, to migration on flat and stiff 2D substrates (Liu *et al* 2015). However, constrictions below approximately 5 μm in diameter require substantial nuclear deformation and hence lead to impaired migratory capacity, which is indicated by reduced migration speeds (Tong *et al* 2012, Fu *et al* 2012, Davidson *et al* 2014, 2015, Lautscham *et al* 2015). In the absence of cell–substrate adhesions through extracellular matrix proteins such as fibronectin, the cells form blebs and perform chimneying between the gel sheets. The orientation of the traction forces and the magnitude of these forces can be determined by traction force microscopy, as expanding blebs push into the surrounding gels and generate thereby anchoring stresses whose magnitude increases with reducing gap size, whereas the migration speed has its highest levels at an intermediate gap size. In order to decipher why an optimal gap size emerges for migration, a computational model has been developed, which revealed that the chimneying speed depends on both the magnitude of the intracellular pressure and on the distribution of blebs on the cell's membrane. Moreover, the model predicts an increase in the optimal gap size due to the softening of the membrane through a weakening of the adhesion strength between the cell membrane and the actin cortex. Indeed, this has been verified by an experiment involving weakening of the cell membrane–cortex adhesion strength through addition of the ezrin inhibitor baicalein. Indeed, the chimneying mode of amoeboid migration is based on a precise balance between the intracellular pressure and cell membrane–actin cortex adhesion strength.

Using various different cell types, it has been shown that nuclear deformability represents a physical limit for the migration through 3D collagen fiber matrices with different pore sizes (Wolf *et al* 2013). In particular, when inhibiting matrix metalloprotease (MMP) activity, which is necessary for the degradation of the extracellular matrix, the cell migration speed is reduced with decreasing pore size, as the nuclei increasingly need to deform (Wolf *et al* 2013).

At pore sizes below 10% of the non-deformed nuclear cross-section, cells encounter their nuclear deformation limit, which leads to completely stalled migration, although the cytoplasm can continue protrusion exertion (Wolf *et al* 2013). In line with this result, there are several other studies using various cell lines and cell culture migration assays, such as microfluidic devices, membranes with defined pores, extracellular matrices and *in vivo* xenografts, which have all revealed a similar model of a nuclear deformation limit that restricts the cell's ability to squeeze through small gaps and spaces when the pore size is below the cross-section of the

nucleus (Davidson *et al* 2014, 2015, Lautscham *et al* 2015, Rowat *et al* 2013, Guzman *et al* 2014, Mak *et al* 2013, Malboubi *et al* 2015, Booth-Gauthier *et al* 2013, Greiner *et al* 2014). When the role of specific physical factors on cell migration in confined environments has been investigated, it has been found that increased nuclear volume (by constant cytoplasmic volume), enhanced nuclear stiffness, decreased cell adhesion and reduced cellular contractility stalled the cellular migration within microfluidic confinements (Lautscham *et al* 2015). As these parameters have been observed commonly in a broad variety of cell lines such as neutrophils, fibroblasts and cancer cells, the exact amount of confinement required to evoke such an effect, and even the magnitude of this effect, varies from cell type to cell type. These differences suggest that a variation in nuclear deformability or the cytoskeletal forces applied to the nucleus are parameters delineating the 'nuclear barrier' effect.

10.9.2 Lamins determine nuclear deformability and migration through confined environments

As mentioned before, the nuclear deformability is largely determined by the nuclear lamin network and the chromatin (Dahl *et al* 2004, 2005). Lamins belong to the type V nuclear intermediate filaments that are subdivided into two subtypes: A-type (A, C, C2) and B-type (B1-3) lamins (Fisher *et al* 1986, McKeon *et al* 1986, Furukawa *et al* 1994, Peter *et al* 1989, Vorburger *et al* 1989, Lin and Worman 1993, 1995, Machiels *et al* 1996). These different lamin subtypes can assemble separate but interdigitating fibrillar networks at the nuclear periphery (Shimi *et al* 2008, 2015). In addition to their regulatory role in the maintenance of the nuclear shape and stiffness (Dahl *et al* 2004, Lammerding *et al* 2004, 2006, Swift *et al* 2013, Schaepe *et al* 2009), they act in the organization of chromatin, DNA damage repair and transcriptional regulation (Shimi *et al* 2008, Solovei *et al* 2013, Solovei *et al* 2013). Cell-stretching and micropipette aspiration measurement revealed that A-type lamins regulate nuclear stiffness more pronouncedly than B-type lamins and thus the nuclear stiffness strongly scales with expression of lamins A/C (Lammerding *et al* 2004, 2006, Swift *et al* 2013, Schaepe *et al* 2009), although elevated expression of lamin B1 also leads to enhanced nuclear rigidity (Ferrera *et al* 2014).

In line with the 'nuclear barrier' hypothesis, it has been found that cells possessing decreased levels of lamins A/C display more deformable nuclei and hence migrate faster through confined spaces than control cells expressing normal lamin A/C levels (Davidson *et al* 2014, Harada *et al* 2014). Inversely, enhanced expression of lamin A or the expression of a mutant lamin (termed progerin, which is a truncated version of lamin A) both enhance the nuclear stiffness and hence stall the transition of cells through these narrow constrictions (Rowat *et al* 2013, Booth-Gauthier *et al* 2013). In turn, the loss of lamins A/C induces the migration of these cells through small constrictions, as a larger nuclear deformation is possible, rather than enhanced nuclear compression, as the nuclear volume is not altered during the nuclear translocation (Davidson *et al* 2015). Moreover, these findings are directly relevant in physiological and clinical investigations, as the downregulation of lamins A/C

during granulopoiesis is crucial for the capacity of neutrophils to squeeze through micron-sized constrictions (Rowat *et al* 2013) and the deregulation of lamins has been frequently observed in various cancer types (Hutchison 2014).

However, there is not much known about the role of B-type lamins in 3D cell migration. The lack of B-type lamins can abolish the migration of neurons, which also lack A-type lamins in the developing brain, and this effect may be caused by defects in the linkage between the nuclear interior and the cytoplasm (Coffinier *et al* 2010, Young *et al* 2014). Based on the results that the lamin A/C levels and the nuclear organization can vary due to substrate stiffness and cytoskeletal tension (Swift *et al* 2013, Ihalainen *et al* 2015, Buxboim *et al* 2014), it is intriguing to hypothesize that the cells may dynamically adapt their nuclear stiffness during their migration.

10.9.3 The role of chromatin in nuclear deformability and migration

Chromatin contains DNA, which is wrapped around the histone octamers and occupies most of the nuclear interior, which then provides the viscoelastic response of the nuclear deformation (Dahl *et al* 2005, Pajerowski *et al* 2007). In particular, chromatin exists in two configurations: the first one is open euchromatin, which is usually transcriptionally active and the second one is closed and more compact heterochromatin, which is transcriptionally inactive (Eissenberg and Elgin 2001). The organization of chromatin is mostly as euchromatin (highly decondensed chromatin) and not as heterochromatin (highly condensed chromatin), which can be induced by treatment with the deacetylase inhibitor trichostatin A (TSA) resulting then in softer and more deformable nuclei through decondensation of chromatin (Pajerowski *et al* 2007). However, the treatment with 50-deoxy-50-methylthioadenosine (MTA), which is a methyltransferase inhibitor facilitating the decondensation of chromatin, abolished cell migration through microchannels (Fu *et al* 2012). It remains elusive whether this counterintuitive effect is based on an increase in nuclear size due to chromatin decondensation, which seems to counteract decreased nuclear stiffness (Lautscham *et al* 2015) or based on altered transcriptional regulation. What is the exact role of the chromatin organization in cellular migration through narrow confinements?

10.10 Cytoskeletal forces pulling or pushing on the nucleus

Molecular components such as the LINC complex that physically links the nucleus with the cytoskeleton have been discovered and subsequently have been determined to be the clutch transmitting the mechanical force through the nuclear envelope (figure 10.15) (Crisp *et al* 2006, Gundersen and Worman 2013, Chang *et al* 2015). However, the mechanism by which the cells translocate the nucleus through narrow confinements is still not well understood. Do cells pull or push the nucleus through the narrow constrictions? An answer cannot yet be given, as it may be that cells apply a varying combination of both mechanisms, which depends on the specific conditions.

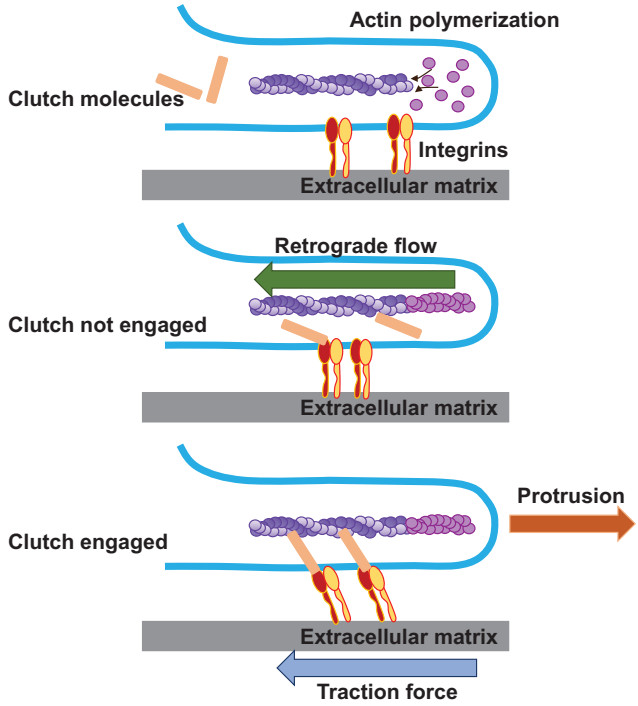

Figure 10.15. Molecular clutch. New actin monomers are added at the barbed end of a pre-existing actin filament at the leading edge of the cell (upper image). Transmembrane integrins are bound to the extracellular matrix. Since the clutch is not engaged to couple actin filaments to the extracellular matrix, the actin polymerization causes a rapid retrograde cytoskeletal flow without leading edge protrusion and traction force exertion the extracellular matrix (middle image). When the clutch is engaged, forces generated by the polymerization of the actin filament cytoskeleton are now transmitted to the extracellular matrix and hence cause an impairment of the retrograde flow, exertion of traction force on the extracellular matrix and the protrusion of the cell's leading edge (lower image).

10.10.1 Pulling the nucleus forward

In most migrating cells, the nucleus is located at the cell's rear end (Gundersen and Worman 2013). It has been proposed that the actomyosin contractility, possibly together with the intermediate filaments (Dupin *et al* 2011, Ketema *et al* 2013), physically pull the nucleus forward through the cell's leading edge during 2D and 3D migration (Wolf *et al* 2013, Wu *et al* 2014). During 3D lamellipodial-based migration, the actomyosin contractility and the integrin-facilitated tractions at the cell's leading edge are necessary to translocate the nucleus through narrow confinements (Wolf *et al* 2013). During 3D lobopodial-based migration, the non-muscle myosin IIA (NMIIA)-containing actomyosin bundles and vimentin filaments pull the nucleus forward by coupling to nesprin-3a of the LINC complex through plectin (Ketema *et al* 2013, Petrie *et al* 2014). Whereas microtubule-associated motors are dispensable for nuclear translocation in 2D cell migration (Wu *et al* 2014), the microtubule-associated motors dynein/kinesin that bind directly to the LINC complex proteins (Chang *et al* 2015) are

required for the interkinetic nuclear migration in neurons (Tsai *et al* 2010). In multinucleated myotubes, dynein and kinesin are also necessary for the nuclear positioning, where nuclei are squeezed, rotated and translocated to build a proper myotube structure (Wilson and Holzbaur 2012). Hence, it is possible that microtubule-associated motors additionally participate in the nuclear translocation during 3D migration of cells through confined space.

10.10.2 Pushing the nucleus forward

For the squeezing of the nucleus of leukocytes through narrow pores, the actomyosin contraction at the cell's rear is necessary (Lämmermamm *et al* 2008). In breast and brain cancer cells, non-muscle myosin IIB (NMIIB) is translocated possibly by nesprin-2 to the perinuclear cytoskeleton and thereby posterior of the cell, where it exerts pushing forces towards the nucleus to enable nuclear translocation through confined 3D microenvironments (Thomas *et al* 2015, Ivkovic *et al* 2012). The depletion of NMIIB, but not the deplection of NMIIA, abolished the migration of breast cancer cells through microfluidic structural confinements and dense collagen matrices (Thomas *et al* 2015), which leads to the suggestion that perinuclear NMIIB actin networks play a distinct role in squeezing the nucleus through these confined spaces. However, it needs to be determined whether NMIIB-dependent actomyosin contraction represents a general mechanism for nuclear translocation, whether it similarly utilizes nesprin-3a and vimentin filaments, and whether enhanced pulling forces can rescue impaired pushing forces and vice versa.

10.11 Physical compartmentalization by the nucleus

During the cell migration through narrow constrictions, the nucleus fills most of the space within the confinement and thus provides no or only little cytoplasmic transport around the nucleus, which divides the cytoplasm into front and rear compartments. Hence, this intracellular compartmentalization is highly crucial for two migration mechanisms: firstly, the osmotic pressure differences are evoked by water permeation and intracellular pressure generated by the nucleus, which functions as an active piston (Petrie *et al* 2014, Stroka *et al* 2014). In particular, the polarized distribution of Na^+/H^+ pumps and aquaporins within the cell membrane at the front and rear of the cell changes the permeation of water and thereby drives the cell migration and nuclear translocation, even in the absence of actin polymerization (Stroka *et al* 2014). As this osmotic pressure-based mechanism has solely been identified in tube-like microchannels restricting the fluid flow between the front and rear of the cells, its importance in more physiological microenvironments is still not well understood. Secondly, during lobopodial-based migration, the actomyosin-driven pulling of the nucleus compresses the cell's front similarly to a piston in a cylinder and hence creates a higher intracellular pressure compartment at the cell front, which enables the extrusion of new lobopodial protrusions at the cell's leading edge (Petrie *et al* 2014). Similarly, the nucleus may counterbalance the directional protrusion of invadopodia, which leads even to a visible indentation of the basal nuclear surface (Revach *et al*

2015). In summary, these findings revealed how cells can take advantage of a large and rigid nucleus to facilitate cell migration in 3D microenvironments.

10.12 Biological consequences of nuclear deformation during 3D cell migration

The cellular nucleus can not only be treated as a passive mechanical element containing the cell's genomic information, it also possesses the sites of DNA replication, transcription and RNA processing. Hence, the substantial mechanical forces and subsequently deformation of the nucleus caused by the migration of the cell through narrow constrictions can have severe impacts on biological processes such as cellular function and viability.

10.12.1 Influences on cell survival and genomic stability

Divergent results have been reported for the cell migration through narrow confinements and its impact on the viability and proliferation of cells. On the one hand enhanced apoptosis has been detected after migration through small pores, such as 3 μm in diameter, which has in particular been observed in cells lacking lamin A/C (Harada *et al* 2014), whereas on the other hand no noticeable enhancement in cell death during confinement has been found (Liu *et al* 2015, Davidson *et al* 2014, 2015, Rowat *et al* 2013). However, these apparent differences may be evoked by the investigation of different cell types and various mechanical confinements such as porous membranes, microfluidic devices and 3D collagen fiber matrices. Pharmacological inhibition of the heat shock protein 90 (HSP90) enabling DNA damage repair (Dote *et al* 2006) causes enhanced cell death after the transmigration through 3 μm diameter pores, which leads to the hypothesis that cells suffering from DNA damage during cell migration are undergoing apoptosis, when the DNA is not adequately repaired (Harada *et al* 2014). The mechanical stress itself is sufficient to induce severe DNA damage (Mayr *et al* 2002), but it still needs to be revealed whether the mechanical stress during the 3D cell migration can indeed cause pronounced DNA damage.

10.12.2 Influences on mechanotransduction signaling and gene expression

The mechanical stress exerted on the nucleus during cellular migration may also stimulate non-lethal alterations, which may further impact cell migration and cellular function. Isolated nuclei subjected to mechanical stress revealed that the nucleus functions as a mechanosensitive element. The force application through the LINC complex causes a rapid phosphorylation of the inner nuclear membrane protein emerin, which enables the recruitment of lamin A/C to the nuclear envelope and finally causes nuclear stiffening (Guilluy *et al* 2014). The shearing of isolated nuclei induces a partial unfolding of lamin A/C, exposing cryptic binding sites, which then may induce mechanotransduction processes (Swift *et al* 2013). Moreover, alterations in the mechanical microenvironment and hence the force application to intact cells can provide the remodeling of chromatin (Li *et al* 2011,

Iyer *et al* 2012) and its unbinding from nuclear protein complexes (Poh *et al* 2012), which then affects both the nuclear deformability and the gene expression levels (Kaminski *et al* 2014, Guilluy and Burridge 2015). However, the suggestion that nuclear deformation during 3D cell migration may impact the nuclear organization, the remodeling of chromatin and gene expression, still requires experimental evidence.

As the nucleus has long been simply seen as a center for genomic information and its processing, the physical impact of the nucleus on cellular function has to be considered, particularly during migration through 3D microenvironments. As the nucleus has a large volume and relative rigidity, it acts as physical barrier when cells enter pore sizes smaller than the nuclear diameter, and hence their migration is reduced or even stalled. In particular, the extent of this nuclear barrier effect depends on the nuclear size and stiffness controlled by the levels of the nuclear envelope proteins lamin A/C and the chromatin organization. These findings are relevant to immune cells and invasive cancer cells, which move through tissues with pore sizes smaller than the nuclear diameter and often display altered expression of lamins and other nuclear envelope proteins. There are still questions which remain to be answered. Are cells able to dynamically adapt their nuclear stiffness to drive cell migration through confined spaces, possibly by phosphorylation and/or degradation of nuclear lamins? Are specific cell types particularly preferred in their capacity to migrate through 3D extracellular matrices, either by displaying more deformable nuclei or by pulling/pushing stronger on the nucleus? What are the underlying molecular mechanisms through which cells enable the translocation of the nucleus through narrow confinements? How are cells undergoing large deformations of their nucleus altered in terms of chromatin organization, DNA damage and gene expression? It can be hypothesized that these mechanically driven events can not only alter the cell migration itself through adapted nuclear stiffness and changed cytoskeletal dynamics, but they may also affect a broad variety of cellular functions including cellular viability. To promote new discoveries, it will be crucial to combine new imaging tools such as fluorescence resonance energy transfer (FRET)-based intracellular force probes (Cost *et al* 2015), with microfabricated microenvironments that mimic physiological microenvironments, while they provide defined geometries and enhanced live-cell imaging conditions, single cell-based assays for cell viability and gene expression analysis as well as epigenetic modification. However, enlightening of the role of the nucleus in 3D migration will not only help to understand the physical constraints during cell migration in physiological microenvironments but also lead to new strategies for targeting invasive cancer cells and abolish the malignant progression through the propagation of the metastatic cascade.

10.13 Nuclear mechanotransduction

The cell nucleus represents a hallmark for eukaryotic evolution, as the gene expression is precisely regulated, the entire genome is replicated and repaired. In addition to these complex molecular processes, the nucleus exhibits severe physical tasks, which require its optical and mechanical properties. The nuclear

mechanotransduction of externally sensed forces and extracellular stiffness cues is driven by the physical connectivity of the extracellular microenvironment, the cell's cytoskeleton and the nucleoskeletal matrix consisting of a lamin network and chromatin. Nuclear mechanosensory components are able to convert the exerted tension into biochemical signals activating the downstream signal transduction cascades. Indeed, mechanoregulatory networks significantly stabilize the contractile cell state that is able to provide feedback to the extracellular matrix microenvironment, cellular focal adhesions and cytoskeletal components and structures. Current advances have provided several mechanistic insights into how the external forces are sensed from the interior of cells, such as the nucleus where the cell-fate decisions are made (Athirasala *et al* 2017).

The process of mechanosensing of externally applied forces towards cells and thus the extracellular matrix stiffness is a key property of solid tissues consisting of adherent cells (Discher *et al* 2005, DuFort *et al* 2011). It enables adhesive cells to migrate towards stiff and scar-like tissues regions (Pelham and Wang 1997), increases the malignancy of cancer cells (Schrader *et al* 2011), induces and directs the differentiation of adult stem and progenitor cells into either soft or stiff tissue lineages (Engler *et al* 2006), regulates the pluripotency of embryonic stem cells (Zoldan *et al* 2011, Chowdhury *et al* 2010) and control the initial differentiation process in mammalian preimplantation embryo development (Samarage *et al* 2015, Maitre *et al* 2016). Indeed, many recent advancements revealed mechanistic insights to the mechanism of how mechanical forces are transferred into biochemical signals within the cell's nucleus and how these emerging signals are transduced via conserved pathways in order to adapt gene expression and create a contractile cell state. There are still some questions that need to be answered: What are the underlying principles for the sensing of forces by the cell? How are nuclear properties contributing to the nuclear mechanotransduction processes?

10.13.1 Structural organization of nuclear lamina

The discovery of the structural organization of the nuclear lamina brought a challenge in the field of cell biology (Aebi *et al* 1986, Stuurman *et al* 1998). In particular, the nuclear envelope can be seen as a highly crowded microenvironment that is filled with cytoplasmic intermediate filaments. In particular, on the cytoplasmatic side many perinuclear cytoskeletal remodeling events occur, whereas on the nuclear side dense formations of heterochromatin, DNA, nuclear lamins and lamin-binding proteins are present (Goldman *et al* 2002, Gruenbaum *et al* 2005). Using high-resolution fluorescence microscopy the protein specificity can be detected and hence it has been revealed that juxtaposed networks comprising either A- or B-type lamins in mammalian nuclei have been shown to cross-interact and are involved in the maintenance of chromatin and the transcriptional regulation (Shimi *et al* 2008, 2015). Due to the great advances in cryo-electron tomography (cryo-TM) techniques, an unparalleled resolution of the nucleus has been reached (Harapin *et al* 2015). The nuclear lamins identified in Hela cancer cells and in mouse embryonic fibroblasts (MEFs) form thin filaments with a diameter of 3.5–4 nm and a persistence length

larger than 500 nm (Mahamid *et al* 2016, Turgay *et al* 2017). Taken together, lamins can assemble into the thinnest cytoskeletal/nucleoskeletal filaments that have been detected, as they are even thinner than the actin microfilaments.

After the identification of lamin filaments a detailed description of their molecular scale organization has been performed using cryo-TM imaging of vimentin deficient MEFs (Turgay *et al* 2017). In particular, it has been found that A- and B-type lamins are laterally decorated by an even longitudinal distribution of Ig-domains. The *in vitro* assembly of A- and B-type paracrystals revealed that lamin dimers polymerize in a head-to-tail orientation through the creation of short overlapping tetrameric edge regions of approximately 6 nm. In the next step, the two half-staggered lamin polymers interact laterally to build a rod with alternating tetrameric-hexameric regions and a 20 nm distance between two adjacent globular domains. The spatial resolution of 20 Å is not sufficient to analyze the polarity of the tetrameric protofilaments. In *Caenorhabditis elegans*, the assembly of lamin reveals the assembly of apolar filaments (Ben-Harush *et al* 2009). However, the structural analysis of mammalian lamin filaments yields both parallel and antiparallel lateral assembly. A non-uniform coverage of the inner nuclear membrane encompasses dense lamin network regions that are separated by sparse areas. Only half of the nuclear lamina area is usually covered by lamins, which are restricted in their location to a thin layer of 14 nm underneath the inner nuclear membrane (Turgay *et al* 2017). Thus, the nuclear lamina seems to assemble a thin and sparse filamentous sheet, which allows regions in the nuclear lamina to be associated with chromosomes in order to provide specific nuclear mechanical properties by regulating its own nuclear stiffness.

10.13.2 Direct transmission of forces to the nucleus

As the cell nucleus is not directed connected to the extracellular matrix micro-environment, the transmission of forces into the nucleus occurs indirectedly through the activation of the nuclear mechanosensory components that is facilitated by the LINC complex (Padmakumar *et al* 2005, Crisp *et al* 2006). The C-termini of the trimeric SUN1 and SUN2 interact with the inner and the outer nuclear membrane protein nesprin, as they extend into the perinuclear lumen and provide through the SUN–KASH-domain association a physical bridge between the nucleoskeleton and the cytoskeleton. These nuclear attachments bind in a specific manner to the main cytoskeletal networks within the cell. In particular, nesprin-1 and -2 bind F-actin through their N-terminal calponin homology domain, nesprin-3 connects to intermediate filaments through plectin and the shortest isoform nesprin-4 associates with kif5b, which is a subunit of the microtubule motor protein kinesin-1 (Rajgor and Shanahan 2013). Similar to cell–matrix and cell–cell adhesions, the LINC complex can be treated as a nuclear adhesion complex that promotes the rapid propagation of tensile forces from the extracellular matrix into nucleus and the reverse through the cytoskeleton towards the extracellular matrix (Buxboim *et al* 2010).

10.13.3 Nucleus deformability dictates mechanosensitive response to fast and slow physical inputs

Cells are able to sense a broad range of forces, which can alter their magnitude and vary in time. The precise enlightening of the nuclear mechanical response is crucial for elucidating the underlying mechanisms of the mechanotransduction signal pathways. In particular, the nucleus is free of a stable 3D fibrous network transversing the nucleoplasm in order to provide the nuclear shape and mechanical integrity interrelated to the cytoskeleton (Pederson 2000, Wilson *et al* 2016, Hancock 2000). The two major mechanical components of the nucleus are the nuclear lamina and the dense-packed interphase chromosomes. The nuclear deformability can adapt vastly different values depending on the cell type. Moreover, the nuclear deformability is pronouncedly reduced during embryonic stem cell differentiation (Pajerowski *et al* 2007, Olins *et al* 2008). In particular, the interphase nucleus of solid tissue cells is usually stiffer and also more viscous compared to the pure cytoplasm (Caille *et al* 2002, Guilak *et al* 2000). Hence, the nuclear deformability is the main sterical hindrance for the migration of cells across physically confined spaces and is pronouncedly regulated by A-type lamins that provide the physical protection of the entire genome (McGregor *et al* 2016, Davidson *et al* 2014, Harada *et al* 2014, Rowat *et al* 2013). Excessive shear stresses on the cells exerted during the cancer cell or immune cell migration across narrow confined spaces can evoke the nuclear envelope rupture, DNA breaks, the mislocalization of chromosomes and the recruitment of the molecular DNA repair machinery (Denais *et al* 2016, Raab *et al* 2016, Irianto *et al* 2017). The depletion of lamin levels increased the nuclear envelope rupture events and hence enhanced the instability of the genome and the occurrence of chromosomal aberrations. In a similar manner, the nuclear tension causes the recruitment of lipid processing enzymes into the stretched nuclear membrane in order to activate the proinflammatory lipid signal transduction (Enyedi *et al* 2016).

The rheology of the nucleus is complex due to its heterogeneous structural organization (Lammerding 2011), and wide-ranging strong and transient interactions with chromatin (Schreiner *et al* 2015). In addition to its high complexity, the global mechanical response of the nucleus to a constant micropipette aspiration can be described mechanically by a simple arrangement of two springs and one dashpot (termed standard linear solid (SLS) model), which accounts for a single dissipative relaxation mode (Guilak *et al* 2000). In swollen nuclei containing disrupted chromatin attachments, this viscoelastic behavior is shifted towards the elastic load-bearing system (Dahl *et al* 2004, 2005). The 3D chromatin cross-interactions can be described by a power-low rheology, accounting for a wide range of timescales and relaxation modes, which are not modeled properly using simple spring-dashpot circuits (Pajerowski *et al* 2007). The viscosity is provided mostly by lamin A/C, whereas the B-type lamina generates the elastic resistance to stretching (Harada *et al* 2014, Swift *et al* 2013). Moreover, the viscosity is caused by the presence of 5%–10% mature lamin A/C as a nucleoplasmic fraction in interphase nuclei, whereas farnesylated B-type lamins are undetectable within the nucleoplasm

(Buxboim *et al* 2014). The precise multimeric nature of nucleoplasmic lamin A/C has not been fully revealed, but has been suggested to transiently interact with chromatin, which seems not to be correlated with specific sequences (Bronshtein *et al* 2015, 2016). Lamins provide stable attachments to long genomic regions termed lamina-associated domains (LADs). These LADs represent up to 35% of the entire genome and are commonly associated with a repression of transcriptional activity (Guelen *et al* 2008). Constitutive LADs (cLADs) are even conserved across species in their genomic localization and size, whereas the sequence of these genomic regions is not conserved among species. Within these cLADs, long A/T-rich segments may provide docking signals to the lamina (Meuleman *et al* 2013), which is an indicator of a functional genome organization. In addition, nucleolus-associated domains (NADs) build nucleoplasmic chromosomal crosslinks (Nemeth *et al* 2010).

10.13.4 Conversion of mechanical forces into biochemical signals

Dissimilar to molecular recognition motifs targeting specific protein domains, the chromatin modifications and nucleic acid sequences targeting mechanical forces act on all interconnected components during their transmission through the cytoskeleton into the nucleus. Hence, only distinct structural components can convert the physical forces into biochemical signals, which are connected in force-bearing hotspots and usually undergo force-induced unfolding and hence expose formerly hidden functional cryptic sites or can even protect other components. Thus, mechanically induced conformational alterations either induce or impair molecular interactions with signaling molecules through elevated or diminished allosteric accessibility of interactions sites. This prominent mechanobiochemical regulation is purely based on the alteration of existing molecular interactions by simply modulating the molecular specificity through triggering or impairing downstream signal transduction pathways.

The exposure of hidden 'cryptic' molecular recognition sites by forced unfolding seems to be a unifying basic principle of extracellular filaments, cell–matrix and cell–cell adhesions and cytoskeletal proteins. In particular, the stretching of fibronectin promotes an extracellular fibril assembly (Krammer *et al* 1999, Smith *et al* 2007, Baneyx *et al* 2002). Similarly, the stretching of integrins alters the ligand binding (Chen *et al* 2012b, Freidland *et al* 2009), the extension of p130Cas enhances the rate of phosphorylation by Src kinase (Sawada *et al* 2006) and the extension of talin induces the recruitment and provides its binding to vinculin (del Rio *et al* 2009), all of which is indeed required for the mechanically induced growth of focal adhesions and the activation of downstream targeted pathways such as Rho–ROCK signaling and other pathways (Janostiak *et al* 2014, Geiger *et al* 2009, Huveneers and Danen 2009). In a similar manner, the strain-induced proliferation of epithelial monolayers is promoted by the activation and nuclear translocation of the E-cadherin-sequestered Yap1 and β-catenin (Benham-Pyle *et al* 2015). The force-driven unfolding of the cytoskeletal proteins such as spectrin, vimentin and myosin IIA evokes the regulation of their own structural organization, polymerization and finally their assembly (Johnson *et al* 2007).

In analogy to cell–matrix adhesions, the LINC complex assembles through dynamic nuclear interactions, which are under constant structural remodeling and are subject to local stiffening due to external force application (Guilluy *et al* 2014). Using magnetic beads coated with anti-nesprin-1 antibodies, a local stiffening of isolated nuclei has been found only when the pulling forces are facilitated through the LINC complex. Hence, the stiffening requires lamin A/C and is associated with the tyrosin phosphorylation of the inner nuclear membrane protein emerin on Tyr74 and Tyr95. Local stiffening is hence based on tension-dependent reinforcement of cytoskeletal–nucleoskeletal interactions, which facilitates a highly efficient transmission of tensile forces inside–out and outside–in (Buxboim *et al* 2010). The forces acting on the nuclear lamina can unfold the Ig-domain of lamin A/C, which has been seen when isolated nuclei are exposed to controlled shear stress and hence protect the nucleus from being ruptured (Swift *et al* 2013). Additionally, the nuclear tensile forces impair the lamin A/C phosphorylation, which encompasses also serine-22 (termed mitotic site) that promotes the disassembly (Buxboim *et al* 2014). Moreover, the tension-dependent suppression of lamin A/C phosphorylation is associated with a mechanically facilitated intermolecular steric shielding impairing the kinase accessibility. In line with this, the cell relaxation evoked by matrix softness, causes adhesion site detachments similar to pharmaceutical inhibitors or myosin contractility, which all revealed a significant elevated level of lamin A/C phosphorylation in interphase cells (Buxboim *et al* 2014). Thus, the nucleoplasmic fraction of lamin A/C is increased and facilitates faster degradation and protein turnover rates. The force-dependent phosphorylation is a nuclear mechanotransduction mechanism, which enables the cell to regulate its nuclear lamina and activate transduction pathways downstream of the nucleoplasmic lamin A/C (Swift *et al* 2013, Buxboim *et al* 2014).

In addition to lamin A/C, the tensile forces are associated with increased protection of extracellular collagen fibrils from proteolytic degradation (Flynn *et al* 2010, Bhole *et al* 2009, Ruberti and Hallab 2005), whereas the relaxation of cells promotes the disassembly of the cytoskeletal intermediate filament vimentin (Johnson *et al* 2007) and non-muscle myosin IIA minifilament (Buxboim *et al* 2014, Raab *et al* 2012). In general, exerted forces are associated with extracellular matrix stabilization and matrix stiffening that have been shown to induce the cell-based generation of traction forces (Lo *et al* 2000, Paszek *et al* 2005, Saez *et al* 2005). The cytoskeletal strain is further enhanced due to lamin A/C stabilization and nuclear stiffening, which created a concentration of stress (Heo *et al* 2016). Cytoskeletal assembly and the internal prestress of cells have been demonstrated to adapt to matrix elasticity alterations (Solon *et al* 2007) in terms of lamin A/C expression levels, which match with the tissue microelasticity (Swift *et al* 2013). This establishes a mechanobiochemical feedback mechanism that maintains the contractile cell state, which is typically found in muscle, cartilage or bone and other mechanically loaded tissues.

10.13.5 Molecular mediators of cellular mechanotransduction

The conversion of mechanical loads into biochemical signals is facilitated by mechanisms such as force-induced unfolding of adaptor and linker proteins and

the tension-driven stabilization of protein filaments. The mechanoregulation of cellular functions needs the propagation of mechano-converted signals through a distinct available repertoire of signal transduction pathways. Several molecular regulatory proteins have been identified that utilize a shared common principle, as their cytoplasmic-to-nuclear translocation is facilitated by mechanical stimulations.

The MKL1 is a transcription coactivator of the SRF transcription factor and is sequestered by cytoplasmic G-actin. A contractile cell state can be induced by serum supplementation (Miralles *et al* 2003), mechanical stress application and other physical signals that lead to the nuclear translocation of MKL1, concomitantly with the polymerization of G-actin into F-actin (Somogyi and Rorth 2004, Iyer *et al* 2012, Connelly *et al* 2010). Within the nucleus, the export of MKL1 is facilitated by nuclear actin (Vartiainen *et al* 2007), where lamin A/C and emerin (representing an actin-capping protein) induce the nuclear actin polymerization to maintain the nucleoplasmic localization of MKL1 and hence the activation of SRF target genes (Ho *et al* 2013). However, the applied strain can also evoke the translocation of the inner nuclear membrane associated emerin towards the outer nuclear membrane, where emerin induces the assembly of perinuclear actomyosin filaments, thereby depletes nuclear G-actin and promotes a remodeling of the heterochromatin (Le *et al* 2016). Thus, SRF seems to be a key regulatory element of structural and motor proteins of the actomyosin cytoskeleton and the entire contractile apparatus of the whole cell (Vartiainen *et al* 2007, Sun *et al* 2006, Olson and Nordheim 2010). In mesenchymal stem cells, a combined transcriptomic–proteomic profiling revealed indeed an impact of lamin A/C knockdown on MKL1-SRF target gene expression (Buxboim *et al* 2014). Moreover, Yap/Taz are mechanically activated transcriptional co-factors of the Hippo pathway that is known to facilitate cell–cell contact inhibition, the regulation of organ size and malignant cancer progression (Low *et al* 2014).

Similar to MKL1, the Yap/Taz translocates into the nucleus due the matrix stiffness, cell spreading and the applied stretching of the cell (figure 10.16) (Dupont *et al* 2011, Aragona *et al* 2013). The nuclear localization of Yap/Taz and its activation are mechanically driven by the non-canonical Hippo pathway, which is independent of the activation of MST-LAST. No direct involvement of lamin has been found, which caps and severs factors of the actin cytoskeleton impairing the Yap/Taz mechanotransduction signaling solely in mechanically relaxed cells (Aragona *et al* 2013). In the nucleus, Yap/Taz can even activate the TEAD family of transcription factors regulating proliferative and apoptosis-inhibitory genes. Indeed, other mechanoregulated nuclear mediators have been revealed. Among them are SHP2, which interacts with Yap/Taz, acts also downstream of Yap/Taz and elevates the Wnt signaling (Tsutsumi *et al* 2013). The retinoic acid receptor gamma (RARG) increases the transcription of the lamin A/C gene, whereas in turn the lamin A/C protein promotes the nuclear localization and activation of RARG, which is possibly provided by SUN2 (Swift *et al* 2013). Subsequently, the matrix softness rather than stiffness evokes the nuclear localization of Nkx2.5, where it suppresses genes that are contributing to the contractile cell state such as smooth muscle actin in mesenchymal stem cells (Dingal *et al* 2015). Taken together, the cell

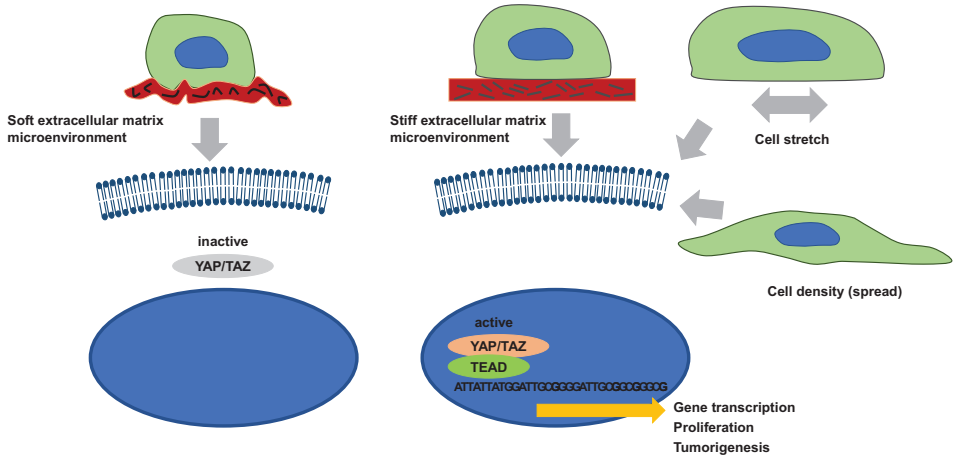

Figure 10.16. The induction of Yap/Taz translocation in the nucleus through matrix stiffness, cell density and stretching (microenvironment) affects cancer cell gene.

mechanotransduction is faciliated via molecular key components adapting gene expression programs and maintaining the contractile state of the cell. LAP2a is another transcriptional and epigenetic regulator which associates with nucleoplasmic lamin A/C, however, its mechanobiological role is not yet fully understood.

10.13.6 Force-dependent genome reorganization modulates transcription

The entire genome of the interphase nucleus is arranged into distinct chromosome territories (Cremer and Cremer 2001) and these substructures are termed topologically associated domains (Dixon *et al* 2012). Long-range interactions between regulatory elements and gene promoters are required for the precise transcriptional regulation. In particular, the interactions of accessory proteins and promoters are strongly cell-type-specific and are even enriched for connections between active promoters and epigenetically marked enhancers. In particular, the promoter interactomes reflect lineage relationships of the hematopoietic tree, which are in line with the dynamic remodeling of nuclear architecture during their differentiation (Javierre *et al* 2016). The lineage-specific genome organization correlates with the transcriptional regulation (Neems *et al* 2016). This leads to the hypothesis that mechanical forces physically deforming the nucleus alter the gene expression independent of molecular relays (such as protein components) by directly stretching the chromosomal sections and/or the opening condensed configurations within the chromosomes. Moreover, mechanobiological functions will then need mechanisms to directly target specific genes in a cell-type-dependent approach. Periodic shear stress exerted to the apical surface of CHO cells using magnetic beads, provides a method to measure the extent of chromatin stretching and correlate it to the rate of transcription of a targeted transgene (Tajik *et al* 2016). Indeed, the transcription rate correlated with stress-induced chromatin stretching, and both are dependent on the amount of applied shear stress and on the angle of the shear stress. Stress-induced

chromatin stretching and transcription has been found to be disrupted when nuclear lamins, emerin, LINC complex proteins, heterochromatin protein-1 (HP1) and barrier-to-autointegration factor (BAF) are knocked down, whereas the knockdown of the lamin B receptor (LBR) has no effect. The stress-induced chromatin stretching and transcription has been seen to be increased in contractile cells and even to be fully suppressed in relaxed cells. However, the effects of applied stress are independent of the activation of cell–matrix adhesion signaling, as the levels of the induction of transcription rates through the stretching of chromatin are not altered when beads are coated with RGD peptide specifically targeting cell–matrix adhesion receptors such as integrins or coated with nonspecifically with PLL. Hence, mechanotransduction is based on the force propagation along the actomyosin cytoskeleton, the LINC complex associations, nuclear lamins and heterochromatin linkages to evoke the stretching of chromatin and elevate the accessibility of the transcriptional machinery (Tajik *et al* 2016).

10.14 Nuclear envelope rupture and repair during cancer cell migration

During the process of cancer metastasis, cancer cells migrate in tissues through tight interstitial spaces, which requires the extensive deformation of the cell and its nucleus. Hence mammalian cancer cell migration has been studied in confining microenvironments *in vitro* and *in vivo*. Nuclear deformation is facilitated by the localized decrease of nuclear envelope integrity, which causes the uncontrolled exchange of the nucleo–cytoplasmic content, herniation of chromatin across the nuclear envelope and subsequently DNA damage. The occurrence of nuclear envelope ruptures of cells is elevated in narrow confinement and associated with the loss of nuclear lamins and nuclear envelope proteins structurally stabilizing the nucleus. Cells can restore nuclear envelope integrity using components of the endosomal sorting complexes required for transport III (ESCRT III) machinery. These findings have shown that cell migration evokes substantial physical stress on the nuclear envelope and its embedded proteins and requires an efficiently strong nuclear envelope and functional DNA damage repair for cell survival (Denais *et al* 2016).

The nuclear envelope is composed of the inner and outer nuclear membranes and contains nuclear pore complexes for import and export of substances and the nuclear lamina, which acts a physical barrier of the nuclear interior and the cytoplasm protecting the integrity of the genome from the cytoplasmic components and hence provides a separate compartment for the synthesis of DNA and RNA and its further processing (Burke and Stewart 2014). A loss of the integrity of the nuclear envelope and the selectivity of the nuclear pore complex is connected to the normal process of aging and a variety of human diseases such as cancer (Hatch and Hetzer 2013). In the malignant progression of cancer, the key steps of cancer cell invasion depend on the ability to deform the nucleus in order to squeeze through the available spaces within the 3D tissues (Weigelin *et al* 2012, Harada *et al* 2014, Thomas *et al* 2015, Wolf *et al* 2013). As the cytoplasm of migrating cells can in principle squeeze

through submicron-sized pores, the deformation of the largest and most rigid organelle, the cell's nucleus, is limited and hence restricts the migration through pores below 25 mm^2 in cross-section (Harada *et al* 2014, Wolf *et al* 2013, Friedl *et al* 2011, Davidson *et al* 2014, Rowat *et al* 2013, Fu *et al* 2012). Hence, it is hypothesized that cell migration through such tight gaps represents a substantial mechanical challenge for maintaining the integrity of the entire nucleus. Hence, it needs to be analyzed whether the migration of cells through confined spaces promotes nuclear envelope rupture and thereby compromises DNA integrity and also how cells can repair these nuclear envelope ruptures during the interphase of the cell cycle.

In order to investigate cancer cell invasion with precise control of cell confinement, a microfluidic device containing constrictions with defined stable height and varying pore sizes, mimicking the interstitial widths have been constructed (Davidson *et al* 2015). In particular, the nuclear envelope ruptures can be detected by using established fluorescent reporters consisting of green (NLS-GFP) or red fluorescent proteins fused to a nuclear localization sequence (NLS-RFP), which rapidly escape into the cytoplasm by diffusion when the nuclear envelope integrity is lost (De Vos *et al* 2011, Vargas *et al* 2012, Hatch *et al* 2013). Breast cancer cells, fibrosarcoma cells and human skin fibroblasts exhibit a transient loss of nuclear envelope integrity, which is associated with the nucleus passing through the constrictions. In addition, nuclear envelope rupture has been shown to be associated with a transient influx of fluorescently labeled cytoplasmic proteins into the nucleus and in turn it can also be detected by the localization of fluorescently labeled DNA-binding proteins such as BAF (Jamin and Wiebe 2015) and guanosine 3′,5′-monophosphate–adenosine 3′,5′-monophosphate (cyclic GMP-AMP) synthase (cGAS) (Civril *et al* 2013) at the nuclear envelope structural ruptures.

Can the nuclear envelope ruptures also be found during cancer cell migration in biological microenvironments? As suggested, fibrosarcoma cells and skin fibroblasts displayed nuclear envelope ruptures during the migration within 3D fibrillar collagen matrices, with kinetics similar to those detected in the confined microchannels. Nuclear envelope rupture usually occurs when the minimal possible nuclear diameter is closely matched by a pore size of 3 μm and thereby nuclear envelope rupture is connected to cellular movement through narrow spaces (Wolf *et al* 2013). In line with this, nuclear envelope ruptures are rarely detected (below 5% per roughly 12 h) for cancer cells migrating on glass, in low-density collagen matrices or through channels that are 15 × 5 mm^2 wide (Vargas *et al* 2012). However, when matrix pore sizes are further reduced to 5–20 mm^2 and even lower by simply increasing the collagen concentration and/or by blocking the cells' ability to degrade collagen fibers and hence widen the pores through the addition of a matrix metalloprotease (MMP) inhibitor, increases the nuclear envelope rupture events by approximately ten-fold. Similarly, the reduction of the pore size of the microfluidic channels below 20 mm^2 enhanced the nuclear envelope rupture more than ten-fold. Independently of the experimental model, the incidence of nuclear envelope rupture is exponentially enhanced with decreasing pore size and reached more than 90% of the cells when the nuclear height is confined to 3 μm. The analysis

of HT1080 fibrosarcoma cells, which invade a collagen-rich mouse dermis in living tumors after orthotopic implantation, even demonstrated that migration-dependent nuclear envelope rupture occurs *in vivo*, particularly by individually disseminating and migrating HT1080 cells. However, the nuclear envelope ruptures have been less frequently observed in cells moving as multicellular collective strands that generally follow linear tracks of least resistance and hence undergo less severe nuclear deformations (Weigelin *et al* 2012, Friedl *et al* 2011).

Nuclear envelope rupture *in vitro* and *in vivo* has been shown to be usually associated with the protrusion of chromatin through the nuclear lamina. Moreover, the occurrence of these 'chromatin herniations' is pronouncedly increased with decreasing pore size. In certain, severe cases, small pieces of the nucleus are pinched off from the primary nucleus when the cells are squeezed through narrow constrictions, leading to an enhanced and persistent fraction of cells with fragmented nuclei. Moreover, cells passing through microfluidic constrictions displayed more nuclear fragments positive for g-H2AX, which is a marker of DNA double-strand breaks (Nakamura *et al* 2010), than cells migrating without constrictions. These results are in line with studies showing that the loss of nuclear envelope integrity in micronuclei may cause DNA damage (Hatch *et al* 2013) and chromothripsis (Zhang *et al* 2015). In addition, increased g-H2AX staining has been seen at chromatin protrusions. In order to investigate whether the DNA damage has been caused by migration-induced nuclear deformation and nuclear envelope rupture, live-cell imaging on cells coexpressing NLS-GFP and fluorescently labeled 53BP1 (RFP-53BP1), another marker of DNA damage has been performed (Bekker-Jensen *et al* 2005, Loewer *et al* 2013). Indeed, nuclear envelope rupture and even more severe nuclear deformation, leads to the rapid formation of new RFP-53BP1 foci when cells squeeze through narrow constrictions, which is consistent with the enhanced activation of DNA damage response genes after compression-based chromatin herniation and nuclear envelope rupture (Le Berre *et al* 2012). To obtain insights into the biophysical processes underlying the nuclear envelope rupture, the timing and location of nuclear envelope rupture with respect to nuclear deformation, local membrane curvature, nuclear envelope composition and cytoskeletal forces have been determined. The nuclear envelope rupture has been seen predominantly (approximately 76%) at the cell's leading edge of the nucleus and precedes the subsequent formation of nuclear membrane protrusions such as nuclear blebs as the nuclei squeezed through the constrictions. Nuclear membrane blebs are usually formed at those sites where the nuclear lamina signal, the lamin B1 network, is rather weak or even absent. These findings demonstrate that blebs are formed when segments of the nuclear membrane can detach from the nuclear lamina and protrude into the cytoplasm. The loss of the lamins A/C and lamin B2 pronouncedly elevates the probability of nuclear envelope ruptures, indicating that lamins are required for the stabilization of the nuclear envelope (Harada *et al* 2014, De Vos *et al* 2011, Vargas *et al* 2012, Le Berre *et al* 2012, Broers *et al* 2004, Lammerding *et al* 2004).

The expanding nuclear blebs are free of GFP-lamin B1 and contain no nuclear pores and initially they possess little or no GFP-lamin A and B2 (Vargas *et al* 2012, Le Berre *et al* 2012, Broers *et al* 2004, Shimi *et al* 2008, 2015, Denais *et al* 2016).

Upon nuclear envelope rupture, the blebs are retracted and collapsed, indicating that the hydrostatic pressure has been released from these fluid-filled blebs. Despite the existence of pores within the nuclear envelope, the nuclear envelope has been found to function as an effective barrier and hence largely promotes the generation of intracellular pressure gradients (Petrie *et al* 2014, Neelam *et al* 2015). The nuclear pressurization seems to be based on actomyosin contraction at the rear of the nucleus and is necessary to push the nucleus through tightly confined spaces (Thomas *et al* 2015, Wolf *et al* 2013). This natural compression of the nucleus indeed effectively mimics cellular compression experiments (Le Berre *et al* 2012, Broers *et al* 2004), in which a precise threshold deformation exists above which the nuclear lamina breaks and reversibly reconstructs, whereas the nuclear volume is altered (Le Berre *et al* 2012). Moreover, altered nuclear deformations are associated with the expression of specific gene sets compromising nuclear factors and mechanotransduction pathway components (Le Berre *et al* 2012). A further support of this hypothesis has been obtained through the treatment of cells with low concentrations of blebbistatin, a myosin II inhibitor, which caused a significant reduction in the occurrence of nuclear rupture without an inhibition of the migratory capacity of these cells through larger channels.

Based on the transient nature of nuclear envelope ruptures, it has been proposed that cells have the capacity to restore nuclear membrane integrity during the interphase of the cell cycle. A rapid (within two minutes) accumulation of GFP-lamin A has been found at the site of nuclear rupture, which is related to the extent of nuclear damage (rupture) and the nuclear damage can persist for several hours. Subsequent ruptures within the same cell have been seen at distinct sites, which leads to the suggestion that there is a local protection by these so-called lamin scars. It has been reported that members of the endosomal sorting complexes required for transport III (ESCRT III) family are involved in the resealing of the nuclear membrane during late anaphase (Olmos *et al* 2015, Vietri *et al* 2015). To investigate whether ESCRT proteins fulfill a similar function in interphase nuclear envelope repair, GFP-fusion constructs of the ESCRT III subunit CHMP4B and the ESCRT III–associated VPS4B, which is necessary for the disassembly and recycling of ESCRT III proteins, are used to reveal their involvement in recruiting other ESCRT III proteins and enabling membrane scission (Hurley 2015). Due to nuclear envelope rupture induced by confined cell migration or laser ablation, CHMP4B-GFP and VPS4B-GFP rapidly (within two minutes) formed transient foci at the site of nuclear membrane rupture. Indeed, superresolution microscopy has demonstrated the recruitment of endogenous ESCRT III proteins to sites of nuclear envelope rupture into complexes of approximately 160 nm in size. The recruitment of the ESCRT III machinery is independent of microtubules. In addition, the depletion of the ESCRT III subunit CHMP2A, CHMP7 or the ectopic expression of a dominant-negative VPS4B mutant (GFP-VPS4BE235Q) preventing ESCRT III subunit recycling significantly prolonged the time necessary for nucleo–cytoplasmic recompartmentalization, which shows that ESCRT III proteins seem to fulfill a crucial role of in restoring nuclear membrane integrity. In order to reveal the functional relevance of nuclear envelope repair, the cell viability has been quantified after nuclear envelope

rupture. Under normal conditions, more than 90% of the cells survived even repeated rupture events of the nuclear envelope. However, the separate inhibition of either ESCRT III–faciliated nuclear envelope repair or DNA damage repair pathways cannot impact cell viability, whereas the inhibition of both repair mechanisms substantially enhanced cell death after nuclear envelope rupture.

In conclusion, cell migration through confined spaces has been shown to alter the integrity of the nuclear envelope and the content of DNA, which may all contribute to DNA damage, aneuploidy as well as genomic rearrangements and subsequently to cell death, when the repair mechanisms are not efficient (Raab *et al* 2016). Finally, a biophysical model can be proposed in which the cytoskeletal-generated nuclear pressure causes the exertion and possible also the rupture of nuclear membrane blebs at membrane sites of high curvature and a softer underlying nuclear lamina. Indeed, these events seem to be crucial in cells with decreased levels of lamins, whose expression is altered in various cancer types and usually correlates with negative outcomes for the patients (Hutchison 2014, Matsumoto *et al* 2015). Although the rupture of the nuclear envelope and the subsequent genomic instability seem to enhance cancer progression, they represent a possible specific weakness of metastatic cancer cells and may provide an opportunity for the development of novel antimetastatic pharmacological drugs by specifically targeting these cells, inhibiting the repair mechanism of the nuclear envelope and the DNA.

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Part V

The impact of the tumor microenvironment on cellular invasion

Among the hallmarks of the malignant progression of cancer, such as transformation of epithelial cells in the tissue, is the alteration of stromal constituents of the surrounding microenvironment. Pre-eminently, the aberrant remodeling of the structural architecture of the extracellular matrix and subsequent matrix stiffening promotes tumor growth and cell survival, which all drive the sequential steps contributing to the metastatic cascade. Cancer cell invasion is not only regulated by the cancer cell's capacity to migrate through the connective tissue. The microenvironment also affects the motility of cancer cells through the extracellular matrix, with respect to structural confinement and mechanical properties. In turn, cancer cells also challenge the matrix's mechanical properties by degrading the extracellular matrix or secreting extracellular matrix proteins and growth factors. Moreover, cancer cells can even exert contractile forces towards the matrix and thereby alter the mechanical properties of the matrix, which responds to the mechanical load possible through strain stiffening. Thus, cancer cells are able to alter the structure, composition and, consequently, the mechanical properties of the extracellular matrix of connective tissues. During physiological processes such as development and wound repair, and upon tissue injury and pathological processes such as cancer, normal and neoplastic cell types can travel through the extracellular matrix, which represents a complex composite of collagens, elastin, glycoproteins, proteoglycans and glycosaminoglycans that all contribute to the arrangement of the entire tissue architecture. Moreover, for tissue-invasive processes, cancer cells can utilize protease-dependent or protease-independent strategies whose selection is based on the characteristics of the motile cell type or subpopulation and additionally on the structural properties of the intervening extracellular matrix. Among the structural and mechanical properties of the extracellular matrix cues are dimensionality, elasticity, crosslinking and pore size, which all contribute to provide a distinct invasion pattern for cells. In addition, cancer-associated fibroblasts embedded in the extracellular matrix of primary tumors can restructure the local tumor microenvironment by contracting the matrix, thus altering its mechanical properties, such as stiffness. Similar to cancer cells, cancer-associated fibroblasts (CAFs) degrade the extracellular matrix, mechanically strengthen the matrix scaffold and secrete cytokines, chemokines, growth factors and extracellular matrix proteins. When designing new experimental approaches for interrogating the invasion programs of cancer cells or when identifying potential cellular targets for next-generation or even personalized therapeutics, knowledge of the extracellular matrix's role in cancer cell invasion may be crucial.

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Cellular and microenvironmental effects
Claudia Tanja Mierke

Chapter 11

The mechanical and structural properties of the microenvironment

Summary

The mechanical properties of the microenvironment (for example, the connective tissue consisting of extracellular matrix proteins) plays an important role in providing the conditions for cellular motility and invasiveness. Type I collagen represents one of the major extracellular matrix components, and is the major contributor to the properties of the extracellular matrix scaffold and its effects on cell migration and invasion. In particular, it serves as a scaffold protein within the stroma contributing to the tissue's mechanical properties, imparting the tensile strength and rigidity to tissues such as skin, breast, kidney, tendons and lungs. Moreover, collagen contributes to the intrinsic spatial heterogeneities, which are based on a distinct fibrillar architecture, pore size and ligand density on the microscale and the bulk mechanical properties of the extracellular matrix on a mesoscale. The hydrogels based on type I collagen can be tuned within a broad range by the polymerization temperature and the concentration to mimic the physicochemical properties of a normal tissue and the surrounding tumor microenvironment. The mechanical properties of the 3D collagen fiber matrices are determined by *in situ* calibrated active microrheology using optical trapping or bulk rheology measurements using either a plate rheometer or magnetic resonance elastography (MRE). This chapter addresses and discusses the question of how the physical limits of the extracellular matrix regulate cellular invasion. In particular, chapter 11 considers how the structural composition, mechanical properties and steric hindrance affect the malignant progression of cancer. Moreover, chapter 11 reveals how each of the parameters of the extracellular matrix facilitates cellular invasiveness. The focus here is on the proteins building up the extracellular matrix, while the effect of the embedded cells is discussed in chapters 12, 15 and 16.

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11.1 Why is the extracellular matrix of connective tissue crucial for the invasion of cancer cells?

In cancer, the neoplastic cells possess the capacity to inappropriately utilize a normal cell function in an optimized manner and escape from their primary localization by engaging the invasive machinery which has been engineered to control the precisely regulated cellular motility modes that are operative in tumor growth and developmental processes (Rowe and Weiss 2009, Kessenbrock *et al* 2010, Wolf and Friedl 2011, Mierke 2015). In particular, the progression of the tumor and cancer cell invasion are often connected though an elevated expression of proteolytic enzymes, which are on the one hand generated and secreted by cancer cells and on the other hand produced by stromal cells, integrated into the matrix scaffold and released in the surrounding tumor microenvironment. The enzymes are capable of degrading the major extracellular matrix macromolecules such as collagen fibers that comprise all extracellular matrices of connective tissues (Rowe and Weiss 2009, Kessenbrock *et al* 2010, Wolf and Friedl 2011, Lu *et al* 2012). Multiple proteases have been identified to function in the extracellular matrix remodeling events which are closely related to cancer, although there exist contradictory results on the issue of whether proteolysis is an essential step in the tissue-invasive process of cancer cells or simply shifts the basic cancer cell invasive capacity to an advanced invasion mode (Rowe and Weiss 2009, Sabeh *et al* 2009a, Sabeh *et al* 2009b, Kessenbrock *et al* 2010, Wolf and Friedl 2011). Whereas multiple groups have revealed that cancer cells can only migrate through the extracellular matrix by the proteolytic degradation of surrounding structural barriers, others have shown that neoplastic cells can even push or squeeze through confined spaces and thereby find a path through extracellular matrix barriers without mobilizing proteases (Wolf *et al* 2003a, 2007, Sabeh *et al* 2004, Sabeh *et al* 2009a, Madsen and Sahai 2010, Wolf and Friedl 2011, Friedl *et al* 2012). Why are the results diverse and even contradictory? How does the extracellular matrix affect the outcome of cancer cell migration? Can the extracellular matrix of the interstitium be remodeled by artificial hydrogels?

The interstitium: a natural microenvironment for cancer cells

The extracellular matrix scaffold of connective tissues contains predominately interstitial collagens, among which type I collagens are present at the highest levels and are even the single-most abundant extracellular proteins detected in mammalian cells (Rowe and Weiss 2009, Grinnell and Petroll 2010). Simultaneously with the secretion of the extracellular matrix by fibroblasts, the collagen propeptide domains are proteolytically digested and subsequently a complex auto-polymerization process is induced (Kadler *et al* 2008). After lysyl oxidase-facilitated crosslinking within the N- and C-terminal nonhelical ends of the secreted and processed collagen molecules such as the telopeptide domains, the collagen fibrils become mature in a mechanically reinforced network of collagen fibers and fiber bundles (figure 11.1) (Eyre *et al* 1984, Christiansen *et al* 2000). Due to the enormous tissue-to-tissue variation in the interstitial collagen content and crosslinked structure (Eyre *et al* 1984, Christiansen *et al* 2000, Kadler *et al* 2008, Wolf *et al* 2009), the trafficking of

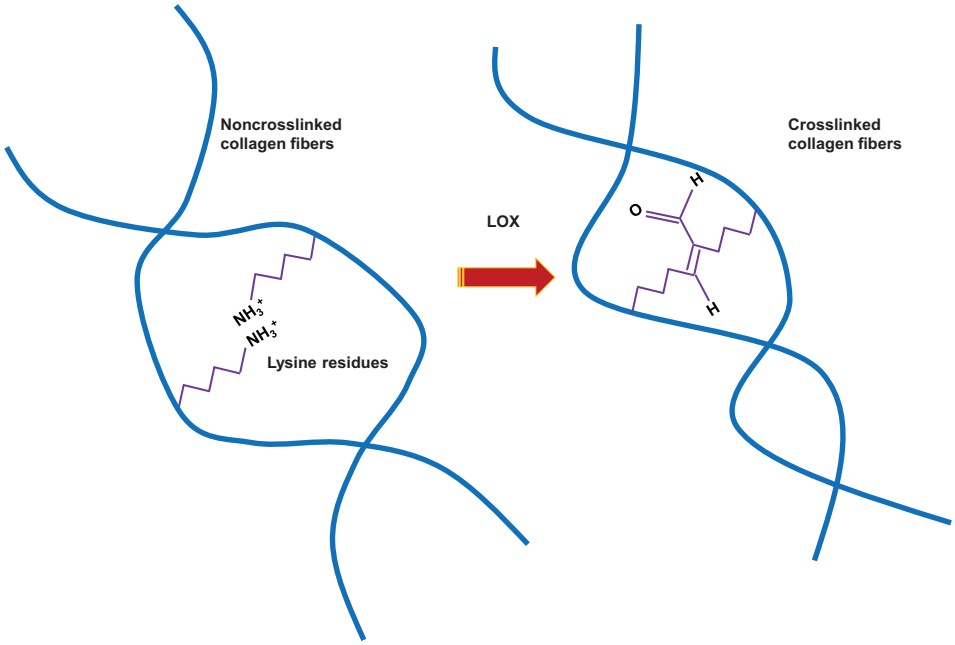

Figure 11.1. Lysyl oxidase (LOX) crosslinks in collagen fiber networks.

cancer cells and the recruited tumor stromal cells are confronted with structurally distinct extracellular matrix scaffold barriers. For the migration and invasion of cells at cell–extracellular matrix interfaces, cells employ a strategy which can be commonly described by a two-body model of invasion. In this model, the elasticity and the size of the infiltrating cell's largest and most mechanically rigid subcellular compartment, which is the nucleus, determines the migration type that cells utilize to migrate or squeeze through the fibrillar network of the interstitial matrix (Nakayama *et al* 2005, Wolf *et al* 2007, Beadle *et al* 2008, Fisher *et al* 2009, Friedl *et al* 2011, Khatau *et al* 2012). When on the one hand the size such as the cross-sectional area of a rigid matrix pore is too small to comply with the size of the nucleus of the migrating cell, the physical barrier will be impossible to overcome unless the extracellular matrix barrier constriction is proteolytically broken down. When on the other hand the pore size of the matrix is larger than the cell's nuclear diameter, the physical barrier to cellular trafficking vanishes and invasion is performed independently of protease-driven remodeling of the extracellular matrix scaffold (Wolf *et al* 2013). When the pore size is too small for passive cellular movement, the cell needs to utilize a complex migration mode—the physical barrier needs to be broken down by at least three different mechanisms. First, the physical barrier is degraded using extracellular matrix-degrading proteases, which simple cut the collagen fibers, hindering cell migration. Second, actomyosin motors and cell–matrix adhesion molecules act together to mechanically bend and distort the surrounding matrix fibers, which then no longer represent a physical barrier (Friedrichs *et al* 2007). Third, the intracellular contractile apparatus deforms and

translocates the nucleus and hence the cells are able to migrate by squeezing through the small gaps of the nondeformable matrix cage barrier (Nakayama *et al* 2005, Beadle *et al* 2008, Friedl *et al* 2011, Balzer *et al* 2012, Khatau *et al* 2012). However, which of these models is preferred for the migration and invasion of cells depends on the cell type, the strength of the confinement and on other not-yet-known regulatory parameters or molecules.

What effects do the mechanical and structural properties of the local tissue micro-environment have on the ability of cancer cells to migrate?

The general description of cellular motility through 3D connective tissue is based on a physicochemical balance between cellular deformability and physical as well as mechanical tissue constraints. Cell migration rates are determined by the capacity of the cells to degrade the extracellular matrix through proteolytic enzymes, such as membrane-bound or secreted MMPs, and mechano-coupling between the integrin transmembrane receptors and the actomyosin cytoskeleton. How these parameters cooperate when the space is confined is not yet well understood. Using MMP-degradable collagen lattices or nondegradable substrates of varying porosity, the limits of cell migration can be quantitatively identified by the physical arrest of the cells within the tissue. In particular, the MMP-independent cell migration decreases linearly with decreasing pore size of the extracellular matrix and with decreasing deformability of the nucleus, which can be deformed up to maximally 10% of the nuclear cross-section until the cell is caught within the matrix (figure 11.2). The limits for cancer cells are cross-sectional areas of 7 μm^2, for T-lymphocytes of 4 μm^2 and neutrophile granulocytes of 2 μm^2 (Wolf *et al* 2013). This suggests that the residual migration under spatial constriction depends upon MMP-dependent degradation of the extracellular matrix through enlarging the matrix's pore diameters and enhancing integrin- and actomyosin-dependent force generation, which together push the nucleus forward through the spatial confinement. The main

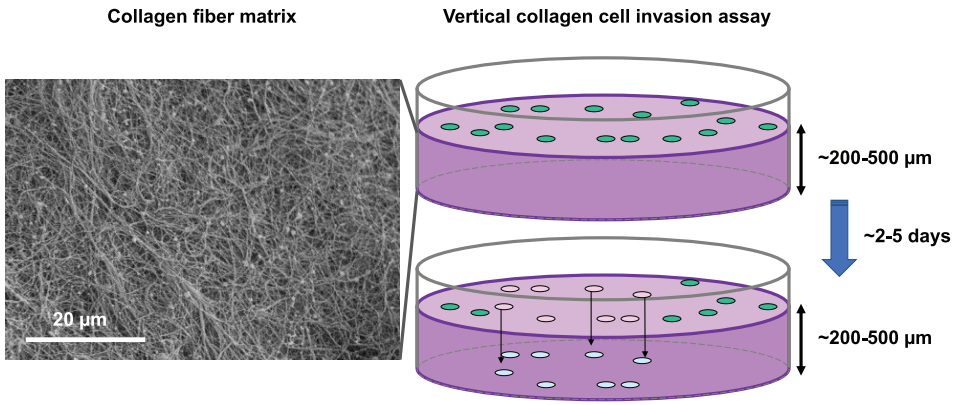

Figure 11.2. The 3D collagen fiber matrix is a physical barrier for cell migration. (A) Scanning electron microscopic image of a 3D collagen fiber matrix (mixed rat and tail collagens with a final concentration of 2.4 mg ml^{-1}). (B) 3D collagen invasion assays. Cells are seeded on top of the matrices and invade for 2–5 days (upper image). After 8 h to 5 days invasion time several cancer cells invaded into the matrix (lower image).

restrictions for interstitial cell migration are scaffold porosity, matrix composition, deformability of the nucleus, pericellular collagen degradation and mechano-coupling between the cell's cytoskeleton, the nucleoskeleton and the extracellular matrix. In principle, the nucleus can be deformed by much more than 10%, as has been reported for endothelial cells cultured on micropatterned substrates (Versaevel *et al* 2012). Moreover, even nuclear rupture events do not necessarily lead to cell death, as most of these nuclear ruptures can be repaired by the cells after passing the confinement.

Cell migration along and through the 3D extracellular matrix is a fundamental process involved in the formation and regeneration of tissues, immune cell trafficking and diseases, such as cancer invasion and metastasis. Interstitial migration is a cyclic process consisting commonly of multiple consecutive steps: (i) actin polymerization-dependent pseudopod protrusion at the cell's leading edge; (ii) integrin-facilitated adhesion to the extracellular matrix; (iii) contact-dependent extracellular matrix cleavage by the cell's membrane-bound proteases; (iv) actomyosin-facilitated contraction of the cell's body increasing longitudinal tension and cellular polarity; and (v) the retraction of the cell's rear followed by the translocation of the cell's body (Ridley *et al* 2003, Friedl and Wolf 2009, Friedl and Alexander 2011). This type of migration is constitutively active in mesenchymal cells such as fibroblasts, stem cells and solid cancer cells (Wolf *et al* 2007, Sanz-Moreno *et al* 2008, Sabeh *et al* 2009b, Grinnell and Petroll 2010, Ciria *et al* 2017). In particular, these mesenchymal cells show prominent protrusions and possess a polarized spindle-shaped morphology, adhere strongly to the extracellular matrix and remodel the tissue proteolytically. In contrast to the mesenchymal movement of cells, the interstitial movement of leukocytes can be described by an ellipsoid cell shape and a rapid deforming of their cellular shape and morphology with short protrusions, weak adhesion strength and no proteolytic degradation of the extracellular matrix (Wolf *et al* 2003b, Sabeh *et al* 2009a). Finally, each migration step is supposed to be adaptive due to cell-intrinsic and extracellular chemical or mechanical signals, such as regulators of adhesion, cytoskeletal dynamics, proteolysis, deformability of the cells and the matrix's extracellular geometry and material properties (Berton *et al* 2009, Lautenschlaeger *et al* 2009, Friedl and Wolf 2010, Friedl *et al* 2011, Tong *et al* 2012, Fischer *et al* 2017, Kunschmann *et al* 2017, Mierke *et al* 2017).

The interstitial invasion of mesenchymal cells such as fibroblasts and cancer cells into highly concentrated collagen-based extracellular matrices is controlled by MMPs. They are particularly membrane-bound, with, for example, MT1-MMP (formerly MMP-14) serving as the most important enzyme for degrading intact fibrillar collagen (Sabeh *et al* 2004, Wolf *et al* 2007 Rowe and Weiss, 2009). Active MT1-MMP is concentrated at the plasma membrane sites at which the migrating cell is in close contact with the extracellular matrix confinements, such as the collagen fibrils, in order to cleave the fibrils acting as steric hindrances for cancer cell migration. Moreover, MT1-MMP is present in the invadopodial protrusions that cancer cells protrude in extracellular matrix confinement (Eddy *et al* 2017). The invadopodia belong to a subset of invadosomes that are engaged in the integration of signals from the tumor stromal microenvironment to promote cancer cell invasion

and their subsequent dissemination. How do cancer cells manage to regulate the plasticity that is required for the invadopodia to assemble and function efficiently in the broad variety of surrounding microenvironments, ranging from tumor stroma to normal tissue extracellular matrix scaffold? Invadopodia play a crucial role in regulating the cancer cell communication with the microenvironment through the productions of localized MMP activity, decreasing stromal pH and the secretion of exosomes. This invadopodia-driven communication can last over short and long distances through alterations of the stromal microenvironment and subsequently the exosome-facilitated establishment of the premetastatic niche (figure 11.3) (Zhang and Wang 2015, Lobb *et al* 2017).

However, inhibition of MT1-MMP eliminates collagen cleavage and remodeling of the extracellular matrix (Sabeh *et al* 2004, Wolf *et al* 2007). Thus, nonproteolytic migration is performed by amoeboid cellular deformation (Wolf *et al* 2003a) or ceases entirely (Sabeh *et al* 2004), depending on the type of collagen scaffold used as a migration substrate (Packard *et al* 2009; Sodek *et al* 2008, Sabeh *et al* 2009a). Collagen scaffolds reconstituted from different collagen animal and tissue sources and collagen types may vary in their physicochemical properties, such as porosity and stiffness (Zaman *et al* 2006, Sabeh *et al* 2009a, Wolf *et al* 2009, Yang and Kaufman 2009, Miron-Mendoza *et al* 2010, Yang *et al* 2010). To date, there have been numerous studies that have investigated the effect of the collagen matrices on cellular motility, but there is no overall integrative concept that explains the differences reported for the particular types of collagen matrices. However, an integrative concept seems to be necessary to understand how the properties of the extracellular matrix allow or restrict the migration of cells due to the MMP activity or the matrix's stiffness.

What are the rate-limiting substrate conditions that regulate the migration of different cell types in 3D extracellular matrices?

In order to address and answer this question live-cell microscopy can be performed to monitor migration rates and the deformation of the cell's body and nucleus in 3D extracellular matrices. These matrices vary from low to high collagen density and the

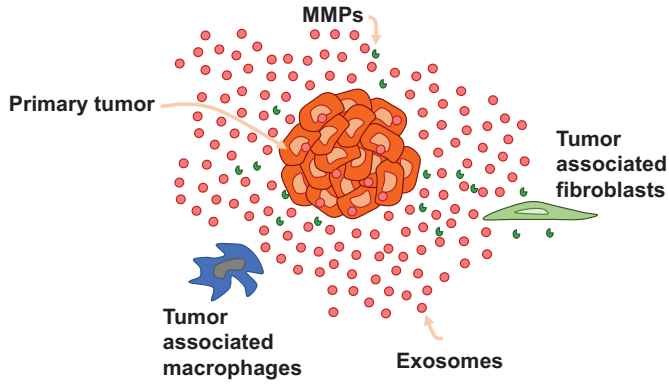

Figure 11.3. Release of exosomes.

degree of crosslinking, which can be provided by either natural collagen crosslinking proteins or chemical substances. Thus, the subtotal and absolute migration limits are mapped in order to address the important molecular regulators of migration efficacy in a constrained environment (Wolf *et al* 2013). Using multiparameter analyses, the ratio between extracellular matrix density and cellular deformability has been identified as key parameters similar to MMP activity, actomyosin-based contractility and integrin-facilitated mechano-coupling, which are modulators of invasion efficacy, because they regulate cellular migration in dense 3D tissue microenvironments.

In vitro reconstitution of 3D collagen matrices

The structure of 3D collagen matrices depends entirely on the type of collagen (such as types I–XIX), the animal source (such as a rat or bovine) from which the collagen is isolated and on the collagen isolation procedure (such as purely acid-soluble based or combined acid- and enzymatic (e.g. pepsin) -soluble based collagen isolation methods). In most studies, the 3D hydrogels are reconstituted from either telopeptide-intact covalently crosslinked collagen obtained from rat-tail tendons using only acid extraction, or telopeptide- and crosslink-reduced bovine dermal collagen isolated using acid and pepsin treatment (figure 11.4) (Wolf *et al* 2003a, Sabeh *et al* 2004, Sodek *et al* 2007, Packard *et al* 2009). In order to investigate the effect of telopeptide-intact covalently crosslinked collagen and telopeptide- and crosslink-reduced collagen, equal collagen concentrations (such as 1.7 mg ml^{-1}) have been compared with respect to collagen fibril assembly speed, fibril architecture, matrix porosity and matrix stiffness. All these parameters can be precisely tuned during the isolation and polymerization of 3D collagen fiber matrices.

First, the *in vitro* 3D fibrillar collagen matrix represents a network of collagen fibrils, which are polymerized from collagen molecules or monomers. The formation of a collagen fibril is induced when soluble collagen is shifted to physiological conditions of neutral pH and warmed to 20 °C–37 °C (Wirtz *et al* 2009,

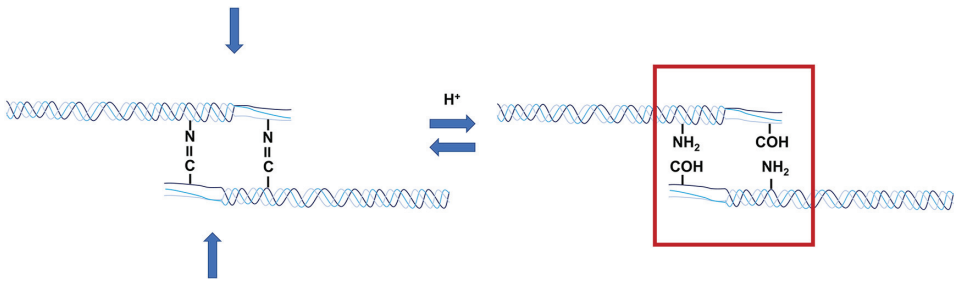

Figure 11.4. MT1-MMP-independent cancer cell invasion through telopeptide excised or crosslink-deficient type I collagen matrices. Schematic drawing of the collagen type I intermolecular crosslinks. *In vivo*, the lysyl oxidase generates aldehyde moieties within N- and C-terminal telopeptide domains of type I collagen, which are arrayed across from epsilon-amino groups (blue region) that condense to conform Schiff base and aldimize crosslinks. During pepsin extraction, N- and C-terminal telopeptides are removed (arrows, left-hand image). Under acidic extraction (right-hand image), Schiff base formation is reversed to generate the starting aldehyde and amine groups.

Gelman *et al* 1979). Inversely, the collagen solution is prepared by solubilizing native fibrillar collagen of pH 2 at a temperature reduced to 4 °C in a buffer solution. In particular, the native collagen type I molecules possess nonhelical telopeptides on the C- and N-ends (figure 11.4). In particular, these C- and N-telopeptides support the collagen fibril alignment and provide additional sites for the crosslinking of collagen fibrils within the 3D extracellular matrix scaffold (Eyre *et al* 1984). The distinct conditions for the collagen monomer preparation from native fibrillar collagen of tissues determine the amount of telopeptides, which can be preserved or cut from the collagen molecule. Hence, the acid extraction of native fibrillar collagen with acetic acid or hydrochloric acid still provides native telopeptides, whereas the extraction of collagen with the enzyme pepsin cuts off the telopeptides. Moreover, it has been observed that cells cultured in reconstituted 3D collagen matrices without telopeptides do not need proteases for the migration and invasion through these matrices (Packard *et al* 2009, Sabeh *et al* 2009a). Thus, the choice of the isolation and polymerization conditions is crucial for the resulting matrix, which predicts the cellular morphology, behavior, cellular protein localization and dynamical remodeling processes.

Second, the concentration of collagen monomers used for the polymerization of fibrillar collagen networks determines the density of the matrix and pore size. Moreover, it has been suggested that the cells even undergo mesenchymal–amoeboid transitions that enable a protease-independent cell migration mode through 3D matrices of low density and high pore size, where cells squeeze through pores in the 3D matrix (Even-Ram and Yamada 2005). However, for the cell to navigate through a dense 3D fibrillar collagen network, it needs to additionally use proteases to cut and degrade collagen fibrils, which then helps to facilitate the squeezing of the cell body through the narrow pores (Sabeh *et al* 2009b).

Third, the morphology of collagen fibrils and alignment of collagen microfibrils within reconstituted collagen matrices are dependent on several parameters such as the ionic strength, pH, temperature and the amount of phosphate (Wirtz 2009). The conditions chosen for the production of 3D collagen matrices are usually dependent on the requirements of the experiment and should produce reconstituted fibrils that mimic native fibrils, but they are limited in their ability to exactly recreate the morphology and alignment of native collagen fibrils, as many other factors are present in *in vivo* tissues. To avoid possible heterogeneity in experimental results due to alterations in collagen fibril morphology, conditions for 3D collagen matrix assembly need to be kept constant and the same batch of isolated collagen preparations should be used to compare the results within an experimental approach.

The fibrillar matrix architecture can be detected by using confocal reflection microscopy and/or by using collagen type-I immunofluorescence. Extracellular matrix fibers usually differ in their refractive index from their surroundings and hence can reflect light. Laser-scanning confocal microscopy in reflection mode can be used to collect the reflected or back-scattered light from collagen fibers for each confocal plane generating 3D structural details of the extracellular matrix, which is not labeled with a fluorochrome. The imaging of an unlabeled collagen 3D matrix

has many advantages such as saving the time and resources required for collagen labeling with fluorescent dye, the elimination of photobleaching that exists for fluorescently labeled 3D collagen matrices and the usage of unmodified collagen matrix for cell culture assays, as labeled 3D collagen matrices may alter the cancer cells' capacity to migrate or even the migration mode. Moreover, confocal reflection microscopy can easily be combined with simultaneous fluorescence confocal imaging, which provides the advantage of accessing multiple fluorescently labeled markers within cells or the matrix.

For controlled preparation and imaging conditions, the collagen matrices are anchored using custom glass chambers. The time needed to polymerize the collagen matrices was monitored and recorded using confocal backscatter microscopy at 37 °C. The rat-tail collagen, which reached the half-maximum polymerization after 30 s, assembled 16-fold faster than the bovine dermal collagen, which reached the half-maximum polymerization after 8 min (figure 11.5). The different speeds of collagen fibril assembly correlate with the different telopeptide contents within the collagen preparations (Helseth and Veis 1981, Sabeh *et al* 2009b). The fibrillar matrix architecture has been observed by confocal reflection microscopy and it has been additionally analyzed using collagen type-I immunofluorescence. Indeed, there is only a negligible detection error from backscatter-negative fibrils in vertical orientation, below 3% of signal-containing pixels. This result is in contrast to another report in which twice as many fibers have been detected with collagen I specific fluorescence compared to confocal reflection microscopy (Jawerth *et al* 2010). Thus, the method for analyzing the collagen fibers within a 3D collagen matrix has to be carefully chosen. Rat-tendon-derived collagen assembles to thin fibrils with a diameter of 20 nm and a narrow pore size range of 2–5 μm^2 (1–2 μm pore diameters), whereas bovine-dermis-derived collagen matrices form fibrils with a diameter of 60 nm and wider pore cross-sections, ranging from 6–30 μm^2 (2–6 μm pore diameters) (Wolf *et al* 2013). In order to control the fibril density-dependent alterations in collagen matrix stiffness, atomic force microscopy (AFM) was used

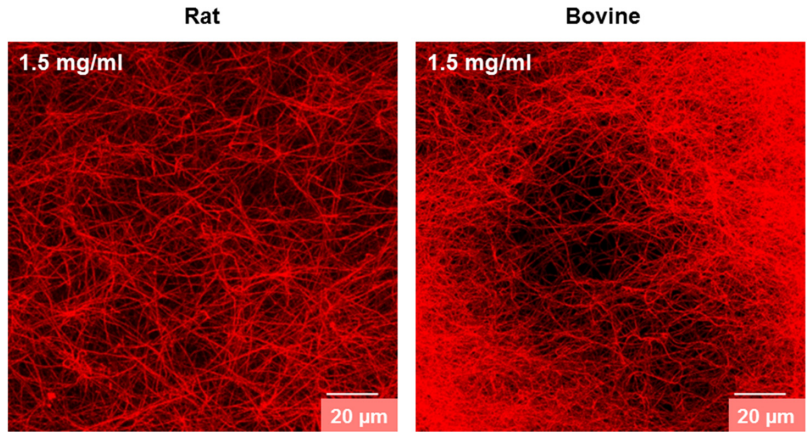

Figure 11.5. 3D collagen fiber matrices of rat (tail) and bovine (skin) collagen type I of a concentration of 1.5 mg ml^{-1}.

with a 10 μm bead as a cantilever probe to approximate the size of a cell. The surface of the collagen matrix was probed by the cantilever with the connected 10 μm bead and then the bead penetration and the force were determined. The AFM measurements revealed a two-fold lower elastic modulus (28 Pa) for a bovine dermal collagen matrix compared to a rat-tail collagen matrix (both at 1.7 mg ml^{-1} (51 Pa)) (Wolf *et al* 2013, Stein *et al* 2008, Yang and Kaufman 2009). Although reconstituted at equivalent 1.7 mg ml^{-1} collagen type I concentrations, the collagen matrices differ substantially in fibril diameter as well as interfibrillar space and consequently in network stiffness.

11.2 3D collagen matrices partly mimic the natural extracellular matrix scaffold

Collagens encompass roughly 30% of the total cell protein in mammalian cells and they have been established as key players among the most promising biomaterials for the formation of novel bioengineering interventions (Fratzl 2008, Tihan *et al* 2016, Walters and Stegemann 2014). The superfamily of vertebrate collagen compromises over 50 collagen and collagen-like proteins (Ricard-Blum 2011, Hulmes 2008), among which collagen type I represents the greatest potential in cell and tissue engineering due to its distinct characteristics, such as the ability for self-assembly, biocompatibility, biodegradability and nontoxicity (Hasirci *et al* 2006). In particular, collagen type I is the most abundant protein in mammals and is the major component of the extracellular matrix (Fratzl 2008) and it is of crucial importance for vertebrate biology (Stylianou *et al* 2012). Collagen has a fibrous nature and its molecules consists of three amino acid chains, which build assemblies of rod-shaped triple helices to form collagen fibrils (Ricard-Blum 2011, Ricard-Blum *et al* 2005). These fibrils are aligned together to assemble even more complex structures such as bundles and fibers (figure 11.6) (Ricard-Blum 2011, Fratzl 2008, Ricard-Blum *et al* 2005). As a special feature, collagen molecules are packed in a quarter-staggered fashion, which creates a repeating banding pattern of about 67 nm in length that is termed the D-periodicity or D-band (Fratzl 2008, Hulmes 2008, Bozec *et al* 2007, Petruska and Hodge 1964). However, the exact length of the D-band depends on the individual tissue (Fratzl 2008, Hulmes 2008, Wallace *et al* 2011, Grant *et al* 2012). In particular, it has been shown that the transverse D-banding periodic pattern is a key player in providing the fibril mechanical properties (figure 11.7), while it has also been associated with pathological conditions and assumed to be critical for cell–collagen interactions (Wallace *et al* 2011, Grant *et al* 2012, Poole *et al* 2005, Stamov *et al* 2013). Indeed, a strong correlation between the orientation of D-band and cell elongation has been found (Poole *et al* 2005). Moreover, collagen type I fibrils represent the elementary building blocks in numerous collagen-rich tissues (Ivanova and Krivchenko 2012, Shoulders and Raines 2009). The collagen fulfills a wide range of functions ranging from the mechanical strength of tissues to the scaffolding for cellular migration and tissue repair after injury (Fratzl 2008, Ricard-Blum 2011, Gordon and Hahn 2010). Moreover, the collagen can have diverse morphologies in different tissues

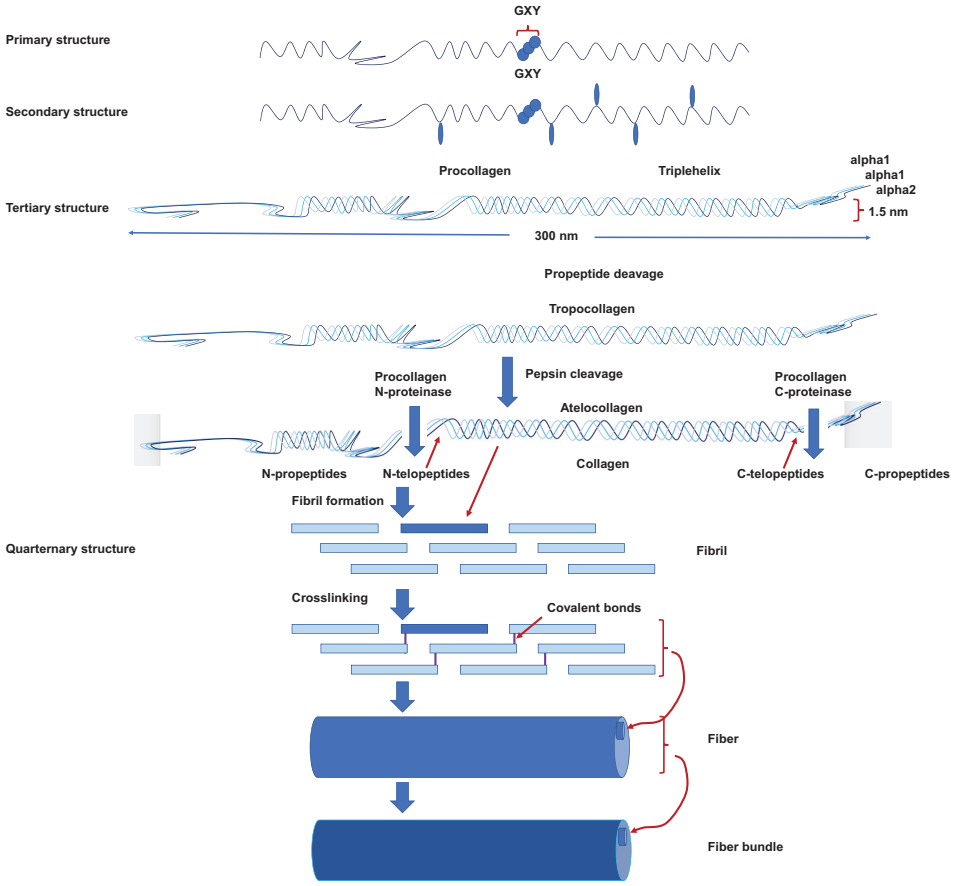

Figure 11.6. Collagen structure principle and pepsin digestion effect.

(Fratzl 2008). In skin dermis, the collagen is arranged in the form of loosely interwoven, wavy, randomly oriented and loosely packed bundles, whereas in tendon, collagen has a highly uniform distribution of fibril diameters with exhibits a tight lateral packing (Cen *et al* 2008). Within all different extracellular matrices, collagen fulfills the same role as it commonly acts to maintain the shape and integrity of tissues (Fratzl 2008).

11.3 Pore size

The pore size of 3D extracellular matrices is important for providing a scaffold in which cancer cells are able to invade. If the pore size is too small, cancer cells are not even able to migrate into the matrix, although they have properly adhered to the surface of the 3D extracellular matrix (Mierke *et al* 2011a, Mierke 2013). Another option for cells is, when the pore size is too small to support cell migration, to degrade and thus restructure the collagen matrix in a manner that allows them to move deeper in. This will be addressed in the following.

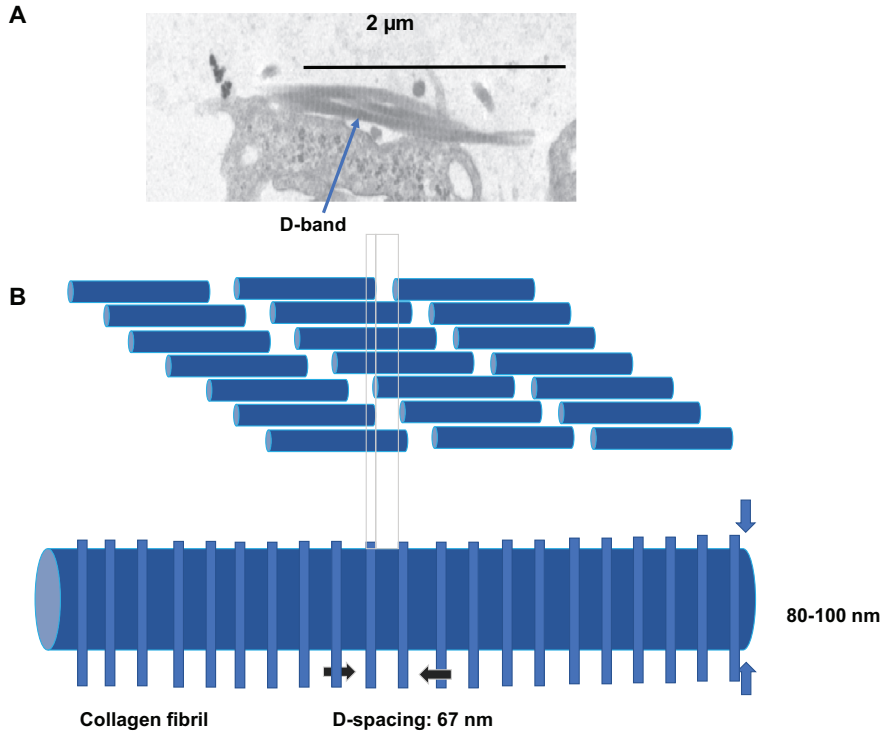

Figure 11.7. Collagen D-band structure.

The role of pore size during proteolytic and nonproteolytic migration of HT-1080 cells

In order to investigate differences in MMP-independent cell migration rates between collagen preparations, MT1-MMP expressing HT-1080 (HT/MT1) cells were used as a model for collagenolytic invasion, which can be inhibited and thus turned to collagenolysis-independent migration after addition of a broad-spectrum MMP inhibitor such as GM6001, or by MT1-MMP silencing (Wolf *et al* 2007, Sabeh *et al* 2009b). The HT/MT1 cells exhibit a higher migration speed of 0.7 μm min^{-1} in bovine dermal collagen matrices compared to a migration speed of 0.3 μm min^{-1} in rat-tail collagen matrices, with a collagen concentration of 1.7 mg ml^{-1} collagen type I for both (Wolf *et al* 2013). These results lead to the suggestion that MMPs support the migration in collagen scaffolds of different pore sizes and the migration speed depends on the pore size rather than the type of collagen. Conversely, the broad-spectrum MMP inhibitor GM6001 inhibited cell motility in rat-tail collagen matrices, whereas no inhibition of cellular motility in bovine dermal collagen matrices was observed. In particular, individual cell migration has been quantified by cell tracking, cell emigration from multicellular spheroids and vertical invasion after seeding the cells on top of the 3D collagen matrices. Moreover, similar results have been obtained using a transient knockdown of MT1-MMP expression,

confirming that MT1-MMP is indeed the invasion-promoting collagenase (Sabeh *et al* 2009b). How can this difference in the inhibition of cell motility be explained?

In order to analyze whether the difference of MMP-independent migration is caused by alterations in the fibril density and/or matrix porosity, different collagen concentrations have been used. Increased concentration of bovine dermal collagen (from 1.7 to 15 mg ml^{-1}) led to significantly smaller pore cross-sections (median 5 μm^2), higher stiffness and a dose-dependent decreased migration (Wolf *et al* 2013). Finally, the migration of HT/MT1 cells in the presence of GM6001 was totally inhibited. This graded response was subdivided into subtotal and absolute migration limits of 90% and 99% speed delay, respectively. Indeed, the decreased migration in bovine-dermis collagen was linearly correlated with the pore size for both MMP-dependent and MMP-independent migration, with similar slopes for the linear functions. The difference shown was in a higher offset with MMP-dependent migration. In contrast, when the concentration of rat-tail collagen was reduced to a minimum of 0.3 mg ml^{-1}, causing decreased collagen scaffold stiffness but providing larger pore cross-sections of 20–30 μm^2, a totally restored MMP-independent migration was observed (the speed distribution was similar to that of the control cells) (Wolf *et al* 2013). The migration correlated linearly with the pore size, but with a significantly steeper slope than for the migration of cells in which the MMP activity was inhibited.

What impact have type I collagen crosslinks on cancer cell invasion?

In contrast to other reports (Wolf *et al* 2003a, 2003b, 2007, Demou *et al* 2005, Carragher *et al* 2006), it has been observed that MT1-MMP-silenced HT-1080 or MDA-MB-231 cells are not able to adopt an invasive, amoeboid phenotype, when these cells are embedded in 3D type I collagen gels (Sabeh *et al* 2009b). However, the 3D collagen fiber matrices used in these studies were polymerized from pepsin-based type I collagen isolations. These isolations are based on a proteolytic process that eliminates the nonhelical telopeptides situated at the N- and C-terminal ends of native collagen molecules by cutting them from the collagen molecules (figure 11.6) (Sabeh *et al* 2009b, Wolf *et al* 2003a, 2003b, 2007, Demou *et al* 2005, Carragher *et al* 2006, Gadea *et al* 2008, Sanz-Moreno *et al* 2008). In the process of collagen fibrillogenesis, the collagen telopeptides play key roles, as they possess critical lysine residues that after lysyl oxidase-dependent oxidation *in vivo* promote the formation of intermolecular covalent crosslinks, which are required for the stabilization of the 3D collagen matrix scaffold (Gelman *et al* 1979, Brennan and Davison, 1980, Eyre *et al* 1984, Woodley *et al* 1991, Christiansen *et al* 2000, Sato *et al* 2000). Thus, it seems to be possible that the cancer cell's ability to migrate by utilizing a proteinase-independent, amoeboid migration mode may be altered by the structural integrity of the reconstituted collagen type I gel used for the *in vitro* motility assays.

When the formation of collagen gels is performed under conditions identical to those employed for full-length, native type I collagen, the pepsin-extracted collagen builds fibrillar networks that cannot be distinguished from those obtained with telopeptide-intact (natural) collagen as analyzed by scanning electron microscopy. As a main difference between telopeptide (native) containing and lacking collagen

gels is that the control (native) collagen gels are insoluble in high salt buffers, whereas collagen matrices prepared from pepsin-extracted material rapidly solubilize, which represents a characteristic physicochemical behavior of crosslinked (native) versus non-crosslinked collagen gels (Gelman *et al* 1979). Moreover, HT-1080 spheroids, which are embedded in pepsin-extracted collagen gels, can migrate into the surroundings of the collagen matrix as single cells with the invasive front displaying a mixture of two morphologically different types, such as spheroid- and elongated-shaped cells. Within 24 h, migrating HT-1080 cells migrate in the pepsin-extracted gels to distances that require an additional 72 h incubation period in full-length type I collagen matrices (Sabeh *et al* 2009b). Neither the MT1-MMP silencing through the addition of the pan-specific MMP inhibitor GM6001 nor the inclusion of a broad-spectrum cocktail of proteinase inhibitors (Wolf *et al* 2003a) can impair the cell invasion of either HT-1080 nor MDA-MB-231 cells (Sabeh *et al* 2009b).

The type I collagen telopeptides can potentially regulate invasion by either altering the linear or lateral stages of the fibrillogenic process, or by supporting the establishment of collagen crosslinks, which in turn increases the stiffness of the matrices (Gelman *et al* 1979, Woodley *et al* 1991, Christiansen *et al* 2000, Sato *et al* 2000). The intact collagen can be acid-extracted in nearly pure form from tissues of young animals, as a subset of the lysyl oxidase-catalyzed collagen crosslinks are formed *in vivo*, which are acid-labile, Schiff base adducts (Eyre *et al* 1984). In particular, at low pH these crosslinks are reversibly ruptured, which leads to the formation of aldehyde-bearing collagen molecules that are fully soluble in acidic buffers of low ionic strength (figure 11.6) (Eyre *et al* 1984). The Schiff base adducts, which connect collagen bundles, are reformed spontaneously when these collagen gels are reconstituted at neutral pH, whereas this crosslinking process can be impaired by chemically reducing the aldehyde moieties before the formation of the gel (figure 11.6) (Gelman *et al* 1979). In addition, when HT-1080 cells are embedded in 3D gels of aldehyde-reduced type I collagen, the cells can even migrate with faster speeds through these matrices of reduced collagen gels than these cells migrating through pepsin-extracted collagen gels. Moreover, neither MT1-MMP silencing nor the addition of GM6001 are able to impair significantly the HT-1080 or MDA-MB-231 invasion (Sabeh *et al* 2009b). As the pore sizes of collagen gels consisting of intact (native) and pepsin-extracted collagens are similar (Demou *et al* 2005), it can be hypothesized that the MT1-MMP-independent invasion only proceeds when the structural pores present in the collagen gel networks are not stabilized by the covalent crosslinks which define the fibril architecture and structural rigidity (Zaman *et al* 2006).

What is the effect of varying collagen concentrations on cellular motility and are there side-effects? Similar experiments have been performed, but without varying the collagen concentrations in order to minimize the indirect effects unrelated to porosity, such as altered density of the adhesive ligands (Zaman *et al* 2006) or uncontrolled collagen crosslink density in bovine dermal collagen, and the porosity of rat-tail collagen matrices was altered by reducing the polymerization temperature (Raub *et al* 2007) while keeping the collagen concentration constant (1.7 mg ml^{-1}). At 9 °C, the fibrillogenesis was delayed in time and, hence, the pore diameters and

related cross-sections increased (median diameter 8 μm and median cross-section 30 μm^2) and the fractal box count decreased, while the diameter of the fibrils (80 nm) and the mechanical stiffness increased. As the pore size increased, MMP-independent migration in rat-tail collagen was rescued by single-cell movement with peak speeds close to the proteolytic migration rates. This behavior was seen for both cell emigration from multicellular spheroids and vertical invasion cultures. As reported previously, the speed of MMP-independent migration was linearly dependent on the pore size, with a significantly steeper slope than for protease-dependent migration. In the method where the pore size and the type of migration (migration mode) were altered, the cell speed was in all cases linearly correlated with the pore size and independent of collagen preparation. The maximum speed is observed at pore sizes that approximate the size of the entire cell body where the matrix scaffold guides the migration without any physical impairment (Jacobelli *et al* 2010). This ability to perform MMP-dependent motility when cells are sterically hindered by small pores that otherwise impede or arrest migration leads to maintenance of the migration.

The role of the deformability of the cell's nucleus during MMP-independent migration in dense extracellular matrices

What types of subcellular compartments regulate migration in dense 3D microenvironments? In order to answer this question, the morphokinetic alterations of the cell's body, leading edge and nucleus were analyzed during cell migration as a function of collagen density. HT/MT1 cells embedded in bovine dermal collagen in the presence of the MMP inhibitor GM6001 moved the leading edge and cell bodies at equal velocities. However, these cells stopped their migration in rat-tail collagen matrices and the cells had a spherical central body with dynamic, dendrite-like extensions that were able to deform the collagen at the cell's front pole without promoting cell movement. In addition, it has been observed that pseudopods can break off from cell bodies and then move with a snake-like morphology (Wolf *et al* 2013). The immobile fraction of the cell's body still consists of cytoplasm and the nucleus, and forms occasional small protrusions pointing toward the extension of the cell's leading edge. In non-moving cells within the matrices, the nuclear prolapses measure 1–3 μm in diameter (1–7 μm^2 cross-section), whereas the nuclei of cells during MMP-independent migration adopt deformations that are 3–7 μm in diameter (7–40 μm^2 cross-section). In contrast, these morphological nuclear structures are distinct from the ellipsoid nuclear shapes, with a diameter of 8–11 μm and a cross-section of 50–100 μm^2 being found in MMP-dependent migrating HT-1080 cells that are able to generate proteolytic tracks with a diameter close to that of the cell's diameter. Independent of the collagen preparation or matrix scaffold porosity, the nuclear diameters of MT1-MMP expressing control cells possess average cross-sections of 40–90 μm^2. However, the addition of GM6001 leads to a decrease in nuclear diameters until the cell sticks within the collagen matrix scaffold without any movement. Then the nucleus displays a single small nuclear prolapse or a non-deformed spherical shape, suggesting that the non-moving nuclei cycle between prolapse and rounding morphology. Finally, hourglass-shaped deformations of the cell's nucleus supported the MMP-independent migration of HT/MT1 cells through

3D collagen matrices with pore diameters above 7 μm^2. In contrast, smaller pore sizes break down the nuclear deformability and lead to physical arrest, while persistent leading edge kinetics, force generation and transmission are still present. Taken together, the MMP-independent migration into dense 3D extracellular matrices is regulated through the deformation of the cell's nucleus. However, the role of the contractile forces in providing cancer cell motility is still controversial. There are reports that have found a connection between cellular motility and the generation of contractile forces (Mierke *et al* 2011a, Mierke *et al* 2011b, Mierke et al 2017, Fischer et al 2017).

The impact of actomyosin contractility and integrin-facilitated mechano-coupling of cells migrating in a dense matrix confinement

Mechano-coupling seems to fulfill an active role in confined spaces, as cytoskeletal activity has been shown to regulate the migration of cells (Mierke *et al* 2008). Thus, it has been investigated whether cell motility is altered by (1) the integrin-facilitated leading edge traction on a substrate (using a β1 integrin-perturbing mAb 4B4) (Wolf *et al* 2003a) and (2) actomyosin-dependent contractility (using a ROCK inhibitor, such as Y-27632, which inhibits myosin light chain (MLC) phosphorylation and hence the contraction of the cell's rear) (Ren *et al* 2004, Laemmermann *et al* 2008). However, these two approaches gradually reduced HT/MT1 cell-facilitated contraction of collagen matrices. When using 3D collagen matrices with a 20 μm^2 pore area, which represents only a moderate physical challenge to nuclear deformability, both mAb 4B4 (inhibiting the function of the β1 integrin subunit) and Y-27632 decreased the migration rates in a dose-dependent manner. In particular, the effect was even more pronounced in the presence of GM6001-treated cells compared to buffer-treated control cells. Mechanically perturbed force generation from mAb 4B4 addition caused alterations in the cell elongation. When the mAb 4B4 was used at a concentration that inhibited the contraction of the collagen matrix by approximately 50% (1 $\mu g\ ml^{-1}$), the MMP-independent migration was almost completely inhibited due to impaired capacity to generate sufficient adhesion and traction force to move the nucleus through the cell's cytoplasm. In line with these observations, reducing space constraints by enlarging pore diameters to approximately 55 μm^2 almost completely rescued the migration of cells. When the migration speed was decreased in a confined 3D collagen matrix (1.7 mg ml^{-1} bovine collagen) in the presence of GM6001, increased nuclear deformation was observed, which could be reversed when the matrix porosity was increased or MMPs were not inhibited by GM6001. When cell–matrix adhesion receptors such as integrins are active, the importance of actomyosin-facilitated cell contraction in pushing the nucleus through the 3D extracellular matrix was mirrored in the time-delayed rear retraction and increased the cell length in the presence of Y-27632. In addition, a concentration of Y-27632 (2 μM) led to a half-maximum collagen contraction, which caused a partial cell migration arrest and at the same time evoked a strong deformation of the nucleus in the presence of GM6001. When the cross-sections of the pores were increased, these two effects (cell motility and nuclear deformation) could be reversed. Taken together, both integrin-facilitated traction force and actomyosin contractility are

needed to squeeze the nucleus forward when a dense extracellular matrix is transmigrated in concert with MMP-facilitated pore generation.

Are there different kinetics and rate-limits in mononuclear and polymorphonuclear cells?

To investigate how different nuclear shape types regulate migration in confined matrices, the nuclear mechanics of different cell types with mononuclear or polymorphonuclear organization were analyzed. The intermediate filament lamin A/C is a central nuclear protein that is required for nuclear membrane organization and stability (Goldberg *et al* 2008), and in addition it is expressed in cancer cells such as HT/MT1, HT/wt and MDA-MB-231/MT1 (MDA/MT1) breast cancer cells, whereas it cannot be detected in human $CD4^+$ T-lymphocytes or polymorphonuclear neutrophils (PMNs). Independently of cell type, migration of mononuclear cells through low- to intermediate-density 3D collagen matrices is not affected by GM6001. Migration is still possible by deformation of the nucleus with cross-section distributions matching the available pore size range of the 3D extracellular matrix. In HT/wt cells and T-lymphocytes migrating through dense 3D extracellular matrices, hourglass nuclear shapes predominate, whereas MDA/MT1 cells show a broader spectrum, from hourglass to cigar-like shapes. When the pore dimensions range from 2 to 5 μm^2, GM6001 leads to a migration stop and the formation of a nuclear prolapse in cancer cells, whereas the T-lymphocyte migration persists with a lower migration speed. Compared to cancer cells, T-lymphocytes can be distinguished by a two- to four-fold smaller nucleus and their inability to proteolytically degrade fibrillar collagen, thus their motility and their ability to cross barriers (such as an endothelial barrier) depends solely on shape changes (Wolf *et al* 2003b). However, all T-lymphocytes become unable to migrate through matrices when they have pore cross-sections of 1–2 μm^2, which is no longer in the spatial range for possible nuclear deformation. In summary, MMP-independent mononuclear cell migration uniformly depends on the ability to deform the nucleus in response to the lateral compression induced by structures of the connective tissue.

Similar behavior has been observed for PMN. PMN migration is not influenced by the absence or presence of GM6001 in low- to intermediate-density collagen matrices. In contrast to the homogeneous deformation of mononuclear nuclei, the segmented nucleus of PMNs is characterized by interconvertible folding states, such as compact configuration or pearl-chain-like complete and partial unfolding. The different folding states lead to migration speed alterations in the PMWs. When the nucleus is compact, the cells are nearly immobile. In contrast, when the nucleus is unfolded, the cells are motile. In high-density rat-tail collagen matrices (3.3 mg ml^{-1}, pore cross-section 2–3 μm^2), PMN migration is still observed independently of MMP activity, however, the migration speed is reduced and the cells are immobile at higher collagen density (6.6 mg ml^{-1}), with pore cross-sections in the range of 0.5–1.5 μm^2, which is below that of the nuclear cross-sections. Similar to cancer cells, immobilized PMNs display collapsed and spherical nuclei with occasional single-segment prolapse in the direction of the leading oscillating pseudopod. In summary, shape change patterns such as the hourglass-like compression of mononuclear nuclei

or the unfolding of polymorphonuclear nuclei are needed for cellular migration through interstitial matrices. In high-density fibrillar 3D collagen matrices with no collagenolysis, the nuclear deformability determines the migration rates as a function of pore size. The cellular motility limit for cancer cells is at a cross-section of 7 μm^2 and for PMNs and T-lymphocytes it is at a cross-section of 2–3 μm^2. Taken together, inhibited cell migration is linearly correlated with pore size, but is independent of the matrix scaffold's stiffness.

Physical confinement of cell migration through nondegradable substrates
Migration rates through dense 3D collagen matrices and broad-spectrum MMP inhibitor treatment can also be regulated by additional parameters that have not yet been addressed, such as residual low-level MMP-independent collagenolysis performed by other classes of proteases that are not inhibited by GM6001. In addition, also structural guidance by occasional gaps present in the assembled matrix scaffolds and mechanical rupture of very small or incompletely polymerized collagen fibrils by migrating cells may alter the motility of cancer cells. In order to exclude such side-effects from interfering with the parameters, a polycarbonate filter model can be used for cell trafficking through nondegradable and hence nondeformable barriers. In this relatively stable system, a subtotal inhibition (90%) of the cell migration was detected at pore cross-sections of 7–10 μm^2 for all mononuclear cell types and 4 μm^2 for PMN. Complete inhibition (>99%) was found at pore dimensions below 5–6 μm^2 for mononuclear cells and 1 μm^2 for PMNs. Similar to 3D collagen matrices, efficient transmigration through polycarbonate filter pores is facilitated through the cytoplasmic protrusion, which is followed by a nuclear deformation within a pore cross-sectional range of 7–50 μm^2. The adhered and immobilized cells above the small pores (0.8 μm^2 cross-section) display a morphology of a long cytoplasmic protrusion extending into the pore, whereas the nucleus is still above the pore and able to squeeze through the pore. This is very similar to the arrested phenotypes of cells observed in high-density fibrillar 3D collagen matrices. In summary, cell migration through nondegradable pores has certain physical limits that depend on pore size and nuclear deformability. Moreover, these physical limits of 3D fibrillar collagen matrices are confirmed by the results of these cell migrations through nondegradable and nondeformable pores.

The rate-limiting physicochemical parameters for cell migration can be obtained through analyzing the differences between MMP-dependent and MMP-independent cell motility with decreasing matrix scaffold porosity and by quantitatively determining the migration speed and the shape of the cell's body as well as the nucleus. The first of these parameters is the availability of spaces between neighboring extracellular matrix fibrils or within the filter pore that can afford the movement of the cell's body, and the second is the deformability of the cell's nucleus due to the requirements of the matrix's or the filter pore's confinement. With decreasing cross-section, interfibrillar pores mechanically hinder cellular migration and evoke a deformation in a cell-type-specific manner until the limit for deformability is reached and the nucleus is mechanically trapped within the matrix's confinement. The balance between cell translocation and arrest is regulated by processes that control

either the pore size or nucleus deformation. The cellular translocation is supported first by effectors modulating the remodeling of collagen fibrils through MT1-MMP to create 'neo-space', and second by mechano-coupling through the integrins and the cell's actomyosin contractility to push the nucleus through confined matrices.

How does the extracellular matrix porosity in vivo and in vitro affect cancer cell motility?

Fibrillar collagen is organized *in vivo* as fibrils and fibers of diverse thickness, orientation and interfibrillar spacing (Starborg *et al* 2008, Wolf *et al* 2009). It has been reported that the interfibrillar space varies largely *in vivo* in loose and dense interstitial tissues, ranging between 2 and 30 μm (Stoitzner *et al* 2002, Wolf *et al* 2009, Weigelin *et al* 2012). In order to have an appropriate model for cellular invasiveness, 3D collagen matrices can be utilized, in which the porosity of 3D collagen matrices can be varied similarly. This large range of porosity serves on the one hand to guide cell migration and on the other hand to mechanically challenge migrating cells that commonly display cross-sections of 30–100 μm^2 in a cell-type-dependent manner.

What are the limits of cell migration in 3D?

It has been shown that cell migration speed reduces linearly with decreasing pore size in a cell-type-specific manner. The reduction in migration speed in response to increasing mechanical confinement is a gradual process that largely depends upon cellular deformability, which is a strategy used by numerous migrating cells (Lautenschläger *et al* 2009, Wolf and Friedl 2011, Tong *et al* 2012, Mierke *et al* 2011a). The maximum speed is obtained at pore sizes that approximate the cell's body sizes, where the matrix substrate guides without physically hindering migration (Jacobelli *et al* 2010). With decreasing porosity of the matrices, cell migration decreases inversely proportionally to their increased capability to deform, leading to a slower migration, which is abolished when the subtotal limit of cellular deformability is reached. Migration may be abrogated when a cell-type-specific maximum of deformation is reached, which is termed the absolute limit. As the cell's shape is highly adaptable to microenvironmental conditions, pores of defined cross-sections differing only in their geometry (such as discontinuous polygonal-shaped pores in 3D fibrillar collagen matrices, flat and broad cleft-like spaces, evenly shaped cylindrical pores of polycarbonate membranes and elongated continuous channels of microdevices) are equally well suited for cell migration (Lautenschlaeger *et al* 2009, Jacobelli *et al* 2010, Rolli *et al* 2010, Ilina *et al* 2011, Balzer *et al* 2012, Tong *et al* 2012). It has been reported that a cross-section of 20–30 μm^2 is near-optimal for the interstitial migration of HT-1080 and MDA-MB-231 cells through complex-shaped spaces in 3D collagen matrices and monomorphic transwell membrane filter pores, or through the 30 μm^2 cross-sectional areas of engineered rectangular microchannels, promoting cell migration with high speed (Tong *et al* 2012). Keratinocytes are good examples for cells displaying cell shape adaptations as they can traverse gaps of 500 nm height and unrestricted width (Brunner *et al* 2006). Hence, it can be concluded that it is only the cross-section of the transmigrated space and not the diameter that regulates the efficacy of cell migration in a confined

matrix. Hence, the limits of cell migration depend on a two-parameter function of the matrix's or substrate's porosity and the cell's deformability.

What role does the nucleus play in the process of cell motility through dense extracellular matrices?

The nucleus is the largest and most rigid cell organelle, because of the chromatin content and the stabilizing nuclear lamina consisting of the intermediate filament lamin A/C (Dahl *et al* 2004, Gerlitz and Bustin 2011, Chow *et al* 2012). Migrating cells display two mechanically distinct forms of nuclear deformation: cell types with mononuclear nuclei show global deformation, resulting in transient hourglass-like or cigar-shaped elongated morphologies, whereas cell types with polymorphonuclear nuclei show the unfolding of polymorphonuclear nuclei in order to display pearl-chain-like configurations without major deformations in the individual nuclear segments. These reversible migration-associated nuclear morphologies are frequently detected *in vivo* during the dissemination of cancer cells (Yamauchi *et al* 2005, 2006, Alexander *et al* 2008, Beadle *et al* 2008, Friedl *et al* 2011) and their transendothelial migration (Feng *et al* 1998, Voisin *et al* 2009). The major restriction for cellular deformability is the stiff nucleus, because the deformability of the nucleus in dense spaces is finite, whereas the cytoplasm is able to squeeze through almost any pore size, including 1 μm^2 gaps in 3D collagen matrices and 0.8 μm^2 pores in the polycarbonate membranes used for transwell membrane assays (Schoumacher *et al* 2010, Shankar *et al* 2010).

Taken together, cell movement through dense 3D extracellular matrices is regulated by at least three properties of the nucleus—size, rigidity and shape (structure)—which regulate an adaptation range of a factor of two to five when mononuclear cancer cells are compared with PMN. A consistent ratio of nearly 1/10 has been found for minimum nuclear cross-sections relative to the nondeformed state, suggesting that the maximal compressibility is an absolute value, independent of cell type, basal nuclear shape and nuclear rigidity. The nuclear lamina seems to regulate the nuclear shape and/or deformability rather than the absolute compression limit, which leads to the suggestion that noncompressible intranuclear components such as chromatin determine the compression maximum and, consequently, provide the physical restriction of cellular migration.

How does proteolysis alter the porosity of the extracellular matrix?

By degrading fibrillar collagen at the cell–matrix interface, MT1-MMP-dependent proteolysis enlarges the collagen pore cross-sections to facilitate cell migration through degradable substrates such as 3D extracellular matrices (Wolf *et al* 2007, Fisher *et al* 2009, Sabeh *et al* 2009b). It has been shown that MT1-MMP increases the migration at confining porosity above critical limits and hence provides slow and persistent migration even in very dense 3D extracellular matrices. However, with porosity high enough to perform migration through the deformation of the cell body, pericellular proteolysis is unnecessary, as the migration persists despite pharmacological or molecular targeting of MMP activity in either cancer cells or leukocyte populations. This suggests that MMPs regulate the dimensions of the

pores, thereby enhancing cell speed, deformation and finally the limits of cell migration in degradable matrix scaffolds. There has been confusion about the role of MMPs in pore size control (Wolf *et al* 2003a, Sabeh *et al* 2004, 2009a, 2009b), but another report using different collagen preparations side by side resolved the different results regarding the role of MMPs for cell invasion through 3D collagen scaffolds of different origin and porosity (Wolf *et al* 2013).

The impact of mechano-coupling on 3D cellular motility

Forces generated between the cell and 3D matrix scaffold may have an effect on cellular deformation and, consequently, on migration through a confined matrix. In particular, β1 integrins act as both the main adhesion receptors to fibrillar collagen and as mechanotransducers in migrating cells (Huttenlocher *et al* 1995, Puklin-Faucher and Sheetz 2009), hence providing the traction forces necessary to push the nucleus through narrow pores in dense 3D extracellular matrices. Rho kinase-mediated actomyosin contractility reinforces the integrin-facilitated cell adhesion and regulates the retraction of the cell's rear (Vicente-Manzanares *et al* 2008), leading to the translocation of the cell's nucleus through small spaces (Laemmermann *et al* 2008). Taken together, MMP-dependent extracellular matrix degradation and integrin- and Rho-mediated force transmission may use complementary mechanisms to secure cellular migration, particularly when space is confined.

What affects the limits of cell migration in an indirect manner?

Additional physicochemical properties of the matrix's substrate may alter migration rates, such as the extracellular matrix's chemical telopeptide status and physical stiffness (Sabeh *et al* 2009b, Miron-Mendoza *et al* 2010, Ehrbar *et al* 2011). It has been shown that high telopeptide content in extracted rat-tail collagen accelerates the fibril polymerization and increases mechanical strength, whereas the fibril thickness and the network porosity are reduced (Helseth and Veis 1981, Elbjeirami *et al* 2003, Sabeh *et al* 2009b, Wolf *et al* 2009), which hinders non-proteolytic cell migration. In contrast, with temperature-dependent slowed-down collagen fibrillogenesis, larger pore sizes are generated, which match nuclear size and deformability better, and hence support nonproteolytic (MMP-independent) cell migration. Finally, independent of the telopeptide content of the collagen preparation used, small pores cause increased nuclear deformability and exclude a MMP-independent migration mode. No influence on cell immobilization through matrix restrictions is observed to come from different substrate stiffness. A stiffness range of 20–700 Pa and larger than 10^6 Pa was investigated for reconstituted fibrillar collagen and polycarbonate membranes, respectively. The elastic moduli of *in vivo* tissues range from 200 to 1000 Pa for mammary interstitium and from 1000 to 10 000 Pa for adipocytes and myofibers, and they can thus be mimicked by the two different artificial microenvironments (Stein *et al* 2008, Butcher *et al* 2009, Levental *et al* 2009, Buxboim *et al* 2010). Finally, when these results are normalized to the pore size, both soft and rigid substrates lead to nearly identical subtotal and absolute migration restrictions, excluding substrate stiffness larger than 20 Pa. These stiff

substrates possess an independent mechanism for regulating cellular deformability and, consequently, physical migration arrest within these matrices.

Taken together, this multiscale analysis of cell–matrix geometries and migration kinetics may help to reveal the biophysical processes involved in the regulation of cellular motility in a confined microenvironment, such as dense 3D extracellular matrices. These biophysical parameters may include the elasticity of the nucleus by regulation of the lamin A/C expression, nucleus–cytoskeleton linkage, intracellular and intranuclear pressure and hydration, chromatin organization and physical sensitivity to repetitive or long-lasting mechanical stresses (such as cellular stress-stiffening behavior). The deformability of the cell's body and cell's nucleus are suitable for migration through 2D and 3D tracks, as well as through gaps that are constitutively present in healthy connective tissues (Wolf *et al* 2009, Weigelin *et al* 2012). However, it is suggested that the penetration of tumor-associated collagen-rich desmoplastic tissue, scarred stroma and the basement membranes involves even more complex invasion signaling pathways (Provenzano *et al* 2006, Rowe and Weiss 2009, Tanaka *et al* 2010, Salmon *et al* 2012). Finally, multiscale biochemical and molecular experiments and analyses of cell migration will together reveal the strategies used by cells to migrate through extracellular matrix barriers encountered *in vitro* and *in vivo*.

11.4 Matrix stiffness

The mechanical properties of tissues in which cells are embedded play an important role in cellular functions such as proliferation, survival, development of tissues, tissue homeostasis, vascularization and organ function. Thus, the impact of matrix stiffness on cellular motility has been investigated in reductionist *in vitro* micro-environment model systems such as artificial extracellular matrices, which mimic specific extracellular matrix functions under highly controlled conditions. In particular, they have frequently served to elucidate the role of cell–extracellular matrix interactions in regulating cell fate. To reveal the interplay of biophysical and biochemical effectors in controlling 3D cell migration, a poly(ethylene glycol)-based artificial extracellular matrix platform was used. The influence of the matrix crosslinking density, represented by the matrix stiffness, on cell migration *in vitro* and *in vivo* was investigated. The migration capacity of single preosteoblastic cells within hydrogels of varying stiffness and susceptibility to degradation by MMPs was analyzed *in vitro* using time-lapse video microscopy. Indeed, the motility of the cells was strongly dependent on matrix stiffness. In more detail, two migration regimes were identified: a nonproteolytic migration mode at a relatively low matrix stiffness and a proteolytic migration mode at higher matrix stiffness. In line with this, *in vivo* experiments revealed a similar stiffness dependence for matrix remodeling by invasive cells; however, the dependence was less sensitive to the MMP sensitivity. Thus, this artificial extracellular matrix model system is indeed well suited to reveal the role of biophysical and biochemical parameters for physiologically relevant cell migration phenomena.

In tissue engineering, the regulation of 3D cell migration within and into biomaterial scaffolds plays an important role. In particular, biomaterials can serve as implants and can be designed to guide endogenous stem or progenitor cells to the site of a tissue defect to facilitate tissue regeneration by promoting repopulation and remodeling of an implant by host cells, which is a process that is regulated by large-scale cell migration (Lutolf and Hubbell 2005). The absence of sufficient cell migration is the most prominent limitation in creating large tissue-engineered constructs. For example, impaired endothelial cell invasion into connective tissue may evoke a lack of vascularization and may then, ultimately, lead to necrosis (Phelps and Garcia 2010). In contrast, in smart biomaterials designed to be carriers for cell delivery to targeted sites, encapsulated cells must be able to leave their delivery system and migrate extensively into 3D extracellular matrices (Mooney and Vandenburgh 2008). Thus, these critical biological and biomechanical requirements for engineered biomaterials containing cells need to be optimized for 3D cell migration properties and they have thus become the focus of biomedical research for cell carrier systems. For this optimization of the cell migration behavior within biomaterials it is necessary to reveal the 3D cell motility mechanisms for physiological and pathological situations as well as proteolytic (mesenchymal) or non-proteolytic (amoeboid) migration strategies (Zaman *et al* 2007, Ilina and Friedl 2009). In contrast to migration in 2D, cells in 3D have to overcome the sterical hindrances and confinements of their surrounding microenvironment. In the proteolytic migration mode, cells secrete active proteases in order to break down macromolecules of the extracellular matrix and thus create macroscopic cavities such as migration tunnels, which facilitate their movement. Matrix degradation occurs in the local microenvironment of the cell and hence it is possible to degrade the extracellular matrix by membrane-bound proteins such as membrane-type (MT) MMPs or secreted proteases such as collagenases.

As an alternative to proteolytic migration through 3D extracellular matrices, a number of inflammatory cell types, such as lymphocytes and dendritic cells, or cancer cells, are known to utilize migration strategies to overcome biophysical matrix barriers by squeezing through the extracellular matrix or even by deforming it without any proteolysis (the amoeboid migration mode). However, the same cell type can use both proteolytic and nonproteolytic mechanisms, depending on the specific extracellular matrix context, such as the constituents and confinement (Friedl and Wolf 2010).

Thus, several approaches for generating synthetic biomaterials that support both types of cellular migration have been reported (Lutolf and Hubbell 2005). The main focus is on designing biomaterials that are beneficial for cell migration through interconnected and pre-existing pores (Moroni *et al* 2008). Indeed, biomaterials have been constructed as artificial extracellular matrices that are sensitive to cell-derived proteases degrading the artificial extracellular matrices that still mimic the *in vivo* extracellular matrix of connective tissue. In more detail, peptidic substrates that are to be cleaved by MMPs or plasmin are placed in the backbone of crosslinked hydrophilic polymer chains, which results in the degradation to specific copolymer hydrogels, as the integrated cleavage sites are cut through (figure 11.8). In particular,

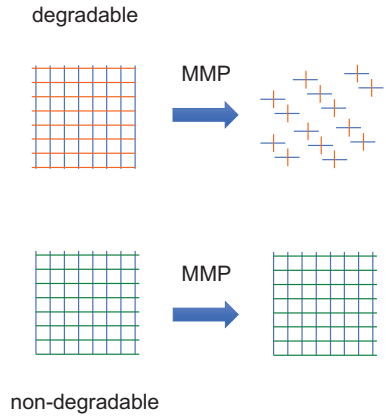

Figure 11.8. Schematic image of the design of PEG-based artificial extracellular matrices. Stoichiometrically balanced (the ratio of lysine to glutamine is 1–4) 8 arm PEG macromers in a buffer solution are enzymatically crosslinked through their pending glutamine acceptor [Gln] and lysine-donor [Lys] FXIIIa substrate sequences to assemble a hydrogel. Through the variation of the linker sequence and the initial precursor concentration, artificial extracellular matrices with different stiffnesses and different MMP sensitivities in the presence of constant RGD concentrations can be obtained.

artificial extracellular matrices are based on a simple design strategy, which includes the incorporation of an integrin-binding peptide such as RGD or other protein components promoting cell adhesion. These resulting advanced extracellular matrices then serve as synthetic extracellular matrix analogs through which the cells can migrate using proteolytic mechanisms. Although artificial extracellular matrices are frequently used in tissue engineering and 3D cell culture to complement naturally derived extracellular matrices, such as collagen matrices or Matrigel (Lutolf 2009, Tibbitt and Anseth 2009), it is not yet clear whether the combination of these different types of matrices affects the various biochemical and biophysical artificial extracellular matrix characteristics in facilitating 3D cell migration and invasion (Zaman *et al* 2007). A key question that has remained unanswered is whether certain artificial extracellular matrix characteristics support proteolytically or nonproteolytically facilitated cell migration modes. However, this has been addressed using an engineered artificial extracellular matrices model system composed of poly(ethylene glycol)(PEG)-based macromers, which are assembled by the addition of transglutaminase (TG) factor XIII (Ehrbar *et al* 2007a, Ehrbar *et al* 2007b) (figure 11.8).

Dissimilar to existing 3D extracellular matrices produced from naturally derived extracellular matrix components such as collagen matrices, these molecularly engineered TG-PEG gels possess no microstructure and are therefore nonporous. Moreover, these matrices are structurally homogeneous. Indeed, these synthetic TG-PEG gels are composed of a molecular meshwork consisting of flexible crosslinked polymers with a mesh size of approximately tens of nanometers (Ehrbar *et al* 2007a). The behavior of cells in 3D extracellular matrix microenvironments can be modulated by varying the crosslink density, altering the matrix degradability, adapting the porosity/topology of the entire matrix, and affecting the cell–extracellular matrix

adhesion. All these parameters can, at least to some degree, be fine-tuned within these synthetic extracellular matrix scaffolds. In particular, the generated TG-PEG hydrogel matrices (Ehrbar *et al* 2007b) are subject to swelling and thus the viscoelastic properties can be varied by different precursor concentrations such as 1.5, 2 and 2.5% v/w. Additionally, the sensitivity to degradation by MMPs is archived by integrating a different linker peptide, which is sensitive to proteolytic cleavage, such as Ac-FKGG↓GPQG IWGQ-ERCG-NH2 (with ↓ marking the cleavage site) instead of the cleavage insensitive linker peptide such as Ac-FKGG-GPQGIAGF-ERCG-NH2. The viscoelastic properties of the swollen matrices are analyzed using small strain oscillatory shear rheometry (Lutolf and Hubbell 2005). The storage moduli (G') at a frequency of 1 Hz is in the range from 94 ± 25 Pa at 1.5% to 482 ± 77 Pa at 2.5% for MMP sensitive matrices and from 62 ± 32 Pa (1.5%) to 347 ± 19 Pa (2.5%) for MMP-insensitive gels (hence the 1.5% gels are soft, the 2.0% are intermediate and the 2.5% are stiff), which indicates that the values of the MMP sensitive and insensitive matrices are highly similar (Ehrbar *et al* 2011). Moreover, these stiff, intermediate and soft TG-PEG hydrogels (1.5–2.5%) display relatively similar elastic properties as 'natural' hydrogel networks such as collagen or fibrin at concentrations of 2 mg ml^{-1} (Raeber *et al* 2005).

As with existing artificial extracellular matrix models (Ehrbar *et al* 2007b, Gobin and West 2002, Halstenberg *et al* 2002, Bryant and Anseth 2002, Shu *et al* 2003, Kim *et al* 2005, Raeber *et al* 2005, Almany and Seliktar 2005, Peyton *et al* 2006), the biochemical and biophysical properties of TG-PEG hydrogel networks are determined by incorporating protease-sensitive peptide domains and cell-adhesive ligands, or by tuning the matrix stiffness through the modification of the precursor polymer architecture or concentration. In order to investigate the modularity of this system, a systematic analysis was carried out to assess how 3D migration of single cells depends on the gel's stiffness, which was adjusted independently of the matrix's degradability.

Surprisingly, it was found that in artificial extracellular matrices with low stiffness, single cells can overcome the confinement of the matrix by performing a degradation-independent 3D migration mode, suggesting that these cells use pre-existing or *de novo*-formed macroscopic gel defects. This particular finding highlights that the engineering of gel defects in seemingly homogeneous polymer gels is a powerful tool for increasing cellular permissiveness in dense artificial extracellular matrices.

The knowledge and optimization of 3D cell migration within and into biomaterial scaffolds is an advantage for tissue engineering. The TG-PEG matrices serve as a suitable model system for examining the impact of matrix stiffness and proteolytic remodeling on 3D cell migration *in vitro* and *in vivo*, without altering the mechanical or structural properties at the start of the cell migration and invasion assay. The structural components of the native extracellular matrix, such as fibrillar collagen, ensure an interconnected microporous network that contains other matrix components such as glycosaminoglycans. 3D cell motility within this complex microenvironment such as TG-PEG relies on proteolysis-dependent and proteolysis-independent mechanisms (Ilina and Friedl 2009), but to distinguish between both modes is not easy. Indeed, 3D cell migration data obtained from *in vivo*

extracellular matrices or *in vitro* models derived from natural extracellular matrix components are difficult to interpret in terms of extracellular matrix characteristics and reproducibility. Hence, it is not easy to uncover the various cell–matrix interactions that determine 3D cell migration and the effect of matrix structural remodeling. Thus, many reports seem to be inconsistent; for example, Sabeh *et al* (2009a, 2009b) showed that cancer cells migrate within noncovalently crosslinked collagen gels independently of proteases, while in covalently crosslinked collagen gels, MT1-MMP activity was essential for movement.

Engineering approaches have led to the formation of artificial extracellular matrices that offer high control over the material microstructure, and in some studies scaffolds have been built that are essentially amorphous and pore-free. Indeed, by using protease inhibitors, it has been demonstrated that 3D migration in dense chemically crosslinked PEG hydrogels is strongly dependent on proteolysis (Raeber *et al* 2005, 2007). Using enzymatically crosslinked artificial PEG-based matrices, a similar protease-dependence across the entire stiffness range has been proposed. From the swelling ratios of all gel conditions (approximately tens of nanometers), the molecular pore sizes can be calculated. Indeed, the molecular pore size is far below the threshold that a cell would be able to penetrate and break through the surrounding physical matrix barrier and hence can be neglected. In line with this, cells encapsulated within relatively stiff gels, polymerized at 2 or 2.5% solid content, were unable to migrate without proteolytic activity, as observed by blocking the proteolysis. A slight decrease in crosslinking density, induced by reducing the solid content to 1.5%, led to matrices through which cells can efficiently migrate by employing a protease-independent mechanism.

In addition to the effects of confined migration in the absence of a microscopic porosity, the viscoelastic matrix properties seem to have a direct impact on the migratory behavior of cells in 3D matrices (Zaman *et al* 2006, 2007, Pedersen and Swartz 2005, Dikovsky *et al* 2008). It has been shown that certain cell types can adopt an amoeboid migration mode that is independent of matrix remodeling and hence is characterized by localized extracellular matrix deformation as well as cell shape alterations (Wolf *et al* 2003b). Indeed, such a mechanism seems to be dependent on the mechanical properties of the microenvironment. Fibroblast-like preosteoblastic cells are expected to migrate using a mesenchymal mode (Friedl and Broecker 2000). An amoeboid migration mode has only been observed in physically crosslinked matrices, which supports material displacement by the exertion of cellular forces. In particular, these PEG-based gels are crosslinked by strong covalent bonds. Alternatively, cells may use existing, macroscopic gel defects or exert sufficiently strong force to induce locally propagating cracks within the hydrogels; however, this is difficult to investigate. Unfortunately, it is well known that these single-component hydrogels, especially those assembled from synthetic polymers such as PEG, are mechanically very fragile, which represents a major problem that can be overcome by choosing more sophisticated multicomponent design strategies (Gong *et al* 2003). However, such cracks within hydrogels seem to be accessible for migrating cells and could result in a migration speed that exceeds that of cells that rely purely on proteolytic migration in higher density hydrogels.

The hydrogel matrices used *in vitro* under well-defined culture conditions were transferred to the more complex *in vivo* situation to assess migratory events during the onset of bone regeneration. Similar to the *in vitro* case, more densely crosslinked matrices led to less migration inside of the gel and thus to bone formation solely on the outside of the gel. Although the differences are significant, the sensitivity of implanted materials to MMPs does not seem to play a major role during the *in vivo* remodeling compared to the *in vitro* 3D migration model.

Many more factors *in vivo* influence the gel performance and thus cannot be ignored, such as the amount and types of proteases, which may be considerably different between *in vitro* cell culture conditions and a wound-healing environment. Moreover, even the interaction of the implant with a larger number of different cells such as specialized inflammatory cell types can remodel the implant rapidly under *in vivo* conditions. Nevertheless, the good correlation between *in vivo* and *in vitro* behavior indicates that the *in vitro* migration model can be successfully applied to predict the response of modulated material characteristics for specific tissue regeneration.

The precise control over *in vivo* cell fates using engineered, artificial extracellular matrices would be an extremely useful capability (Chan and Mooney 2008). Through the variation of the network's stiffness or proteolytic susceptibility and hence specificity, recruited cells may be confined to the tissue–material interface or strongly directed to migrate into the material. By comparison to wound-healing processes, which depend on surface erosion, the inside–out generation of new tissue from an implant might be considerably faster by employing extracellular matrix-based cues. These implant scaffolds are developed and adapted to mimic the extracellular matrix properties by providing structural support and simultaneously facilitate the attachment, proliferation and differentiation with the final major aim of yielding functional tissues or organs. Indeed, it has generally been found that the most efficient bone reformation in long-term experiments occurs with extracellular matrix gel compositions that provide efficient cell migration. Taken together, soft gels, which are subject to very fast gel degradation, are not helpful in supporting complete wound healing, as the implant stability cannot sustain the mechanical properties needed for the formation of bone tissue.

11.4.1 Plate rheometer

In cancer, the primary tumor development and its dissemination into neighboring tissue parenchyma is based on a loss of well-defined tumor–stroma boundary at the invasive front of the growing tumor mass (Provenzano *et al* 2006, Weber *et al* 2006). At this tumor–stroma interface, the presence of tumor desmoplasia, which is caused by aberrant synthesis of many extracellular matrix proteins, an imbalance of matrix remodeling and/or enzymatic degradation, serves as an indicator for the poor prognosis of cancer such as cancer metastasis (De Wever and Mareel 2003). The tumor growth is encouraged by the interaction of transformed stromal and recruited immune cells within the transformed tumor extracellular matrix microenvironment. Since type I collagen is among of the most abundant extracellular matrix proteins in

the stromal microenvironment, its architecture on the micrometer length-scale and the viscoelastic properties represent biomechanical signals towards the primary tumor that in turn promote the initiation of a cascade of molecular and biochemical events that may help to transfer and finally overcome tumor suppressive cues enabling malignant tumor progression (Egeblad *et al* 2010, Friedl and Alexander 2011). In skin cancers such as cutaneous melanoma (the deadliest form of skin cancer) and squamous cell carcinoma, desmoplasia is based mainly on reactive fibroblasts, which are altered partly by infiltrated leukocytes to initiate the excessive production and enhanced assembly of fibrillar collagens (Kalluri and Zeisberg 2006, van Kempen *et al* 2003). In breast cancer, elevated deposition of collagen has been reported, which is accompanied by alterations in both topographical and viscoelastic properties (Barcus *et al* 2013, Gilkes *et al* 2013, Levental *et al* 2009, Mouw *et al* 2014, Ng and Brugge 2009, Plodinec *et al* 2012). In particular, normally curved fibers progressively thicken, lengthen and linearize in order to form even stiffer bundles of several micrometers in width and length, which can serve as migration highways to support the tumor haptokinesis and the dissemination of cancer cells (Conklin *et al* 2011, Friedl and Wolf 2009, Provenzano *et al* 2006). At first view, these highways provide additionally beneficial adaptive and innate immune responses, as T cells and recruited monocytes can more easily enter the primary tumor along these highways. However, the activation of T cells and the polarization of macrophages is driven by the rigidity of the substrate. In particular, stiffer substrates impairs the function of tumor suppressive T cells and instead induces the pro-tumorigenic M2 polarization of macrophages (Leight *et al* 2012, Pickup *et al* 2014). These results lead to the hypothesis that the development of a pro-tumor microenvironment is based on a precisely balanced interplay between the physical properties, topography and the viscoelasticity of the collagen scaffolds. In order to discriminate between the contributions of viscoelasticity from those due to topography, it needs to be understood how these physical and structural signals are providing the malignant progression of cancer.

The metastatic dissemination is additionally affected by the physicochemical properties of the collagen-rich microenvironment of the primary tumor. In particular, cancer cells can adopt different migration modes to migrate through stromal and connective tissues based on the porosity, stiffness and linearity of the surrounding extracellular matrix microenvironment, as has been reported in substituted biomimetic assays and murine *in vivo* models (Alexander *et al* 2013, Brábek *et al* 2010, Entenberg *et al* 2013, Pathak and Kumar 2011, Patsialou *et al* 2013). The *in vitro* 3D cell migration is partly provided by nucleus-based anisotropy in pressure, actomyosin-based traction forces and/or the proteolytic degradation of the extracellular matrix to support the movement of cells through substituted or cell-derived extracellular matrix confinements (Friedl and Wolf 2003, Petrie *et al* 2014, Stroka *et al* 2014). In addition, *in vitro* migration studies in reconstituted extracellular matrices such as collagen I hydrogels have revealed that the cell migration efficiency depends on parameters such as speed, distance traveled and persistence, which themselves depend on the physicochemical properties of the matrices such as protein density, pore size, fiber thickness, crosslinking and alignment (Brábek *et al* 2010,

Doyle *et al* 2015, Entenberg *et al* 2013, Fraley *et al* 2015, Pathak and Kumar 2011, Patsialou *et al* 2013). All these processes rely on physical and chemical properties of the cell such as deformability, adhesion and contractility. Cellular and microenvironmental factors are interdependent and are highly dynamic, as the cell's cytoskeleton and local extracellular matrix actively and passively modify one another in a complex and reciprocal fashion (Kasza *et al* 2007, Kolahi and Mofrad 2010, Xu *et al* 2009b). When the specific phenotypic features facilitating cancer metastasis are identified, it should be possible to develop anti-metastatic targeted cancer therapies (Tanner and Gottesman 2015). Although some factors involved in cancer cell migration have been revealed, the interaction between physicochemical parameters is not yet clearly determined. Moreover, the characterization of cell–extracellular matrix interactions, which play a major role in cellular migration and invasion of epithelial cancer cells through stromal extracellular matrices, will help to widen our understanding of the mechanical phenotype of metastasis.

At the single-cell level, the mechanical cues challenge intracellular processes, such as signal transduction pathways, the regulation of the cell cycle, gene transcription, protein synthesis and cell–extracellular matrix crosstalk, on highly dynamic timescales ranging from several milliseconds to minutes and on various length scales ranging from nanometers to micrometers (Tanner and Gottesman 2015). One of these interactions is the cells' ability to generate and transmit traction forces on the surrounding extracellular matrix, which is in turn also defined by the mechanical properties of the surrounding extracellular matrix microenvironment and hence accounts for the cellular plasticity (Wolf *et al* 2013). The 3D collagen type I hydrogels have been extensively employed to mimic the fractal *in vivo* scaffold of a fibrillar and porous architecture of human tumors (Cukierman *et al* 2002, Wallace and Rosenblatt 2003). In particular, the basic elements of these self-assembled polymer networks are collagen molecules, which polymerize to left-handed proline helices. Three left-handed proline helices are intertwined and thereby stabilized by weak hydrogen bonds to form subsequently a right-handed triple helix approximately 300 nm in length (figure 11.6) (Guthold *et al* 2007). As the collagen polymer is thermodynamically more stable than monomeric collagen, it starts to spontaneously polymerize. The triple helices exhibit a characteristic 67 nm D-periodicity and build a microfibril consisting of ten surface and four core collagens (Shoulders and Raines 2009). Moreover, these fibrils may assemble into arrays of fibrils with a diameter of 20 to hundreds of nanometers, which are termed collagen fibers. The elastic moduli of collagen fibrils and fibers are in the GPa range. The fibrils and fibers display nonlinear mechanical responses due to various magnitudes of the applied stresses or strains (Carlisle *et al* 2010, Fratzl *et al* 1998, Münster *et al* 2013, Shen *et al* 2008, Storm *et al* 2005, Sun *et al* 2002). For the straightening of kinks, the gliding and stretching of subunits within fibrils and the disruption of fibril or the entire fiber organization different forces are necessary and hence provide nonlinear stress–strain relationships. When individual fibers are macroscopically deformed, the nonlinear stress–strain relations are described by the toe, heel, linear and failure regimes (Fratzl *et al* 1998, Roeder *et al* 2002). Although individual collagen fibers possess Young's moduli in the GPa range, the bulk mechanical measurements of reconstituted 3D collagen hydrogels are in the Pa to

kPa range and hence display several orders of magnitude more compliant behavior (Gentleman *et al* 2003, Roeder *et al* 2002). The bulk mechanical properties of 3D collagen matrices are largely affected by network architecture and network component interactions. In particular, in reconstituted collagen hydrogels, the rotational and translational degrees of freedom at crosslinks and entanglements between the fibers dominate over the individual fiber properties. Due to the hierarchical structure of the collagen fiber structure, the viscoelastic properties depend on the length and time-scale analyzed. Moreover, cells sense and react to mechanical cues from both the individual collagen fibers as well as from the bulk matrix and hence it is required to determine the mechanical cues provided by the collagen matrix at different force-, length- and time-scales to obtain a more complete picture of cellular mechanotransduction.

The collagen microarchitecture is provided by the ligand density, fiber alignment, pore size, and intra-fibril crosslinking and extra-fibril crosslinking evoked by fibronectin or other substances. During the *in vitro* assembly of collagen type I gels, the collagen concentration directly determines the matrix elasticity and pore size (Mickel *et al* 2008, Motte and Kaufman 2013). The parameters, such as fibril width, pore size, overall architecture and local fibril stiffness, can be additionally varied by alterations of the collagen polymerization temperature (Raub *et al* 2007, Wirtz 2009). The variation of the collagen matrix assembly conditions also impacts the mechanical properties, which provides the collagen matrix gels with elastic moduli ranging between 1–10^4 Pa. Hence, collagen can be tuned to mimic the *in vivo* physical properties of tissues, ranging from normal, through neoplastic transformation, to fully restructured tumor-associated stroma. In fractal hydrogels, the exerted shear force can be utilized to align collagen fibers parallel to the applied force. Subsequently, this behavior reduces the macroscopic mechanical properties of the gel compared to unaligned gel. In contrast, pre-alignment of collagen fibrils enhances the bulk gel stiffness (An *et al* 2004, Guthold *et al* 2007, Pedersen and Swartz 2005, Sun *et al* 2002). It has been found that topography itself seems to be sufficient to guide cells and facilitate subsequently their alignment parallel to the direction of the nanofibril (Doyle *et al* 2009, Kim and Wirtz 2015). Due to these mechanical characteristics, an individual cell may sense different signals depending on the local architecture or stiffness of the individual fiber.

Using AFM, the elastic modulus of individual collagen fibers and collagen-rich tumor stroma *ex vivo* have been determined (figure 11.9). AFM micro- and nanoindentation provides a higher spatial resolution compared to bulk rheology-based approaches, which enables the analysis of individual fibers and/or superficial mechanical mapping of tissues. It has been shown that an increase in Young's modulus is a mechanical marker for the malignant transformation during human breast tumor progression, as the highest stiffness levels have been detected at the invasive front. In particular, a positive correlation with enhanced stiffness at the invasive front and more aggressive subtypes of human breast cancers have been revealed (Acerbi *et al* 2015). In addition, nanoindentation can be used to map the stiffness of excised breast tumor tissue surfaces. However, weaknesses of the AFM method are that AFM cannot probe the sample interior (beyond a few micrometers) and also cannot measure forces below several piconewtons, and dynamic analyses

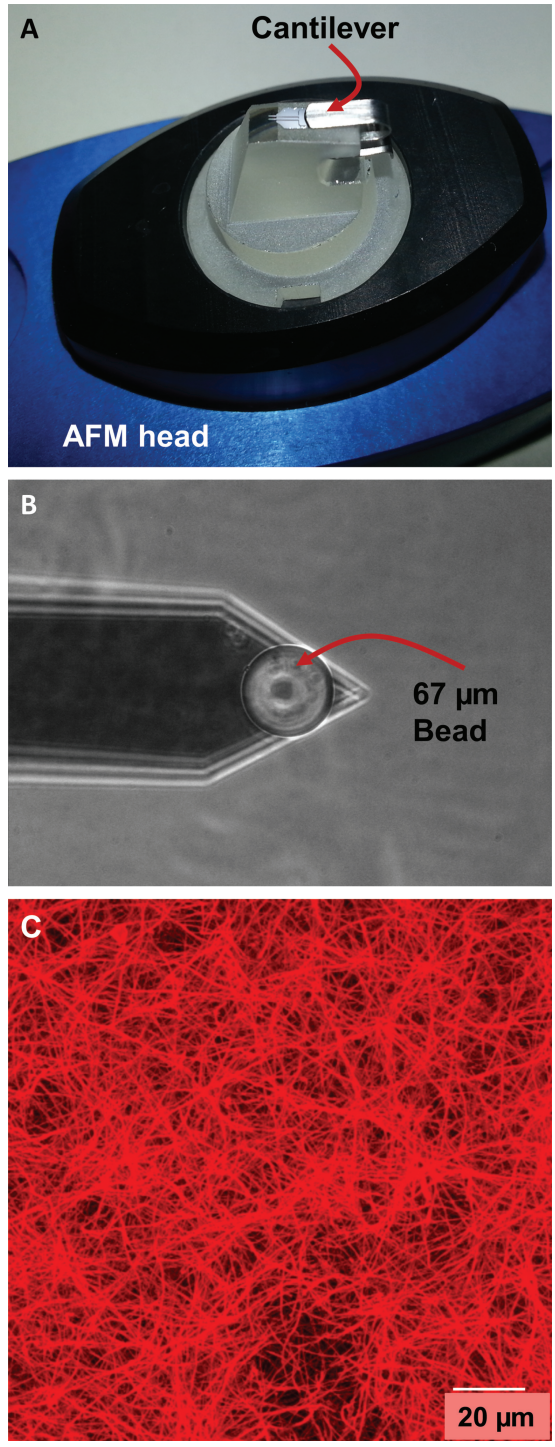

Figure 11.9. AFM can be utilized to measure the mechanical properties of 3D extracellular matrices. (A) AFM head with double cantilever arm. (B) AFM cantilever with a bead (67 μm in diameter) glued to cantilever. (C) Typical 3D collagen matrix used for AFM stiffness measurements with the bead cantilever.

are restricted to only hundreds of hertz. However, when using shear wave spectroscopy to discriminate benign from cancerous tissue and to detected intratumoral heterogeneities, a length-scale of millimeters to centimeters is accessible (Deffieux *et al* 2009). Thus, in order to close to gap between AFM and bulk rheological methods, optical trap active microrheology is performed using *in situ* calibration in order to assess the resistance to flow (viscosity) and the resistance to deformation (elasticity) in these complex viscoelastic materials as functions of the oscillation amplitude (2–20 nm) and frequency (2–15 000 Hz) (Blehm *et al* 2015, Fischer and Berg-Sørensen 2007).

Compared to passive techniques (the observation of thermal fluctuations), active microrheology (the observation of the response to an applied force) can provide a measurement tool for the analysis of nonlinear effects at different length and energy scales and is even less susceptible to thermal noise (Breedveld and Pine 2003, Kotlarchyk *et al* 2011, Kotlarchyk *et al* 2010). The method is based on the analysis of spherical beads acting as local mechanical sensors to which spring-like harmonic forces are applied by using an optical trap. The induced displacements in the tissue microenvironment can be used to determine the local mechanical properties. The spatial resolution is limited by the size of the bead and a spatial resolution of approximately one micron can be achieved. A key step is the determination of the trap stiffness, which will then enable the correct calculation of the applied forces. When using optical-trap-based active microrheology, the calibration of trap stiffness at each single bead is required as otherwise a discrepancy of the viscoelasticity of as much as a 20-fold overestimation of complex modulus can be obtained by repeated measurements of different preparations of the same probe or different beads (Blehm *et al* 2015). Collagen fiber length, pore size and fiber width are analyzed to determine the microscopic determinants of the collective bulk mechanical properties. In addition, the method has been applied to freshly excised murine tumors in order to map heterogeneities within the tissue's mechanical properties with high spatial resolution. Indeed, mouse melanoma tumors and human breast tumors exhibit elastic moduli ranging from approximately 5 to 1000 Pa. In addition, they increase with the frequency and show a nonlinear stress–strain response by displaying a strain stiffening behavior. A direct calibration of trap stiffness for each probe enables the measurement of absolute forces, which is needed to identify local heterogeneities in collagen fractal gels and in murine tumors. Using optics-based active microrheology it is possible to obtain a mechanical biopsy, which provides a diagnostic tool for designing novel therapeutics complementary to those based on standard histopathology.

11.4.2 Effect of the temperature on collagen type I polymerization

Acid-solubilized collagen type I can self-assemble into fibrillar networks *in vitro*. In particular, these networks are built from lateral and end-to-end polymerization of collagen monomers, which usually possess a width of 1.5 nm and a length of 300 nm. In the next step, these structures further assemble into fibrils of approximately 15–200 nm in width, which are stabilized by both hydrophobic interactions and

covalent bonds. These individual fibrils can further bundle into collagen fibers creating a network of fibrillar and porous architecture. The microarchitecture and micromechanical properties of hydrogels depend strongly on the physical and chemical conditions, which are chosen for the polymerization. The properties of high concentration rat-tail type I collagen are regulated by the initial collagen concentration and the temperature used for the polymerization. The latter variable has the greatest impact on the topological features. The lower polymerization temperatures such as 4 °C and 20 °C (instead of 37 °C) decrease the number of nucleation events during polymerization, which means that there are fewer polymers available for monomers to interact with. Finally, this leads to the assembly of fewer, longer and thicker fibers, containing larger pore sizes. These features are quantified through the collection and processing of oversampled images using a so-called curvelet transform fiber recognition algorithm (CT-FIRE) (Bredfeldt *et al* 2014b). The fiber length, width and pore area are quantified. The fiber architecture is dominated by fibers of a length between 2.5–5 μm. Hydrogels of low collagen concentration (2 mg ml^{-1}) display a broader distribution of the fibers than for hydrogels of higher collagen concentration (6 mg ml^{-1}). Taken together, the largest distribution of fiber lengths ranges from 2.5 to less 10 μm and has been observed for hydrogels polymerized at 4 °C and a collagen concentration of 2 mg ml^{-1}. Fibers longer than 10 μm are observed in gels polymerized at 4 °C and a concentration 6 mg ml^{-1}. Collagen assembles a hydrogen network of smaller fibers polymerized at 37 °C and with 6 mg ml^{-1}. Instead, the fiber width increases with decreasing polymerization temperature ranging from 0.2 μm to 0.5 μm. Fibers with a width of more than 0.3 μm are found in the hydrogels polymerized at 4 °C and with 2 mg ml^{-1}. However, hydrogels can display similar distributions of fiber length and width, although different concentrations and polymerization temperatures have been used. Thus, it is not suitable to compare different collagen matrices simply based on the conditions used for the polymerization and requires further in-depth characterization.

Pore sizes on the order of the size of a cell nucleus (64 μm^2) or larger are absent in hydrogels polymerized at 37 °C and final concentrations of 2 or 6 mg ml^{-1}, in which the fibrillary network is built by smaller fibrils. Instead, these larger porous areas can be detected to more prevalent (25% of the distribution) for hydrogels polymerized at 4 °C and in even in greater abundance for hydrogels of 2 mg ml^{-1} than 6 mg ml^{-1} collagen concentration polymerized at 20 °C. A similar trend has been seen for pore sizes on the order of a large cellular protrusion (9–36 μm^2). The heterogeneity of spatial structure has been assessed as a function of the length-scale by performing gliding box scans on the images to detect the (dimensionless) lacunarity, which is a measure of size-dependent self-similarity. In this plot, a straight line indicates a structure generated by a single multiplicative process (so-called monofractal). Alterations in the slope occur at lengths at which a change in the scaling occurs and the curvature suggests an interaction between multiplicative processes at many scales (so-called multifractal) (Plotnick *et al* 1996). In collagen network self-assembly, alterations in the slope can occur at critical length scales characteristic of competing polymerization processes or for example a probable branching distance. In particular, curves of gels with different concentrations but the same

polymerization temperature display similar slopes, which indicates similar topography. Both positive and negative curvatures can be found in the various gels. Hydrogels polymerized at 37 °C possess the lowest lacunarity, as they begin at 3 μm to curve upwards with decreasing length-scale. In contrast, hydrogels polymerized at 20 °C and a concentration of 6 mg ml^{-1} display a higher lacunarity but similar curvatures, whereas hydrogels polymerized at 4 °C and a concentration of 6 mg ml^{-1} show similar lacunarity with less curvature (the curves intersect at approximately 0.75 μm). In line with this, hydrogels polymerized at 4 °C and 20 °C and a concentration of 2 mg ml^{-1} displayed the highest lacunarity.

11.4.3 Comparison of active microrheology and bulk rheology

The 3D collagen matrices may possess microrheological properties that reflect the fractal network of the gel at the micrometer length-scale, which seem to be highly distinct from the bulk rheological properties. Can the rheological properties of the collagen matrices be compared by using the optical-trap-based active microrheology and small angle oscillatory shear (SAOS) bulk rheological measurements for overlapping frequencies (1–10 Hz) of each technique? In order to answer this question, hydrogels with a final concentration of 2 mg ml^{-1} are polymerized at 37 °C. These hydrogels possess an overall shear modulus of approximately 35–50 Pa and a loss modulus of approximately 10 Pa across all frequencies using SAOS. Indeed, in the overlapping frequencies, the active microrheology measurements on the same gels revealed remarkably similar results. Moreover, the optical-trap-based active microrheology method can probe mechanical response at higher frequencies. From 2–200 Hz, an elastic plateau is present with storage moduli that increase only gradually to 40–70 Pa. At higher frequencies, the slope increases and rises to approximately 350 Pa at frequencies greater than 10 kHz. Additionally, the loss modulus was approximately 10 Pa at 2–30 Hz and increases to approximately 1 kPa spanning the range of 30–10 kHz. In particular, the crossover frequency (the storage and loss moduli are equal in magnitude) is roughly 500 Hz. The loss modulus displays a local minimum at approximately 10 Hz, which is characteristic for entangled fiber networks (Abidine *et al* 2015, Granek and Cates 1992).

The active microrheology compared to bulk rheology measurements revealed significantly different microscale complex shear moduli at hertz to kilohertz frequencies and two orders of magnitude of strain amplitude. The access to higher frequencies provides the observation of transitions from elastic to viscous behavior occurring at between 200 Hz and 2750 Hz, which seems to be largely dependent on the tissue architecture that is outside the dynamic range of acquisition accessible with SAOS bulk rheology. In particular, mouse melanoma tumors and human breast tumors possess complex moduli of approximately 5–1000 Pa, which increase with frequency and exhibit a nonlinear stress–strain response. Taken together, it seems to be feasible to take a so-called mechanical biopsy serving as a diagnostic tool for supporting the design of therapeutics complementary to those based on standard histopathology.

11.4.3.1 Using in situ *calibration for analysis of rheological properties as a function of polymerization temperature*

The calculation of the complex modulus using active microrheology requires knowledge of the applied force and hence the effective spring constant or the trap stiffness. This trap stiffness can usually be determined from sample materials (synonymously termed proxy material) such as glycerol and water from which the physical properties are known. However, trap stiffness may vary within a sample due to optical and mechanical heterogeneities and thus applying a trap stiffness that is determined in a sample material may reveal discrepancies in the determined mechanical properties. These optical heterogeneities also affect the sensitivity of the detection of the probe displacement (needed to determine the applied force), hence it needs be calibrated for each measured probe. Both the passive and active spectra yield the trap stiffness and finally the moduli (Blehm *et al* 2015). This technique can be utilized to probe microscale mechanics at frequencies not accessible by SAOS. Using this *in situ* calibration, the effect of polymerization temperature on micromechanical properties is analyzed. In order to identify the differences between values determined from *in situ* and water trap stiffness calibration, the moduli of the gels are compared using the trap stiffness as determined from water to that obtained using *in situ* calibration. The comparison of storage and loss moduli using the values calibrated in water reveals that these values exceed the moduli calculated using an *in situ* calibration by 300%–1000% (as averaged across all frequencies).

Indeed, the hydrogels of final concentration of 2 mg ml^{-1} displayed storage modulus that varied significantly as a function of polymerization temperature, with moduli rising approximately 20–350 Pa (at 37 °C), approximately 90–1500 Pa (at 20 °C), and approximately 200–2500 Pa (at 4 °C) over 2 Hz–12 kHz. On average across the frequencies, the storage moduli of hydrogels polymerized at 4 °C gels were two- to threefold greater than those obtained for hydrogels polymerized at 20 °C or 37 °C. Similar to 2 mg ml^{-1} collagen gel, hydrogels with 6 mg ml^{-1} displayed significantly different storage moduli, as a function of polymerization temperature. In summary, hydrogels polymerized at 4 °C and 20 °C were 2.5-fold and 1.5-fold stiffer than those polymerized at 37 °C. A similar trend has been detected for the loss moduli, which also varied significantly with temperature. These results indicate that micromechanics of the polymer network depends on polymerization temperature.

11.4.3.2 Nonlinear stress–strain behavior

Cells respond to both chemical and physical cues of the surrounding stroma microenvironment. The chemical cues are largely characterized by ligand density, regulated by both the overall collagen concentration and the local structural architecture. Hence, the signaling cues are intertwined with the architecture and mechanical cues sensed by the cells. Thus, these two parameters cannot be decoupled easily. Maybe this can be examined by analyzing hydrogels of the same final collagen concentration but differing architectures (which are polymerized at different temperatures) and those of different final collagen concentration but comparable architectures (which are polymerized at the same temperature). In the next step the stress–strain behavior of these hydrogels is determined using active microrheology.

For an optical trap, there exist two ways to alter the stress and strain applied to the probe's microenvironment: first, by altering the trap position oscillation amplitude or, second, by altering the laser power. As the applied force F and the displacement of the bead from the trap center Δx are related to the trap stiffness k by $F = -k\Delta x$, the change of the oscillation amplitude or trap stiffness affects both the stress and strain applied to the collagen matrix. When the laser power is kept constant, but the trap oscillation amplitude is modulated for the aforementioned hydrogel conditions, an emergence of nonlinearity for the measured complex moduli across all frequencies, when stress and strain are increased by changing the oscillation amplitude from 10 to 20 nm, can be detected. In particular, complex moduli measured at amplitudes of 10 nm and 20 nm are significantly altered. For hydrogels polymerized at 37 °C and a concentration of 2 mg ml^{-1}, the complex moduli are determined at a lower strain, which are induced by an oscillation amplitude of 10 nm, and exhibited approximately 60% of the value measured at a higher strain (induced by 20 nm oscillation amplitude). For hydrogels polymerized at 4 °C and a concentration of 2 mg ml^{-1}, the storage and loss moduli measured at a lower strain (induced by an oscillation amplitude of 10 nm) were approximately 60% and 70% of the values obtained at higher strain, respectively. For hydrogels polymerized at 4 °C and a concentration of 6 mg ml^{-1}, the storage and loss moduli measured at lower strain were approximately 70% and 90% of the values obtained at higher strain increasing to 750 Pa and 450 Pa at 12 kHz, respectively.

For each of these conditions, the crossover frequency ω_c has been analyzed, which occurs at a higher frequency when higher strain is exerted. In particular, the crossover frequency can be seen at the transition from the elastic plateau regime to liquid-like behavior and has been described as a glass transition (Alcaraz *et al* 2003, Sollich *et al* 1997, Wirtz 2009). Both the ω_c at lower and higher strains are elevated between hydrogels with the same collagen concentration and diverse architectures. When the architecture is similar and the collagen concentration is dissimilar, ω_c even exhibits the same values at lower strains, whereas at increasing strain a greater shift in high collagen concentrations than in lower concentrations is observed.

The flexible polymer networks should have complex moduli that exhibit power-law frequency dependence $G^* \propto \omega_\alpha$ with $\alpha = 0.5$, whereas α exhibits a value of 0.75 of semi-flexible polymers. Do these values display a power-law frequency dependence? Indeed, all hydrogels showed a weak power-law frequency dependence in $G^* \propto \omega_\alpha$, with power-law exponents α ranging from 0.66–0.74.

Moreover, it has been hypothesized that intracycle nonlinearities in viscoelasticity can also be interrogated by determining the higher order harmonics of the elastic modulus, which can be decomposed by an appropriate choice of basis functions, in which the addition of higher order terms does not impact lower order terms (Ewoldt *et al* 2008, 2010). In more detail, the second order term reflects transient behavior, whereas the third order term reflects steady state behavior and the sign of the term displays the mechanical behaviors, such as intracycle strain stiffening (positive sign), softening (negative sign) and linearity (zero). When applying this analysis to the above-mentioned data, both the second and third order terms are close to zero.

11.4.4 Active microrheology (optical trapping) of primary murine melanoma

The most prominent collagen in the extracellular matrix is type I collagen, which is a major component of the extracellular matrix and functions as an important scaffolding protein in the stroma contributing broadly to the mechanical properties of the tissue. The aberrant deposition and remodeling of the stroma represents a hallmark for the malignant progression of cancer, which is mirrored in the occurrence of malignant tissue. Histological staining and confocal microscopy of *ex vivo* tumors showed increased amounts of type I collagen in excised melanoma. In order to reveal similar stages of tumor progression for each sample, no distant metastasis has been found using bioluminescent imaging and visual examination of organs at necropsy, when the complex moduli of the tumors have been determined using optical-trap-based active microrheology. In particular, B16-F10 melanoma tumors displayed a nonlinear mechanical response with storage moduli significantly different with various oscillation amplitudes (2 nm, 5 nm, 10 nm, 20 nm). Moreover, the complex moduli (G^*) exhibited a weak power-law frequency dependence with power-law exponents of between 0.52 and 0.70. The storage and loss moduli increased monotonically between 5 Pa–1000 Pa over frequencies of 3 Hz to 15 kHz. The invasive MDA-MB-231 breast tumor samples possess similar mechanical properties to B16-F10 melanoma cells.

Indeed, there exists a strong clinical association between the tissue mechanical properties and pathological diseases (Keely 2011, Mouw *et al* 2014, Schedin and Keely 2011, Tanner and Gottesman 2015). The aberrant deposition and remodeling of the stromal extracellular matrix microenvironment are both markers for a transformed and tumor promoting microenvironment (Werb and Lu 2015). Collagen type I contributes pronouncedly to the biochemical signaling and mechanical cues that affect both tumor and stromal cells within the extracellular matrix milieu (Lu *et al* 2012). Indeed, the biochemical signaling cues are tightly connected with the mechanical cues sensed by cells. How can they be decoupled? The decoupling of the mechanical and biochemical contributions of the extracellular matrix scaffold requires the analysis of these mechanical and biochemical properties on various length scales and is subsequently key to the understanding of mechanosignaling processes in cancer. In the tumor microenvironment a broad variety of molecular processes occur such as protein–protein interactions, molecular motor activity, cytoskeletal remodeling, signal transduction, cell motility, cell proliferation and the assembly of multicellular structures. All these processes feature dynamics which are present over various time and force regimes. The identification of the governing principles of these phenomena still required a lot of effort, as they occur on overlapping scales and feature additionally reciprocal interactions across these scales, which will lead to emergent properties. The knowledge of the mechanical properties and responses of tissue and extracellular microenvironments for a wide range of frequencies and forces seems to be a prerequisite to unravel the complexities of cellular behaviors such as mechanotransduction processes and cellular motility in physically diverse microenvironments.

The mechanical properties of biomaterials such as collagen hydrogels have been extensively analyzed using an array of biophysical instrumentation. In particular, bulk rheometers, atomic force spectroscopy (AFM) and nanoindentation, x-ray scattering, microelectromechanical systems, and others have been employed to probe various mechanical properties (Arevalo *et al* 2010, Carlisle *et al* 2010, Fratzl *et al* 1998, Gutsmann *et al* 2004, Motte and Kaufman 2013, Shen *et al* 2008, Wenger *et al* 2007). In contrast, bulk rheology associates the mechanical contributions of network crosslinking and entanglement at the millimeter scale under the assumption that the entire material is homogeneous and functions as a continuum. Hence, this physical approach is not able to address the mechanical properties of microdomains within this material. However, cells can utilize distinct strategies to migrate in a 3D collagen microenvironment. In particular, a tumor cell crawling along these rather thick and stiff fiber bundles will sense a different mechanical signal than cells squeezing through the pores of the matrix. Moreover, leukocytes migrating into the primary tumor and its surrounding stroma will encounter their own distinct mechanical signals. Indeed, it has been detected that 3D adhesion dynamics are locally controlled by extracellular matrix rigidity. In general, bundled, stiffer collagen fibrils increase single-cell adhesion, whereas more compliant (soft) matrices favor the retraction of cell adhesion (Doyle *et al* 2015). Thus, regional heterogeneities within the 3D extracellular matrix scaffold seem to dramatically alter the cell's ability to adhere to a *de novo* extracellular matrix milieu, which is a key requirement to foster the metastatic spread of tumors. Hence, these microscale heterogeneities need to be resolved and cannot be neglected, as they largely impact the behavior of embedded and interacting cells. The main advantage of microrheological methods in physical biology is that they probe the cellular length-scale and hence can access the local heterogeneities within the microenvironment directly in the presence of living cells (Liu *et al* 2006). Instead, the passive microrheological techniques measure the mechanical properties such as the complex modulus by tracking the thermal motion of tracer particles (Mak *et al* 2014, Mason and Weitz 1995, Mason *et al* 1997, Squires and Mason 2010; Svoboda and Block 1994), which can be even critical when living cells are in close neighborhood to these markers, as they can phagocytize them, which in turn affects dramatically their migratory capacity (Mierke 2013). Passive microrheology and active microrheology in combination with confocal microscopy have been utilized to characterize fibrin gels (Kotlarchyk *et al* 2010, 2011).

Optical trapping (optical tweezers)

In the field of optical micromanipulation, an important breakthrough has been the invention of a single laser beam gradient that can be used as a force trap. The method has now been established as an essential biophysical technique termed optical tweezers (Ashkin *et al* 1986). In optical tweezers, a single laser beam is very tightly focused through a lens with high numerical aperture and hence can provide gradient forces counteracting the scattering forces in the direction of propagation. This rather simple implementation of an optical trap delivers the stable, 3D optical trapping of dielectric particles. In terms of geometric ray optics, optical tweezers can

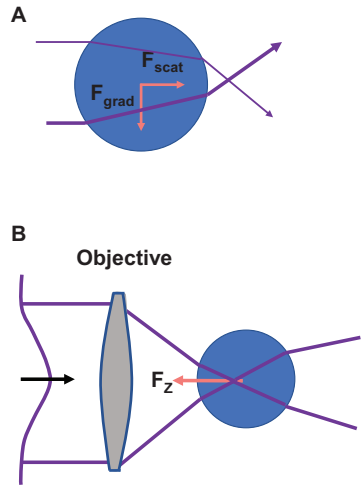

Figure 11.10. General optical trap. The basic principle of optical tweezers in the geometric optics regime. (A) A transverse intensity gradient results in a gradient force F_{grad} pointing to the region of highest intensity. (B) A strong focusing of the laser beam through an objective causes a resulting backwards force (F_Z) parallel to the optical axis.

indeed be qualitatively assessed. In particular, a spherical, transparent particle is considered in a light field that possesses an inhomogeneous intensity distribution in a plane transverse to the optical axis such as a collimated Gaussian beam. Moreover, any light ray is associated with linear momentum flux of $p = n_{\text{med}} P/c$ (P is the power of a ray traveling in a medium with the refractive index n_{med}). When tracing two rays of different intensities that are incident symmetrically on the sphere (figure 11.10(a)), it is easy to reveal that the vector sum of the momentum flux points away from the region of highest intensity. Hence, the entire sphere will experience a force along the intensity gradient, which is termed the gradient force F_{grad} (Ashkin *et al* 1986). The gradient force is accompanied by the scattering force F_{scat} along the optical axis, which is increased by the reflection from the surfaces and absorption.

In the next step, a tightly focused beam is considered (figure 11.10(b)), which is typical of an optical tweezers approach. In particular, the spherical particle functions as a weak positive lens and alters thereby the degree of divergence or convergence of the focused light field. When the angle of the incident rays is high enough, it can result in axial forces F_z pointing backward, only if the particle is located behind the focus of the rays. Hence, a stable trapping position for the particle is achieved, which means that any (small) displacement of the particle will evoke a restoring force holding the particle back in the equilibrium position (Ashkin *et al* 1986, Ashkin 1992).

Geometric optics delivers a qualitative picture, whereas it can also describe optical tweezers quantitatively, when the limits of the regime are respected. In general, geometric optics can only poorly describes the light field in close vicinity of the focus and additionally ignores any effects of diffraction and interference (Nieminen *et al* 2010, Stilgoe *et al* 2008). In summary, geometric optics can only

be used to describe the limiting regime of particles that are relatively large compared to the wavelength of the light field ($d \gg \lambda$) (Ashkin 1992). In order to obtain quantitatively accurate results, usually the smallest dimension of the particle should be at least 20 times the optical wavelength (Nieminen *et al* 2007).

However, an alternative approximate description of optical tweezers is the consideration of particles, which are relatively small compared to the wavelength ($d \ll \lambda$). In this so-called Rayleigh regime, the particles are regarded as infinitesimal induced point dipoles interacting with the external light field. Indeed, a sphere of radius r in a homogeneous electric field $\vec{E}$ is polarized and has an induced dipole moment of (Nieminen *et al* 2007):

$$\vec{p}_{\text{dipole}} = 4\,\pi n_{\text{med}}^2\,\epsilon_0 r^3 \left(\frac{m^2-1}{m^2+2}\right)\vec{E}. \tag{11.1}$$

The relative refractive index of the particle is $m = n_{\text{part}}/n_{\text{med}}$ and ε_0 represents the dielectric constant in a vacuum. Based on this dipole moment, the particle experiences a force in a non-uniform electric field (Harada and Asakura 1996), which is given in equation (11.2)

$$\vec{F}_{\text{grad}} = \pi\, n_{\text{med}}^2\,\epsilon_0 r^3 \left(\frac{m^2-1}{m^2+2}\right) \nabla\,|\vec{E}|^2. \tag{11.2}$$

For small particles, the equation (11.2) is also valid for a time-varying electric field and, hence, the force can be written in terms of the intensity I of the light field, as here in equation (11.3):

$$\vec{F}_{\text{grad}} = \frac{2\pi n_{\text{med}} r^3}{c}\left(\frac{m^2-1}{m^2+2}\right)\nabla\vec{I}. \tag{11.3}$$

The potential energy is derived by integrating equation (11.3) by assuming that the gradient force is conservative. This gradient force depends obviously on the gradient of the intensity and thus naturally is termed the gradient force. It increases the gradient for $m > 1$, for example for high-index particles. In a static field, this expression provides the total force (Nieminen *et al* 2007). For time-varying fields, the oscillating dipole can be assumed as an antenna radiating (dissipating) energy. The (vectorial) difference between the energy removed from the incident field and energy reradiated (dissipated) by the particle causes an associated amount of alteration within the momentum flux and finally leads to a scattering force that has a magnitude of (Harada and Asakura 1996):

$$F_{\text{scat}} = \frac{8\pi n_{\text{med}} k^4 r^6}{3c}\left(\frac{m^2-1}{m^2+2}\right) I. \tag{11.4}$$

The wavenumber is given by $k = 2\pi/\lambda$. When the particle has absorbing properties, an additional force arises that also depends on the intensity but is proportional to r^3 rather than r^6 (Nieminen *et al* 2010). The sum of these forces (which also contains

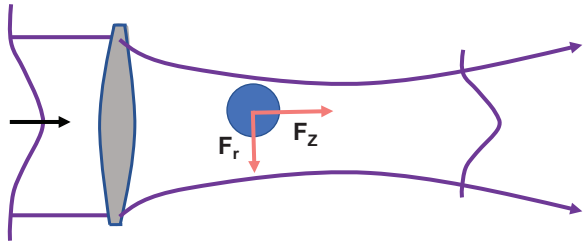

Figure 11.11. The basic principle of the optical tweezers in the Rayleigh regime. A particle exposed to a light field, such as a slightly focused Gaussian laser beam, experiences a transverse force F_r and a force parallel to the beam axis F_Z.

the gradient force) can be separated into a transverse component F_r and axial component F_z (figure 11.11, Rayleigh regime).

With an elevated degree of focusing, the 3D intensity gradients increase, the (axial) gradient force becomes stronger than the scattering force, and 3D trapping is possible. When comparing the scaling of the gradient force (equation (11.3)) and the scattering force (equation (11.4)) with the particle radius, it can be hypothesized that small particles below a certain threshold are always trapped. However, this hypothesis is wrong, as there is an additional force evoked by the Brownian motion of the particle. The thermal kinetic energy of the Brownian motion is $k_B T$ (with the Boltzmann's constant k_B and the temperature T). This energy has to be compared to the depth of the optical trapping potential well, which is generated by the conservative gradient force 1:

$$U = -\frac{2\pi n_{\text{med}} r^3}{c}\left(\frac{m^2-1}{m^2+2}\right) I + C. \tag{11.5}$$

In this equation, C is an arbitrary integration constant. In particular, the drag force due to the dynamic viscosity η, which is $F_{\text{drag}} = -6\pi\eta r v$ for a spherical particle with radius $r = d/2$ and velocity v, decreases with the radius and thereby less efficiently damps the Brownian motion. Both the geometric optical and the Rayleigh approximation provide an understanding of the physical principles of optical trapping, whereas their quantitative validity is strongly restricted for typically trapped particles, whose sizes are usually in the order of the optical wavelength (d is nearly equal λ). In this intermediate regime, a more rigorous treatment based on fundamental electromagnetic theory is needed for the quantitative assessment of optical tweezers.

Indeed, active microrheology using an optical trap (aM-OT) is capable of probing physical properties of tissues and artificial 3D collagen matrices by analyzing the resistance to flow (the viscosity) and the resistance to deformation (the elasticity). In aM-OT, the equations needed to reveal the local viscoelasticity depend on the properties of the probe such as mass and radius. Moreover, the spatial resolution is characterized by the bead size. Using aM-OT, individual fiber bundles can be analyzed. The 1 μm microspheres are not observed to freely diffuse in the samples on experimental time-scales, which indicates that the microspheres are larger than the

local pore size of the microenvironments. Hence, the protein polymer network can be analyzed, which creates the local extracellular matrix microenvironment of the cell and not only the surrounding fluid phase. Finally, heterogeneities on micrometer length scales can be detected.

The microarchitecture and micromechanical properties of 3D collagen hydrogels are highly sensitive to the physical and chemical alterations during the polymerization. The collagen assembly *in vivo* involves many factors such as enzymes and cellular activities, and *in vitro* hydrogels are distinct from native collagen structures. Although a lot of effort was on defining the distinct roles of these parameters in the resulting collagen network, the interconnection of the parameters makes it highly difficult. The collagen assembly *in vitro* depends on pH, ionic strength, temperature, concentration, presence of chemical crosslinkers, hydration state and various other physical and chemical factors. During *in vitro* self-assembly, the fibrillar architecture is affected by the final concentration of collagen (available ligand density), pH and polymerization temperature (Raub *et al* 2007, 2008), which enables fine tuning of the gels to mimic normal and tumor tissue. There are several imaging devices available for the visualization of collagen networks at different length scales such as electron microscopy, second harmonic generation imaging and two photon microscopy (Cox *et al* 2003, Kadler *et al* 1996, Zoumi *et al* 2002). The advantage of reflection microscopy is that it is noninvasive and label-free, which is useful for analysis of human tissue samples requiring rapid mechanical biopsies. However, the penetration depth is strongly limited. For thicker samples, the nonlinear multiphoton optical methods can be utilized. Another disadvantage is that brightness of the fibers depends on the fiber orientation (Jawerth *et al* 2010). The simultaneous fluorescence and reflectance microscopy revealed that some fibers are even invisible when visualized in reflectance mode, hence some of the 'pores' may not be simply fluid filled. Thus, imaging between 10 μm and 50 μm from the coverslip to minimize glare and scattering are commonly performed. Using confocal reflection microscopy, an approximately 250 nm optical lateral resolution can be obtained. The ctFIRE algorithm can be used to quantify fiber properties (Bredfeldt *et al* 2014a, Bredfeldt *et al* 2014b, Khanna *et al* 2015, Tilbury and Campagnola 2015). The alteration of the initial collagen concentration determined porosity, fiber width and fiber length and altered the microscale mechanics, which is varied as a function of polymerization temperature. In particular, the pore area, fiber width, and fiber length decreased with increasing polymerization temperature and are similar to those of other studies (Bredfeldt *et al* 2014a, 2014b, Khanna *et al* 2015, Tilbury and Campagnola 2015).

For gels featuring a fine network with pore sizes below 1 μm^2, a good agreement between bulk rheology and aM-OT for both storage and loss moduli for the overlapping frequencies have been observed. Complex moduli of polyacrylamide gels calculated from bulk rheology and AFM-based microrheology measurements also displayed a good agreement in the overlapping frequency range (Abidine *et al* 2015). The aM-OT method is capable of probing a broadband frequency range spanning 1 Hz–15 kHz, which enables interrogation of the frequency-dependent viscoelasticity beyond most other methods. A limitation of this method is that below 1 Hz, a low frequency acoustic noise becomes problematic in most experimental

conditions, hence for these time-scales other methods should be employed that are better suited. However, across all probed frequencies for low and high collagen gel concentrations that are selected to mirror normal and tumor tissue, the complex moduli have been revealed to be inversely related to the polymerization temperature. In contrast, bulk rheological measurements did not reveal such results, as the gels get stiffer with increased polymerization temperature (Raub *et al* 2007, 2008). This discrepancy may be simply due to the resolution for each approach. The gels polymerized at 4 °C assemble large fibril bundles of several micrometers. Thus, a bead of a 1 μm diameter will sense a stiffer fiber and subsequently the entire gel seems to be stiffer than a gel polymerized at 37 °C, in which the fibers are much smaller. In bulk rheology, the porosity additionally affects the overall tissue mechanics, hence when there are larger pores, the hydration effects lead to an overall decrease in complex moduli, as observed for gels polymerized at 4 °C with large pore size.

In particular, when combined with a high numerical aperture and long working distance water objective, optical-trap-based microrheology can even be used to probe thick specimens. A disadvantage of aM-OT is that the optical trap stiffness varies throughout the sample, when employed in optically and mechanically heterogeneous tissues, and hence needs to be calibrated for each probe. The *in situ* calibration at each probe has shown to be sufficient to improve the accuracy and temporal and spatial resolution necessary to resolve *in vivo* tissue mechanical heterogeneities (Berg-Sørensen and Flyvbjerg 2004, Blehm *et al* 2013, 2015, Fischer and Berg-Sørensen 2007). The fluctuation–dissipation theorem (FDT) method can be used to calibrate the trap stiffness *in situ*. By taking a passive and an active measurement, the three unknowns can be measured in the cellular trapping environment: the local elasticity, the local viscosity and the trap stiffness. The passive measurement yields the trapped bead's amplitude of thermal motion in the trap. The active measurement utilizes an oscillating trap laser and simultaneously measures the bead's response at multiple frequencies (f_n). When the phase delay and the amplitude measured in the active calibration are combined with the amplitude measured during the passive calibration at every frequency f_n, the three unknowns are obtained to calibrate the trap *in vivo*. Indeed, it has turned out that in collagen samples there is a discrepancy of an order of magnitude in the overestimation of complex moduli calculated with trap stiffnesses calibrated in water ($\kappa\omega$) over those calibrated *in situ*. The advantage of *in situ* calibration is that the probe materials can be analyzed without knowledge of local optical and/or mechanical properties of the microenvironment. This seems to be useful for predicting the establishment of a lesion through the measurement of the mechanical properties of the tumor permissive organ (so-called soil) during the early stages of the colonization of distal organs (the onset of metastasis formation), which is based on the classical seed and soil hypothesis (Fidler 2003, Paget 1989).

Another advantage of active microrheology is the ability to exert forces in order to measure nonlinear effects at different length and energy scales, and thereby the stress–strain relation is investigated beyond thermal perturbations. As these biomaterials are very often nonlinear, this is crucial for cell migration and other active

processes (Levental *et al* 2007). Dissimilar to passive techniques, active microrheology is less susceptible to thermal noise (Breedveld and Pine 2003). Discrepancies in the frequency dependence have been detected in active and passive measurements (Brau *et al* 2007). For an optical trap, there are two ways to alter the stress and strain applied to the probe's microenvironment: the first approach is by changing the trap position oscillation amplitude and the second approach is by changing the laser power. For the first parameter, a maximum of 200 nm for the oscillation amplitude is the limit for ensuring that the probing is still in the linear regime. For the second parameter, the local heating effects are limited to laser powers that are not locally affecting denaturing or softening of gels or tissues (Peterman *et al* 2003). When using this approach, the nonlinear mechanical response of collagen gels can be determined as a function of architecture and ligand density.

Collagen is known to be more thermodynamically stable as a polymer than a monomer, which facilitates the rise of various different forms of networks. Collagen forms fractal networks with microdomains possessing distinct microrheological properties such as complex hierarchical structures, which possess distinct mechanical properties and furthermore reveal distinct mechanical responses to perturbations at different force scales. In collagen a doubling of the stress amplitude leads to nearly doubling of the complex modulus. The frequency dependence of the complex modulus is similar to semi-flexible polymers (MacKintosh *et al* 1995, Storm *et al* 2005). Since the frequency dependences are similar at high frequencies among different gels, differences at low and intermediate frequencies become apparent as a function of the applied stress and strain. Some viscoelastic materials display a characteristic crossover frequency at which the loss modulus equals the storage modulus, which indicates a transition (termed glass transition temperature) between a low frequency elastic plateau regime and a high frequency liquid-like regime characterized by power-law frequency dependence that is indicative for complex dynamics without a characteristic time-scale. These materials can often be well-described by structural damping models in which the viscous component is directly coupled to the elastic component (Alcaraz *et al* 2003, Fabry *et al* 2003, Jamali *et al* 2010). The same behavior can also be revealed and modeled, when assessing the dynamics of a soft glass beyond the crossover frequency (Sollich *et al* 1997, Sollich 1998). When fitting the collagen data to the structural damping model, good fits are obtained at frequencies just below the crossover and beyond, whereas in the elastic plateau region the actual storage and loss moduli are higher and lower, respectively, than those predicted by the damping model (Alcaraz *et al* 2003). The discrepancy may be partly due to the local minima in G'' characteristic of entangled fiber networks (Abidine *et al* 2015, Granek and Cates 1992). A concentration dependence of the crossover frequency in polyacrylamide gels has been detected (Abidine *et al* 2015). In collagen gels of equal concentrations but distinct topography, tuned by polymerization temperature, similar crossover frequencies at low stress are determined, suggesting that the fiber size and architecture and not solely the collagen density play a key role. Indeed, the scale free rheological behavior at high frequencies has also been connected to the type of fractal topographical structure found in the collagen gels (Fabry *et al* 2003). Gels with longer, thicker fibers, larger

pores, and greater lacunarity display structural features across a greater range of length scales and have been found to exhibit elastic plateaus, which extend towards higher frequencies, but exhibiting a slightly greater slope in the elastic plateau regime. Moreover, these collagen matrices possess larger shifts in the crossover frequency with the onset of liquid-like behavior occurring at higher frequencies due to larger stress and strain applications. *In vitro* collagen hydrogels are appropriate surrogates for mimicking the *in vivo* tissue, whereas they still differ from native collagen structures such that there are different scaffold topologies in ordered tissues *in vivo*. The acid-extracted telopeptide-intact collagens enable a more ready polymerization of hydrogels from reconstituted protein, as they can more easily polymerize and crosslink through these telopeptides than the commonly found pepsin-extracted collagen structures from tissues. The source (bovine-, rat- or cell-derived tissue explants) and preparation method of the collagen (such as acid solubilization and pepsin extraction) determines their stiffness and porous architecture (Wolf *et al* 2013).

Taken together, these findings may be specific to acid-solubilized rat-tail collagen matrices. The ability to probe extracellular matrix mechanics directly *in vivo* or *ex vivo* on a freshly isolated tissue specimen cannot be predicted. Until now, a few mechanics measurements have been performed on tumor tissue material at the microscale. In particular, AFM nanoindentation on murine breast tumor tissue sections has been employed to measure the Young's modulus of different regions in small force maps (Plodinec *et al* 2012). Using AFM microindentation, 20 μm cryopreserved thin sections of human breast tissue from cancer patients of different molecular subtypes have been investigated (Acerbi *et al* 2015). AFM indentation with spheroconical probes has been utilized to measure the elasticity of cells embedded in 3D collagen I matrices (Staunton *et al* 2016). For tumor samples, the sectioning process itself can alter the superficial surfaces measured in the AFM indentation. With microrheology analysis, mechanical properties can be probed ~10–400 μm underneath the sheared surface. However, the usage of multiple techniques is recommended to provide complementary and supplementary information such as different force and frequency ranges. The active microrheology measurements of the murine tumor microenvironment seem to be the first of their kind. The usage of fluorescent beads and fluorescence microscopy to positively identify them and to avoid inadvertent measurement of vesicles and other small round objects within the tumor. Indeed, these objects can also be trapped and measured, but careful consideration of their surface moieties and size is required to determine accurate displacement–viscoelasticity conversions. AFM indentation measurements with sharp tips on human breast tumors revealed that at frequencies around 0.8–1 Hz a trimodal distribution of Young's moduli with peaks at approximately 1, 2, and 6 kPa (Plodinec *et al* 2012). AFM indentation measurements on human breast tumors at 20 μm s^{-2} with a 5 μm spherical probe determined Young's moduli of approximately 1 kPa (Acerbi *et al* 2015). However, the mouse melanoma cell and human breast cancer cell tumors measured by am-OT are two orders of magnitude softer at comparable frequencies. Moreover, they also displayed nonlinear stress–strain behavior, with a frequency dependence shifting from semi-flexible to flexible behavior with increasing stress–strain amplitude similar

to collagen hydrogels. In addition, the mouse melanoma tumors and human breast tumors possess elastic moduli of 5–1000 Pa which increase monotonically with frequency. Mouse melanoma tumor samples also showed a nonlinear stress–strain behavior, with a frequency dependence shifting from semi-flexible to flexible behavior with an increasing stress–strain amplitude. Collagen matrices displayed distinct properties between the elastic and viscous moduli, however, tumor samples revealed relatively equal elastic and viscous moduli that monotonically elevate due to frequency. The integration of multichannel fluorescence will enable specific labeling of cancer cells, extracellular matrix proteins, or microvasculature and hence stiffness measurements can be attributed to specific structural components.

How can these mechanical properties be compared with standard histopathological analyses and help to reveal the role of tumor progression and extracellular matrix mechanics? The effects of polymerization temperature and collagen concentration on the network architecture and on the microscale have revealed complex shear moduli of type I collagen hydrogels ranging between two orders of magnitudes of the stress–strain amplitude at Hz–kHz frequencies. Indeed, the decreasing polymerization temperature correlated with increasing pore area, fiber width, and fiber length distribution. Lower temperatures delivered stiffer gels, as measured at the microscale. Using *in situ* calibration, absolute rheological measurements in optically heterogeneous materials such as tissue can be obtained, which cannot be observed using calibration techniques in sample materials. The modulation of the stress–strain amplitude by a factor of two significantly altered the response of all gels. Moreover, the complex modulus is similar toward values predicted for semi-flexible polymer networks. Similar to extracellular matrix networks, mouse melanoma tumor samples also possess a nonlinear stress–strain behavior in the tumor, with a frequency-dependent shifting from semi-flexible to flexible behavior by an increasing stress–strain amplitude. In summary, a mechanical biopsy can provide a novel diagnostic tool for cancer therapy and seems to act complementarily to those relying on standard histopathology.

11.4.5 AFM for 3D collagen matrices

AFM measurements of the Young's modulus of the specific alginate gels have been performed with an MFP-3D system using silicon nitride cantilevers. In particular, the stiffness has been calibrated from the thermal fluctuations of the cantilever in air and cantilevers with a stiffness of roughly 13 pN nm^{-1} have been used. These cantilevers are moved towards the stage at a rate of 1 $\mu m\ s^{-1}$ and the resulting force-indentation curves are interpreted using the Hertzian model for a pyramidal indentor (Bilodeau 1992).

The regulation of the collagen self-assembly process provides the formation of collagen-based biomaterials serving as *in vitro* models of different collagen-rich tissues (Stylianou 2017). In order to further increase the collagen biomaterials' performance and widen their applications, a precise characterization of their structural and mechanical properties is recommended at a nanoscale level. In particular, the mechanical characterization can be performed by AFM (Stylianou

and Yova 2013, Stylianou 2017). In principle the AFM represents a scanning probe microscope (SPM), which monitors the interactions between a probe (cantilever tip) and the sample surface. AFM is now a fundamental technique in the fields of surface and biomedical science. Moreover, AFM is unique and possesses advantages over the other microscopy techniques, such as scanning or transmission electron microscopy and optical microscopy (including fluorescent and confocal laser-scanning microscopy). First, the AFM generates topological information at the nanoscale level, which cannot be measured by other microscopes. For example, the AFM can analyze the mechanical properties of the samples at a nanoscale level qualitatively and quantitatively. Second, the AFM can be utilized to perform nanoscale imaging/characterization without the need for specific conditions such as a vacuum or special treatment of the specimen, such as sample labeling with antibodies/fluorescent labels or surface coating (Stylianou *et al* 2012, Morris *et al* 2008, Allison *et al* 2010). The AFM-based analysis of the matrices can be performed without destroying the fibrillar structure of collagen. Moreover, AFM can be used for a wide range of collagen-based structures such as collagen molecules, separated fibrils and fibers, and collagen-based nanobiomaterials (Hasirci *et al* 2006, Cisneros *et al* 2007).

During AFM force spectroscopy, the cantilever-tip assembly can function as a force sensor and as such it provides several modes for measuring mechanical properties of the probe. By using the AFM nanoindentation approach (Stolz *et al* 2004, 2010, Oliver and Pharr 2004) or the so-called force scanning mode (Darling 2011) the stiffness or softness of the samples can be analyzed and even Young's modulus maps of surfaces can be obtained (Mierke *et al* 2017, Fischer *et al* 2017, Kontomaris *et al* 2015a, 2015b, Braunsmann *et al* 2014, Stylianou *et al* 2014). In particular, using the AFM nanoindentation method, force-indentation curves are revealed, which can then be fitted using mathematical models such as the classical Hertz model to calculate values that are required for measuring the sample's Young modulus and for presenting a material's properties maps (termed Young's modulus maps) (Hertz 1882, Mackay and Kumar 2013).

AFM uses a powerful microscope, however, there are many technological challenges, such as instrumentation, experiment setup, and procedures, which need to be overcome for a better measurement outcome (Müller and Dufrene 2008, 2011, Li *et al* 2015, Shi *et al* 2012, Variola 2015, Ohler 2010). The majority of AFM procedures are relatively time consuming and require intensive laboratory effort, which represents a significant drawback, as the achievement of statistically significant results is very challenging and required for obtaining reliable results (Li *et al* 2015). Another approach to further to improve this method, is to refine the existing mathematical models that are generally used for the acquisition of quantitative data with AFM modes (Kirmizis and Logothetidis 2010).

11.4.6 MRE

Since many rheological methods are restricted to surface measurements, they are not suitable for assessing the mechanical properties within thick 3D collagen fiber matrices or tissues resections or biopsies. In contrast, dynamic magnetic resonance

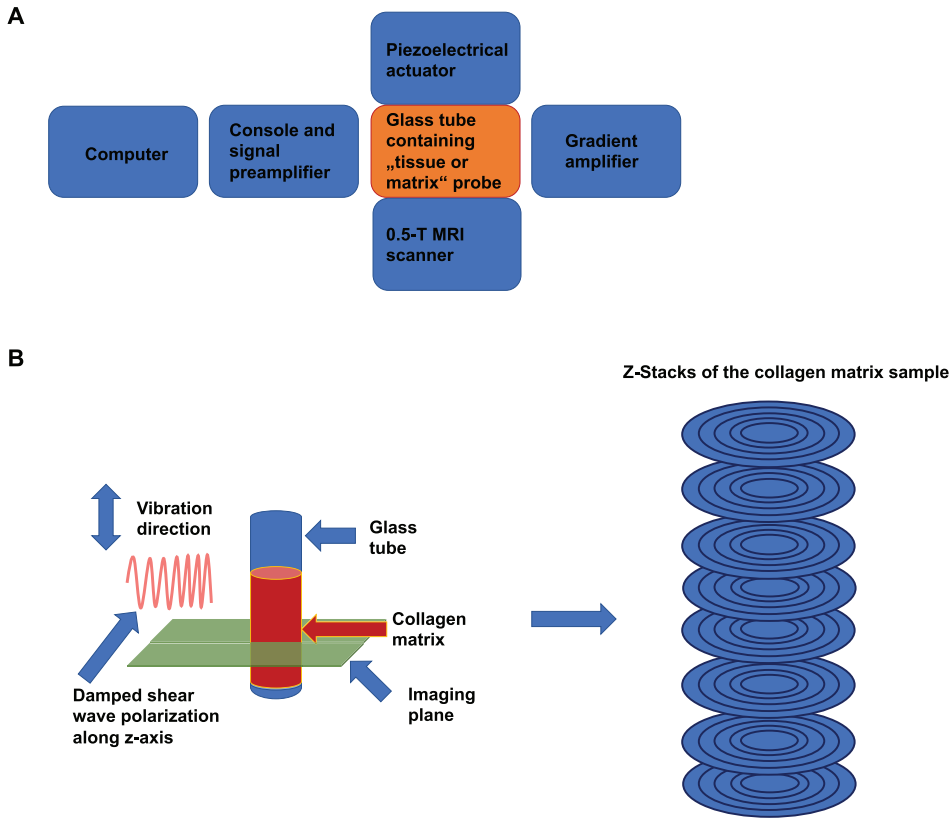

Figure 11.12. MRE setup. (A) Device setup. (B) Measurement principle and expected result when analyzing collagen matrices.

elastography, such as MRE (figure 11.12) is a noninvasive imaging technique that combines wave generation by a non-magnetic, piezoelectric driver with an MRI scanner, which is able to measure deep inside the probe. In more detail, MRE can induce shear waves inside artificial extracellular matrices or connective tissue, and thus turned out to be ideally suited for diagnostic applications (Braun *et al* 2018, Sinkus *et al* 2000, Mierke *et al* 2017). The MRE is a phase-contrast MRI technique that integrates a 0.5 T permanent magnet MRI system, which has been customized by including a piezoelectric actuator, an external gradient amplifier and an inside wavefield-processing cylinder. In particular, the piezoelectric driver was fed with a sinusoidal alternating current of maximum 90 V (depending on the suitable vibration amplitude) and frequencies between 200 and 5700 Hz from the gradient amplifier. Glass tubes with radius of 3.75 mm and 15 cm length were mounted onto the piezoelectric driver (figure 11.12). In more detail, the vibrations inside the glass cylinder are polarized along the main tube axis due to the confined axial motion direction of the actuator. In the next step, shear waves are applied to the tissue samples from the cylinder walls, which leads to the propagation of concentric cylinder waves emanating from the outer sample boundaries towards the center. The

confined cylindrical shear vibrations limit the motion of the field acquisition by uniaxial z-component encoding.

The profiles of the propagating shear waves through living matter, such as tissues, when harmonically (mechanically) excited, can be visualized and determined quantitatively (Braun *et al* 2018), and can be described by Bessel functions and analytically solved. A viscoelastic spring–dashpot model is fitted to the data, in order to obtain directly the relevant parameters related to stiffness, such as the shear wave speed and the shear wave penetration rate (Braun *et al* 2018). The MRE sequence is based on 3D multiple spin echo sequences, which operate in a 2D single echo mode. In summary, the MRE technique can usually be divided into three steps: first, the generation of shear waves in the tissue evoked by the MRI system with a 10 mm bore and 0.5 T permanent magnet, second, the acquisition of MR images showing the propagation of the induced shear waves and, third, image processing of the shear waves to gain quantitative tissue stiffness maps, which are termed elastograms (Mariappan *et al* 2010). Hence, the local quantitative values of the complex shear modulus (stiffness, tissue elasticity or Young's modulus) can be determined. This physical examination provides an assessment of tissue stiffness and seems to be indeed highly suitable for future screening of tumors in living tissues. However, the MRE method is limited, as only accessible parts of the human body can be screened. In summary, MRE seems to be highly promising and suitable for palpation by imaging to detect primary solid tumors of the breast, kidney, lung, liver or prostate tissue. In addition to the initial detection of tumors, the determination of the disease state and, subsequently, the assessment of rehabilitation, seem to be possible future applications.

The main difficulty in assessing the mechanical properties of soft matter, such as living tissues, is that none of the classical non-invasive imaging methods, apart from ultrasound and optical coherence tomography (OCT), which is based on low-coherence interferometry using near-infrared light, has enough sensitivity to measure physical parameters, such as elasticity, in sufficient depth without disturbance of the structure of the probe. Thus, MRE utilizes low-frequency mechanical waves for application to living tissues of mammals or artificial extracellular matrices including cell spheroids, and hence represents a motion-insensitive technique similar to ultrasound methods. A common set-up is presented schematically (figure 11.12). In particular, the stress–strain relation can be locally solved and, subsequently, a complex shear modulus of the probe is obtained. Based on the tissue or cell spheroid type, different frequency regimes can be used. However, the nearly static limit for single individual frequencies is between 10 and 100 Hz and, for the entire frequency spectrum, the static limit lies between 100 and 500 Hz. For soft matter probes, the MRE has been cross-validated with ultrasound-based transient elastography (Oudry *et al* 2009). Indeed, both methods lead to a similar fit of the stress–strain curves and hence MRE has been validated. The MRE measurement is limited for rather stiff tissue near 8 kPa and hence it is optimal suited for softer tissues (Oudry *et al* 2009).

11.5 Matrix composition

The effect of matrix components such as proteins can strongly affect the structure and the mechanical properties of the 3D extracellular matrix. The effect of embedded cells is excluded in this chapter, as it is instead addressed in great detail in chapters 12, 15 and 16. The extracellular matrix, which is a non-cellular component, is still present in all kinds of tissues and organs. Moreover, it provides essential physical scaffolding for the storage of cellular constituents, while it is also responsible for some crucial biochemical and mechanical properties of tissues, which are required for their morphogenesis, differentiation and the maintenance of homeostasis. The importance of the extracellular matrix is mirrored in the wide range of syndromes that arise from genetic abnormalities in extracellular matrix proteins (Jarvelainen *et al* 2009).

The extracellular matrix is fundamental in providing organ and tissue functions, and is mostly composed of water, followed by proteins and polysaccharides. Due to the tissue type, the extracellular matrix displays a unique composition and topology, which is built up during tissue development through a dynamic and reciprocal biochemical and biophysical remodeling between the various cellular components, such as epithelial cells, fibroblasts, adipocytes and the endothelial cell linings of blood or lymphoid vessels. All these diverse alterations lead to a cellular and protein microenvironment that surrounds cells, tissues and organs. Although the physical, topological and biochemical composition of the extracellular matrix has been reported to be tissue-specific, it is also pronouncedly heterogeneous within the same tissue or even in the same tissue of different individual organisms.

The extracellular matrix is a network of secreted extracellular macromolecules (Lu *et al* 2012). In more detail, it consists of a variety of proteins and polysaccharides which are secreted locally and assembled into an organized network in close neighborhood to the surface of the cell that produced them. The extracellular matrix of connective tissue is frequently more abundant than the cells it surrounds and thus it regulates the overall tissue's mechanical properties. Connective tissues form the framework of the organism, but the quantities vary due to their location in organs such as cartilage and bone. In these two tissues, the connective tissues are the major component, but in the brain and spinal cord they are only minor constituents (Galtrey and Fawcett 2007). The cells that secrete the surrounding extracellular matrix can also help to organize the extracellular matrix structurally. In particular, the orientation of the cytoskeleton inside the cell regulates the orientation of the extracellular matrix produced outside (Mierke *et al* 2011a). In most connective tissues, the extracellular matrix's macromolecules are mainly produced and secreted by fibroblasts. In several specialized types of connective tissues, such as cartilage and bone, the matrix's secreting cells are chondroblasts, which build cartilage, and osteoblasts, which assemble bone. However, both cell types still belong to the family of fibroblasts.

Two main classes of extracellular macromolecules assemble the matrix. The first consists of polysaccharide chains called glycosaminoglycans (GAGs). In more detail, these are usually covalently linked to protein in the form of proteoglycans. The second class consists of fibrous proteins such as collagen, elastin, fibronectin and

laminin. All these proteins possess both structural and adhesive functions. In particular, the proteoglycan molecules of the connective tissue build a highly hydrated, gel-like basal substance, in which the extracellular matrix proteins, growth factors, cytokines and chemokines can be embedded. Moreover, the polysaccharide gel can withstand compressive forces on the extracellular matrix while providing the diffusion of nutrients, metabolites and hormones between the white blood cells and the connective tissue cells. The collagen fibers strengthen the matrix scaffold and thus structurally organize the matrix. The rubber-like elastin fibers provide the linear elasticity of the matrix. In addition to a large number of proteins, the connective tissue also contains cells and hence it provides the morphological structure and stability to support cell survival, proliferation and differentiation, and it additionally stores nutrients for cells (Adams and Watts 1993, Hay 1993). Moreover, the extracellular matrix also plays an important role in wound-healing processes (Schultz and Wysocki 2009).

The most abundant protein in the extracellular matrix of connective tissue is collagen (Shoulders and Raines 2009). Collagen monomers can assemble into fibrils and networks and are thus involved in providing cell–matrix adhesion through binding to collagen receptors on the cells. Moreover, they provide the structural scaffold and stability of the extracellular matrix (Halper and Kjaer 2014). There are other proteins embedded in the extracellular matrix, such as fibronectin, laminin and elastin (Halper and Kjaer 2014). Fibronectin and laminin are cell-adhesion proteins that can bind to cell-surface receptors such as integrins and thus they play a role in cell migration, differentiation, primary tumor formation and malignant cancer progression, such as cancer metastasis (Mierke 2014, Wess 2005). Elastin is a structural protein that affects the architecture of the extracellular matrix scaffold, matrix stability and elasticity (Halper and Kjaer 2014). Cell adhesion to the extracellular matrix is facilitated by several groups of extracellular matrix receptors, such as integrins, discoidin domain receptors and syndecans (Harburger and Calderwood 2009, Humphries *et al* 2006, Leitinger and Hohenester 2007, Xian *et al* 2010). The adhesion of cells facilitates the connection of the cell's cytoskeleton to the extracellular matrix (mechano-coupling) and is a major driving factor in cell migration through the extracellular matrix (Schmidt and Friedl 2010). Moreover, the extracellular matrix seems to be a very dynamic structure that is constantly remodeled and restructured enzymatically or non-enzymatically, and its molecular constituents are also altered by numerous post-translational modifications. Through these physical and biochemical characteristics, the extracellular matrix generates the biochemical and mechanical properties of each organ, such as tensile and compressive strength and linear elasticity, and it also provides protection by performing a buffering action, which helps to maintain the extracellular homeostasis and water retention. Apart from collagen fibers, proteoglycans are also an important component of the extracellular matrix. In particular, they are associated with collagen fibrils and other proteins such as elastin, which influence the extracellular matrix's structure and regulate water distribution within it (Halper and Kjaer 2014). In addition, proteoglycans play a role during the assembly of collagen fibers from collagen fibrils (Kalamajski and Oldberg 2010).

Cell–matrix adhesions are facilitated by extracellular matrix receptors to ensure cell survival and enable proliferation, differentiation and motility (Wess 2005). Integrins are the most common extracellular matrix receptors. They are associated with proteins of the cytoskeleton, such as actin, through the focal adhesion and mechano-coupling proteins vinculin and talin, which support cell adhesion by binding integrins to their ligands, such as the extracellular matrix proteins fibronectins, laminins and collagens (Ginsberg 2014).

Finally, water is a key component of the extracellular matrix, influencing processes such as osmosis, filtration swelling and diffusion. Its distribution through the extracellular matrix is modulated by proteoglycans and glycoproteins (Kalamajski and Oldberg 2010). In addition, the extracellular matrix regulates the essential morphological organization and physiological functions through embedment of growth factors and its binding to cell-surface receptors, inducing the induction signal transduction processes as well as the regulation of gene transcription. The biochemical and mechanical protective and organizational properties of the extracellular matrix tissues can vary between different types, for example between lung, skin and bone tissue, and even within a single tissue, as in the renal cortex and medulla; they also differ between different physiological states, for example, between normal and cancerous states.

It has been suggested that cancer cells are influenced by their surrounding microenvironment, such as the extracellular matrix of connective tissue. Alterations in protein distributions of both the cell–matrix adhesion proteins of cancer cells and the structural proteins within the extracellular matrix have an effect on cellular behavior and function (Wolf *et al* 2009). Moreover, the extracellular matrix regulates many of the same cellular responses that have been identified as key hallmarks of cancer (Hanahan and Weinberg 2000, 2011, Pickup *et al* 2014). In addition, growth factors regulate the expression and suppression of genes, which can lead to alterations in several cellular processes (Normanno *et al* 2006). The extracellular matrix affects cancer disease by stimulation through proteins or molecules. The structural and mechanical properties of the extracellular matrix are also known to alter the properties of cancer cells and promote the malignant progression of cancer. Indeed, there are studies that report that matrix stiffness and structure have an effect on cellular processes such as cell migration (Wolf *et al* 2009). In turn, cells have the ability to restructure the extracellular matrix by expressing an enzyme that can degrade the extracellular matrix. MMPs (Fingleton 2005), a disintegrin and metalloproteinases (ADAMs), a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) (Rocks *et al* 2008) and other proteolytic enzymes expressed by the cells can alter the extracellular matrix. Moreover, cancer cells can also express proteins such as fibronectin, collagens and laminin to crosslink extracellular matrix structures and provide ligands for their own cell–matrix adhesion molecules, which hence restructures the extracellular matrix by embedding additional extracellular matrix proteins (Huang and Charrabarty 1994, Mierke *et al* 2011a, Sporn and Roberts 1985).

In the following, the main molecular components of the extracellular matrix are described and compared. For example, the differences between the properties of the

extracellular matrices of a normal simple epithelial tissue and those found within a pathologically modified tissue (such as aged tissue, wounded or fibrotic tissue and tumors) are examined. The focus is mainly on the composition and architecture of the extracellular matrix and interactions with its cellular constituents, and also on common post-translational modifications that facilitate defined topological and viscoelasticity alterations in the tissue. In addition, the functional consequences of extracellular matrix remodeling on cellular behaviors such as altered growth factor sensitivity evoked by changes in the tension of the extracellular matrix are discussed. The particular focus is still on the interstitial stroma of simple glandular epithelial tissues and the basement membranes are excluded here.

The role of the composition of the extracellular matrix in cell motility

The extracellular matrix is composed of two main classes of macromolecules: proteoglycans and fibrous proteins (Jarvelainen *et al* 2009, Schaefer and Schaefer 2010).

Structural and functional properties of proteoglycans

Proteoglycans consist of glycosaminoglycan (GAG) chains that are covalently linked to a specific protein core (one exception is of hyaluronic acid) (Iozzo and Murdoch 1996, Schaefer and Schaefer 2010). In more detail, proteoglycans have been classified according to their core proteins, localization and GAG composition. Thus, the three main families are: small leucine-rich proteoglycans (SLRPs), modular proteoglycans and cell-surface expressed proteoglycans (Schaefer and Schaefer 2010). The GAG chains on the protein core are unbranched polysaccharide ones composed of repeating disaccharide units, such as sulfated *N*-acetylglucosamine or *N*-acetylgalactosamine, D-glucuronic or L-iduronic acid and galactose (–4 *N*-acetylglucosamine-β1,3-galactose-β1), which can be subdivided into sulfated (chondroitin sulfate, heparan sulfate and keratan sulfate) and non-sulfated (hyaluronic acid) GAGs (Schaefer and Schaefer 2010). All these molecules are extremely hydrophilic and hence adopt highly extended conformations that are necessary for the formation of hydrogels and yield to matrices with the capacity to withstand high compressive forces. It has been demonstrated that many genetic diseases have been connected to mutations in proteoglycans genes (Jarvelainen *et al* 2009, Schaefer and Iozzo 2008). In particular, SLRPs have been involved in many signaling pathways, as they activate the epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGFIR) and low-density lipoprotein-receptor-related protein 1 (LRP1) through their binding, and moreover, they regulate the inflammatory response reaction through the binding and activation of TGFβ (Goldoni and Iozzo 2008, Schaefer and Iozzo 2008, Schaefer and Schaefer 2010). Modular proteoglycans can regulate many important cellular processes, such as cell adhesion, migration and proliferation (Schaefer and Schaefer 2010). Basement membrane modular proteoglycans, such as perlecan, agrin and collagen type XVIII fulfill a dual function as pro- and anti-angiogenic factors (Iozzo *et al* 2009). Cell-surface expressed proteoglycans, such as syndecans and glypicans, are able to act as

co-receptors facilitating the ligand bindings to signaling receptors (Schaefer and Schaefer 2010).

The synthesis of the extracellular matrix proteins collagen and fibronectin
To date, 28 different types of collagen have been identified in vertebrates (Gordon and Hahn 2010). The majority of these types of collagen molecules assemble a triple-stranded helix, which may subsequently even assemble into supramolecular complexes, such as fibrils and networks. The general structural feature of collagens is the presence of a triple helix that can cover either most of their structure such as up to 96% for collagen I or solely a small part of the structure such as less than 10% for collagen XII. Whether fibrils or networks are formed depends on the type of collagen. In particular, fibrous collagens build the backbone of the collagen fibril bundles found within the interstitial tissue stroma, while network collagens are detected within the basal membrane. The synthesis of collagen type I includes several enzymatic post-translational modifications (Gordon and Hahn 2010, Myllyharju and Kivirikko 2004), such as the hydroxylation of proline and lysine residues, glycosylation of lysine and N- and C-terminal cleavage of propeptides. After cleavage, collagen fibrils are strengthened through covalent crosslinking between the lysine residues of constituent collagen molecules mediated by lysyl oxidases (LOX) (Myllyharju and Kivirikko 2004, Robins 2007).

Fibronectin is secreted by cells as a dimer connected by two C-terminal disulfide bonds. In addition, it has several binding sites to other fibronectin dimers, collagen, heparin and cell-surface expressed matrix adhesion molecules, such as integrins (Pankov and Yamada 2002). In particular, cell-surface binding of the soluble fibronectin dimer is essential for its assembly into longer fibronectin fibrils. Moreover, cell contraction provided by the actomyosin cytoskeleton and the resulting clustering of integrins induces fibronectin–collagen fibril assembly by exposing cryptic binding sites, which bind one another (Leiss *et al* 2008, Mao and Schwarzbauer 2005, Vakonakis and Campbell 2007).

The main fibrous proteins of the extracellular matrix are collagens, elastins, fibronectins and laminins (Alberts *et al* 2007). In contrast, first proteoglycans fill the majority of the extracellular interstitial space within the tissue by forming a hydrated gel (Jarvelainen *et al* 2009). In addition, proteoglycans have a wide variety of functions such as their unique buffering, hydration, binding and force-resistance properties, which all mirror the important properties of native tissues. In particular, in the basement membrane of the kidney glomerular, perlecan plays a prominent role in glomerular filtration (Harvey and Miner 2008, Morita *et al* 2005). However, in ductal epithelial tissues, decorin, biglycan and lumican can bind to collagen fibers and consequently generate a molecular structure within a certain region of the extracellular matrix that is essential for mechanical buffering and hydration. Moreover, the binding of growth factors to the matrix provides an easily accessible storage sink within this substructure (Iozzo and Murdoch 1996).

Fibroblasts are a major source of the bulk of the secreted interstitial collagen resident in the stroma and even more fibroblasts can be recruited to collagen-rich stroma from neighboring tissues (De Wever *et al* 2008). Through the exertion of

tension on the extracellular matrix, fibroblasts are help to organize collagen fibrils into sheets and cables and, consequently, determine the alignment of collagen fibers. Although there is a heterogeneous mixture of different collagen types within a tissue, one type of collagen usually predominates.

Collagen interacts with elastin, which is a second major extracellular matrix fiber. Elastin fibers provide elastic repulsion to tissues that undergo repeated stretch. However, the elastin stretch is crucially limited by tight binding to collagen fibrils (Wise and Weiss 2009). Secreted tropoelastin (precursor of elastin) molecules can assemble into fibers and are strongly crosslinked to one another through their lysine residues by members of the lysyl oxidase (LOX) enzyme family, such as LOX and LOXL (Lucero and Kagan 2006). In more detail, elastin fibers are covered by glycoprotein microfibrils, such as fibrillins, which are fundamental for the integrity of the elastin fibers (Wise and Weiss 2009).

A third fibrous protein is fibronectin and this is important in directing the organization of the interstitial extracellular matrix. Additionally, fibronectin is crucial in facilitating cell adhesion and function. Indeed, fibronectin can be stretched repeatedly over its resting (persistence) length by cellular traction forces (Smith *et al* 2007). This force-dependent unfolding of fibronectin leads to the exposure of cryptic binding sites for integrins and, consequently, pleiotrophic alterations in cellular behavior. Due to these results, it has been suggested that fibronectin acts as an extracellular mechano-regulator (Smith *et al* 2007). As expected, the tensed fibronectin affects the catch bond, force activation and adhesion assembly of the $\alpha 5\beta 1$ integrin through the exposure of its synergy-binding site (Friedland *et al* 2009). Fibronectin is a major driving factor for cell migration and tissue invasion during development and has been implicated in cardiovascular disease and malignant cancer progression, such as tumor metastasis (Rozario and DeSimone 2010, Tsang *et al* 2010, Mierke *et al* 2011a). As with fibronectin, other proteins of the extracellular matrix such as tenascin exert multiple unrelated effects on cellular behavior, such as the promotion of fibroblast migration during wound healing (Trebaul *et al* 2007, Tucker and Chiquet-Ehrismann 2009). Indeed, levels of tenascins C and W are increased in the stroma of certain transformed tissues where they impair the interaction between syndecan4 and fibronectin in order to facilitate tumor growth and cancer metastasis (Tucker and Chiquet-Ehrismann 2009).

The extracellular matrix and tissue homeostasis under normal conditions

Normal glandular epithelial tissues are composed of a simple layer of epithelial cells displaying apical–basal polarity, where the basal side adheres to the basement membrane and the apical side is exposed to the fluid-filled lumen. In some glandular epithelium there is a basal or myoepithelial cell layer, which separates the luminal epithelium from the interstitial extracellular matrix (Barsky and Karlin 2005). The homeostasis of epithelial tissue depends on the maintenance of tissue organization and provides a dynamic exchange with the surrounding stroma, mainly embedding non-activated fibroblasts and adipocytes and a stable unaltered population of transiting, non-stimulated leukocytes (Ronnov-Jessen *et al* 1996). Thus, non-activated tissue fibroblasts secrete and organize type I and III collagens, elastin,

fibronectin, tenascin and a specific set of proteoglycans, such as hyaluronic acid and decorin, which all keep the structural and functional integrity of the interstitial extracellular matrix in constant balance. Most of the glandular epithelial tissues, such as the breast, saliva gland, lung and prostate, are in tensional homeostasis and their normal state is reported to be highly mechanically compliant (Paszek and Weaver 2004). In a compliant tissue, the extracellular matrix is composed of a relaxed network of collagens type I and III and elastin, which together with fibronectin build a relaxed fiber network. These fibers are surrounded by a hydrogel of glycosaminoglycan-chain-containing PGs (Bosman and Stamenkovic 2003). Hence, the relaxed collagen and elastin fiber network empower the healthy extracellular matrix to resist a wide range of tensile stresses.

In addition, a functionally competent normal tissue can also easily withstand compressive stresses due to the binding of the hydrated glycosaminoglycan (GAG) to the network of the fibrous extracellular matrix proteins (Scott 2003). The extracellular matrix of tissues is consequently a highly dynamic scaffold that is continuously remodeled, as the precise microstructure is crucial for the maintenance of its normal function (Egeblad *et al* 2010, Kass *et al* 2007). Tissue homeostasis is facilitated by the coordinated secretion of MMPs by fibroblasts (Mott and Werb 2004), which is also counterbalanced by tissue inhibitors of metalloproteinases (TIMPs) located in the extracellular matrix (Cruz-Munoz and Khokha 2008) and the controlled activity of other enzymes, such as LOX and transglutaminases, which can crosslink and hence increase the stiffness of the extracellular matrix (Lucero and Kagan 2006). Numerous growth factors are bound to the extracellular matrix and regulate these processes (Friedl 2010, Hynes 2009, Macri *et al* 2007, Murakami *et al* 2008, Oehrl and Panayotou 2008). These extracellular matrix-bound growth factors are able to differentially affect cell growth and migration and upon their release into the matrix they are part of a tightly controlled feedback cycle that is essential for the homeostasis of normal tissue (Hynes 2009).

The extracellular matrix under tissue aging conditions

When a tissue ages, the levels of junctional proteins such as cadherin, catenin and occludin decrease in their expression and subsequently this reduction can compromise junctional integrity, as gaps between the adjacent epithelial cells can occur frequently (Akintola *et al* 2008, Bolognia 1995). Moreover, old tissue is characterized by a thinning of the basement membrane, which may be caused by elevated MMP-driven degradation, as well as reduced synthesis of basement membrane proteins (Callaghan and Wilhelm 2008). In particular, the resident fibroblasts in aged tissues are non-proliferating and can even resist apoptosis, which is indicative of senescence (Campisi and d'Adda di Fagagna 2007). Indeed, senescent fibroblasts express increased amounts of fibronectin, MMPs, growth factors, interleukins, cytokines, plasminogen activator inhibitor (PAI) (Coppe *et al* 2010) and mitochondrial-related reactive oxygen species (ROS) (Untergasser *et al* 2005). Due to this secretion, the extracellular matrix is frequently in a state similar to that of chronic inflammation. Indeed, the combined action of chronic inflammation and elevated levels of MMPs, PAI and ROS may destroy the integrity of the elastin network and

cause alterations to the collagen fiber network, while reduced levels of tissue-associated GAGs also compromise the integrity of the basement membrane (Callaghan and Wilhelm 2008, Calleja-Agius *et al* 2007, Nomura 2006). Paradoxically, in an aging tissue the collagen fibers are frequently inappropriately crosslinked by glycation, by products of lipid oxidation, and upon exposure to UV light (Robins 2007). Glycation increases with age and several advanced glycation end-products additionally act as crosslinkers, which contribute to the progressive insolubilization of the extracellular matrix and the enhanced stiffness of collagens in aged tissues (Avery and Bailey 2006). The combination of elevated and inappropriate collagen crosslinking causes tissue stiffening, which results in a mechanically weaker and less elastic but also more rigid aged tissue compared to a young tissue (Calleja-Agius *et al* 2007, Robins 2007, Schulze *et al* 2012). This aberrant mechanical state can severely compromise the organization of the extracellular matrix, modify the epithelial organization and function, and also potentially support age-related diseases such as cancer (Coppe *et al* 2010, Freund *et al* 2010, Sprenger *et al* 2008).

The role of tensional homeostasis and fibrosis

The acute injury of tissues activates the fibrogenic processes and induces wound healing. An early event, which characterizes a wound-healing response, is vascular damage and the associated formation of a fibrin clot, which then induces the infiltration of monocytes to the damaged sites of the extracellular matrix. When monocytes bind to extracellular matrix-degradation products and cytokines, they rapidly differentiate into macrophages (Clark 2001). These activated macrophages in turn secrete and release multiple growth factors, MMPs and cytokines, which induce angiogenesis and stimulate the motility of fibroblasts and induce their proliferation (Schultz and Wysocki 2009). Thereafter, recruited fibroblasts start to synthesize and deposit large quantities of extracellular matrix proteins, such as collagen type I and III, fibronectin and hyaluronic acid, which all affect the mechanical and structural properties of the extracellular matrix. Moreover, the increased mechanical stress associated with this profound extracellular matrix deposition can lead to the transdifferentiation of fibroblasts and other tissue-resident cells, which means they switch from an epithelial to a mesenchymal phenotype (the epithelial–mesenchymal transition (EMT)). Another example for a transdifferentiation are circulating bone-marrow-derived mesenchymal stem cells, which switch and hence differentiate into myofibroblasts (Schultz and Wysocki 2009, Velnar *et al* 2009). These myofibroblasts possess a high capacity to synthesize extracellular matrix components and are highly contractile. Moreover, they promote the formation of large, rigid collagen bundles that, when crosslinked by LOX enzymes, mechanically strengthen and stiffen the connective tissue (Szauter *et al* 2005). This injured and hence stiffened microenvironment disrupts the basement membrane that surrounds the epithelium and compromises the integrity of the tissue, as indicated by the loss of apical–basal polarity and destabilized cell–cell adhesions. The remodeled extracellular matrix additionally promotes the directional migration of cells within the tissue to the site of the inflammatory wound (Schafer and Werner 2008). In some

cases, the release of transforming growth factor β (TGF-β) caused by tension and secretion of MMPs induces the EMT of the resident surrounding epithelium (Schultz and Wysocki 2009, Wipff *et al* 2007, Xu *et al* 2009a). In a healthy tissue, once the wound has been repopulated, specific feedback mechanisms are induced that lead to the re-establishment of tissue homeostasis and the dissolution of fibrosis (Schultz and Wysocki 2009, Velnar *et al* 2009). Under extreme conditions, such as repeated injury or when normal feedback mechanisms are compromised, continuous extracellular matrix synthesis, deposition and remodeling becomes permanent and myofibroblasts remain in the tissue, where TIMP production prevails over MMP synthesis. However, these aberrant conditions may lead to chronic vascular remodeling and elevated extracellular matrix crosslinking, which may cause aberrant fibrosis. Finally, the connective tissue will not be able to heal properly. This aberrant wound-healing scenario leads to altered mechanical stability and decreased elasticity, which is a typical feature of scarred tissue (Kisseleva and Brenner 2008). In special, extreme cases, a chronic wound is able to promote a tumor phenotype (De Wever *et al* 2008).

How is the extracellular matrix altered near primary tumors?
Cancer can be described as the loss of tissue organization and the abnormal behavior of cellular components. Thus, cell transformation caused by genetic mutations and epigenetic alterations further supports the malignant progression of cancer. In addition, primary tumors seem to be similar to wounds that fail to heal (Schafer and Werner 2008). The tumor stroma only exhibits certain characteristics found in an unresolved wound (Bissell and Radisky 2001). One characteristic feature of tumors is that they are usually stiffer than the surrounding normal healthy tissue. The stress stiffening of primary tumors is induced by extracellular matrix deposition and remodeling through resident tumor-associated fibroblasts and through the enhanced contractility of the transformed epithelium (Butcher *et al* 2009, Levental *et al* 2009). Moreover, chemokines and growth factors (De Wever *et al* 2008) can also induce inflammation and thereby modify the number of infiltrating T-lymphocytes (Tan and Coussens 2007). Tissue inflammation leads to stromal fibroblast activation and induces the transdifferentiation of fibroblasts into myofibroblasts, thus increasing and promoting tissue desmoplasia (De Wever *et al* 2008, Desmouliere *et al* 2004). Myofibroblasts deposit large quantities of extracellular matrix proteins, secrete growth factors and exert strong contractile forces on the extracellular matrix (De Wever *et al* 2008, Desmouliere *et al* 2004). Thus, newly deposited and remodeled collagen as well as elastin fibers are re-oriented and remodeled after crosslinking by LOX and transglutaminase. Finally, larger and more rigid fibrils are present that further stiffen the tissue extracellular matrix (Butcher *et al* 2009, Erler and Weaver 2009, Levental *et al* 2009, Lucero and Kagan 2006, Payne *et al* 2007, Rodriguez *et al* 2008). MMPs can be secreted and activated by cancer cells and myofibroblasts (De Wever *et al* 2008, Kessenbrock *et al* 2010) and are then able to remodel the basement membrane surrounding the primary tumor, releasing and activating extracellular matrix embedded growth factors (Bosman and Stamenkovic 2003, Kessenbrock *et al* 2010). The release of growth factors such as vascular endothelial growth factor

(VEGF) enhances the vascular permeability of blood vessels and thus induces the growth of new vessels, which then generate additional interstitial tissue pressure. There is an amplifying cycle involving cancer-associated extracellular matrix stiffening, reciprocal extracellular matrix resistance induced by resident cancer cells and myoepithelial cells, and cell-generated contractility, which act as a positive-feedback loop to potentiate tumor growth and survival. This induces neoangiogenesis and cancer cell invasion, which can foster cancer metastasis (Butcher *et al* 2009, Erler and Weaver 2009, Paszek and Weaver 2004, Paszek *et al* 2005).

What are the challenges encountered with natural and synthetic extracellular matrices? As the extracellular matrix influences and regulates numerous fundamental cellular processes, many tissue-culture models have been developed to investigate the impact of these microenvironmental biochemical and biophysical properties and to obtain insights into the molecular origins of cellular behaviors controlled by the ligation of the extracellular matrix. In order to assess the fundamental process of cell adhesion and its impact on cell behavior, the majority of cancer research studies have been based on coated tissue-culture dishes (plastic or glass), with purified preparations or mixtures of extracellular matrix proteins for the growth of 2D monolayers (Kuschel *et al* 2006). However, to analyze extracellular matrix rigidity, functionalized polyacrylamide (PAA) gels crosslinked with reconstituted basement membrane generated from Engelbreth–Holm–Swarm mouse carcinoma (Matrigel™), collagen type I, fibronectin or extracellular matrix peptides such as RGD peptide, have become the general experimental setup (Johnson *et al* 2007, Pelham and Wang 1997). However, these experimental strategies do not mirror the behavior of cells within tissues under *in vivo* microenvironmental conditions in an appropriate manner, as no real 3D situation can be investigated, but the extracellular matrix can be readily restructured. To reveal the impact of 3D and extracellular matrix remodeling, natural extracellular matrix and reconstituted extracellular matrix gels can be analyzed in terms of tissue-specific differentiation and architecture. The reconstituted basement membrane, which mimics several of the biochemical and biophysical properties of endogenous epithelial basement membranes, has been used in 3D organotypic culture assays, for xenograft manipulations or tissue engineering to investigate tissue-specific morphogenesis, such as the branching and acini formation and differentiation (Kleinman and Martin 2005, Kleinman *et al* 1986). However, basement membrane preparations such as Matrigel™ are well suited for determining normal epithelial or endothelial behavior and distinguishing between the normal and malignant behavior of certain tissues, although they have a complex and rudimentarily defined composition that does not reconstruct the physical state of the *in vivo* interstitial extracellular matrix. Another weakness of these preparations are large batch-to-batch variations, which may lead to decreased reproducibility of the results, when working with different batches. In addition, fibrin has been used as a natural biodegradable scaffold in vascular tissue engineering. A disadvantage of fibrin is that it lacks the mechanical strength and durability of native interstitial extracellular matrix (Blombaeck and Bark 2004, Shaikh *et al* 2008). In contrast, collagen type I is useful and can be combined with a reconstituted

basement membrane (such as purified laminin or fibronectin) to determine certain biological aspects of normal and diseased interstitial extracellular matrices (Friess, 1998, Gudjonsson *et al* 2002). In particular, collagen type I can assemble into a mechanically tensed network of fibrils that can be oriented, functionally modified, and enzymatically as well as chemically crosslinked and hence stiffened. Thus, collagen I gels are suitable substrates for determining the role of collagen and fibronectin stiffness and organization in the pathogenesis of tumor progression and invasion (Levental *et al* 2009, Provenzano *et al* 2009a). Nevertheless, collagen gels have been reported to be quite heterogeneous and modifying of their architecture alters the entire organization, pore size and ligand concentration, which hence complicates the interpretation of the data in terms of differentiating between the effects of each individual component and generalized effects resulting from the usage of this kind of natural scaffold (Johnson *et al* 2007). To overcome this, denuded extracellular matrix scaffolds from various tissues have been isolated (Macchiarini *et al* 2008). These scaffolds contain colonies of stem cells which may reconstitute normal tissues with reasonable fidelity (Lutolf *et al* 2009). Indeed, extracellular matrices have been isolated and extracted from various tissues, including that of the small intestine, skin, pancreas and breast (Rosso *et al* 2005), and these extracellular matrices have been used to engineer skin grafts (Badylak 2007), increase wound-healing processes and investigate the malignant tumor progression. An example is porcine-derived small intestinal submucosa, which has been proved to be a successful treatment for patients with hernias (Franklin *et al* 2002, Badylak 2007). Although these purified extracellular matrices have certainly been successful in special applications, they are difficult to obtain, as they need well-defined micro-environments in tissue regeneration and stem cell transplantation, in which animal byproducts and contaminants are eliminated. In order to reveal the molecular and biophysical mechanisms through which the extracellular matrix supports special effects in cellular differentiation and morphogenesis, it is important to use chemically and physically defined modular extracellular matrices, which can be reproduced reliably. In this respect, it has been demonstrated that synthetically produced matrices have defined and tunable composition, organization, mechanics and extracellular matrix remodeling capabilities (Ayres *et al* 2009, Dutta and Dutta 2009, Lutolf and Hubbell 2005, McCullen *et al* 2009, Rosso *et al* 2005, Zisch *et al* 2003). For example, polyethylene glycol (PEG) hydrogels are used frequently as biologically compatible synthetic matrices facilitating cell adhesion, supporting cell viability and growth (Lutolf and Hubbell 2005). Indeed, these matrices can be covalently crosslinked with extracellular matrix ligands and can bind collagenase-degradable peptides and store growth factors (Ehrbar *et al* 2007a, Zisch *et al* 2003), although they do not mirror the structural and architectural features of native collagen gels and their pore size often impedes cell migration. In contrast, peptide-based hydrogels such as peptide-amphiphiles assemble into secondary structures that rebuild the collagen triple helix and subsequently facilitate stem cell growth and viability as well as direct multicellular morphogenesis (Hauser and Zhang 2010, Sieminski *et al* 2008, Smith and Ma 2004, Ulijn and Smith 2008). In more detail, these peptide-amphiphiles are modified by covalent binding of native proteins and

MMP-degradable extracellular matrix peptides. As an alternative matrix, polylactic-co-glycolic acid (PLGA), a copolymer of glycolic acid and lactic acid (McCullen *et al* 2009), which is biodegradable because it can be hydrolyzed into lactic and glycolic acid, has been conjugated to various extracellular matrix ligands and peptides, or coated with collagen or chitosan, in order to induce cell adhesion and provide viability and growth. Another further development is modular biocompatible extracellular matrices, which contain ligand-binding cassettes and have tunable stiffness features supporting a precise patterning of cell adhesion in 2D and 3D microenvironments (Serban and Prestwich 2008). The organization of the extracellular matrix's structure is crucial for cellular functions and hence has led to the development of new methodologies that lead to extracellular matrices whose fiber size, orientation, stiffness, ligand-binding function and remodeling potential can be controlled and varied (Zhang *et al* 2009). Anisotropically nanofabricated substrates assembled from scalable biocompatible PEG (Kim *et al* 2010, Smith *et al* 2009) are a novel development in the biomaterials field, and their only major advantage seems to be their ability to address the lack of functional assessment in physiological culture assays and animal models.

The main constituent of the extracellular matrix: collagen

The most abundant fibrous protein collagen of interstitial extracellular matrices delivers up to 30% of the total protein mass of mammals. Collagens constitute the main structural element of the extracellular matrix and provide tensile strength, regulating cell adhesion, chemotaxis and migration, and consequently direct tissue development (Rozario and DeSimone 2010). In more detail, collagens are major proteins in the extracellular matrix of connective tissue and are the most prevalent component of mammals, and are mainly produced and altered by fibroblasts. These structural proteins are part of tissues in bones, tendons, ligaments, skin, blood vessels and the cornea of the eye. Hence, their broad distribution displays the wide range of properties that comes from their functional requirements (Wess 2005). Minerals are included in bones to stiffen and stabilize the matrix. In tendons and ligaments the collagen tissue needs to be more elastic and store energy. In the cornea of the eye, visible light should be able to pass through the collagen matrix that needs to be transparent. All these different properties are caused by special structural arrangements of collagen monomers, fibrils, fibers and interaction with other molecules, such as minerals, proteoglycans and glycosaminoglycans. All collagen types have a common primary structure: collagen monomers can assemble to three left-handed helical polypeptides (called α-chains), which then associate to form a right-handed helical fibrillar collagen monomer (Ramachandran and Kartha 1954, Rich and Crick 1955a). There are at least 40 genes encoding various α-chains, which assemble into different collagen types (Stamov and Pompe 2012). In more detail, the α-chains consist of triplets made up of a glycine molecule and two non-equivalent amino acids Gly-X-Y (Rich and Crick 1955a). These amino acids are often (2S)-proline (28%) and (2S,4R)-4-hydroxyproline (38%) (Shoulders and Raines 2009). Both the N-terminal and C-terminal ends of an α-chain terminate in a propeptide, thus this α-chain is also called the protocollagen chain. Three protocollagen chains

Table 11.1. The five main collagen classes.

• Type I. Skin, tendon, vascular ligature, organs and bone.
• Type II. Cartilage.
• Type III. Reticulate, commonly found alongside type I.
• Type IV. Forms basal lamina, the epithelium-secreted layer of the basement membrane.
• Type V. Cell surfaces, hair and placenta.

bind together through the establishment of hydrogen bonds (Rich and Crick 1955b). The resulting right-handed chain is called procollagen. After intracellular assembly, these chains are transported out of the cell. Finally, parts of the propeptide ends are cut off by specific MMPs, leaving telopeptide ends. The propeptides prevent fibrillation inside the cells, whereas the N- and C-terminal telopeptides support it. Thus, these telopeptides play an important role in the extracellular assembly of collagen fibrils and network structures. The resulting chain, tropocollagen, is the monomer of collagen structures.

Collagen classification

At least 28 different collagen types (and other proteins consisting of a collagen-like structure) have been identified in vertebrates (Ramachandran and Kartha 1954, Ricard-Blum 2011). There are five main classes into which they can be divided (Birk and Bruckner 2005): type I fibrillar collagens, type II network-forming collagens, type III fibril-associated collagens with interrupted triple helices (FACITs), type IV membrane associated collagens with interrupted triple helices (MACITs) and V collagens with multiple triple-helix domains with interruptions (MULTIPLEXINs) (table 11.1).

Fibrillar collagens

The monomers of fibrillar collagens aggregate to fibrils, which further assemble into fibers, bundles and whole networks. The most important members of this class of fibrillar collagens are types I and II. Collagen I is more widely distributed than collagen II, as it is found in the dermis, bone, tendon and ligaments. By contrast, collagen II is found in the cartilage and vitreous of the eye. As mentioned above, a fibrillar collagen monomer is a right-handed helical chain assembled by three left-handed polypeptides. Each fibrillar collagen polypeptide chain consists of 338–343 Gly-X-Y triplets (Ramshaw *et al* 1998). Collagen monomers have a length of approximately 300 nm and a thickness of up to 1.5 nm. They aggregate by establishing covalent bonds between the C-terminal telopeptide and the helical domain within fibrils, which show a repeating banding pattern with a periodicity of 64–67 nm (named D-periodicity). This banding pattern can be seen in high-resolution images and is caused by the gap-overlap structure of regions with high and low electron densities within collagen monomers.

The composition of the three α-chains differs between the fibrillar collagen types: the monomers of collagen I are heterotrimer (one α-chain has another triplet composition), whereas collagen II is a homotrimer (α-chains have the same triplet composition). In addition, the number of amino acid triplets within the protocollagen chain can change.

Collagen fibrils are able to aggregate into fibers or networks with different structural and mechanical properties. Other molecules and proteins also play a role in fiber aggregation, including collagen III, collagen V, fibronectin, glycosaminoglycans, glycoproteins and proteoglycans (Di Lullo *et al* 2002). For example, interactions between proteoglycans or between a proteoglycan molecule and other glycosaminoglycans regulate the interfibrillar interaction (Wess 2005). In addition, collagen V is important for collagen I fibrillation and hence matrix organization (Wenstrup *et al* 2004), and inclusion of collagen III leads to alterations in the network structure (Lapiere *et al* 1977).

Network-forming collagens

This class of collagens assembles into network scaffolds. Important members of the class of network-building collagens are collagen IV located in the basement membrane (Glentis *et al* 2014) and collagen VI.

There are different aggregation modes of network-forming collagens, such as rectangular networks (collagen IV) and hexagonal lattice structures (collagen VI, VIII, V), which are formed by head-to-head interactions among and also between N-terminal and C-terminal domains. In some cases, they can even form fibril-like structures (collagen VI), where two monomers assemble into a dimer through the interaction of the C-terminal domain with the helical structure of the second monomer via the formation of hydrogen bonds. Then, two dimers form a tetramer, which is the building block for further, more complex structures. Moreover, they can assemble into beaded microfibrils, broad-banded fibrils or hexagonal lattice structures by head-to-head interactions of the N-terminal regions. Indeed, interruptions in the triplet sequence of the polypeptide chains are observed and can lead to more flexibility in the overall structure. Interactions between collagens such as the fibrillar (collagen I and II) are possible. In more detail, collagen types such as collagen I and collagen IV interact in the basement membrane (Glentis *et al* 2014).

Fibril-associated collagens with interrupted triple helices (FACITs), MACITs and MULTIPLEXIN collagens are associated collagens. In particular, FACITs occupy the surface of fibrils such as collagen I or collagen II. The structural difference of several triple-helical domains interrupted by nonhelical sequences supports the modification of the connective tissue. They lead to bridges in the extracellular matrix, inducing organization and stabilization of the extracellular matrix (Shaw and Olsen 1991). MACITs also possess helical structure domains, which are interrupted by nonhelical domains such as FACITs and can bind to network-forming collagens such as collagen VI. They can also be found in the basement membrane associated with collagen IV, which alters the structural properties. FACITs cannot form fibrils by themselves, but they are able to associate with the surface of collagen fibrils. In particular, collagen IX is covalently linked to

the surface of cartilage collagen fibrils composed mostly of collagen II (Olsen 1997) and collagens XII and XIV are found to be associated with collagen-I-containing fibrils. In addition, collagen XV is located in close association with collagen fibrils at the basement membrane and thus forms a bridge that links large, banded fibrils such as those containing collagens I and III (Amenta *et al* 2005). Similarly, MULTIPLEXIN collagens are frequently associated with membranes and consist of several interrupted collagenous domains.

Collagen type I

Collagen type I is the most abundant collagen in mammalian tissue, as it accounts for more than 90% of the total collagen found in nature (Gobeaux *et al* 2008). Moreover, collagen I is the main protein of the extracellular matrix and it occurs in tendons, ligaments and the dermis. Monomers of the fibril forming collagen type I are assembled to heterotrimers consisting of two identical $\alpha 1$ chains and an $\alpha 2$ chain, which differs slightly from the $\alpha 1$ chain. The $\alpha 1$ and $\alpha 2$ chains are encoded by the COL1A1 and COL1A2 genes, respectively. They differ solely in their triplet structure Gly-X-Y. Collagen chains can vary in size from 662 to 3152 amino acids for the human $a1$(X) and $a3$(VI) chains, respectively (Ricard-Blum *et al* 2000, Gordon and Hahn 2010). In more detail, each α-chain consists of 338–343 uninterrupted amino acid triplets (Ramshaw *et al* 1998). However, the total number of triplets depends on the collagen source. Apart from the existence of 28 different collagen types, further diversity occurs within the collagen family due to the existence of several molecular isoforms for the same collagen type, such as for collagens IV and VI, and hybrid isoforms composed of chains belonging to two different collagen types, for example type V/XI hybrid molecules (Ricard-Blum 2011). Moreover, there also exist many alternatively spliced isoforms of several collagen subtypes (Ricard-Blum 2011).

The fibrillation behavior of single collagen I fibrils is well understood. The aggregation of monomers and the lateral growth of fibrils is temperature-dependent (Gelman *et al* 1979, Wolf *et al* 2013). Increasing temperature causes a decrease in the fibril diameter and length (Liu *et al* 2005). In addition, the pH of the non-polymerized collagen solution influences the structure of the fiber and the entire network. Thus, it has been reported that with increasing pH the fibril diameter increases (Christiansen *et al* 2000). Moreover, the collagen concentration influences fibril formation, as with increasing collagen concentration, the fibrillation rate and fibril diameter are enhanced (Wirtz 2009, Gobeaux *et al* 2008). However, what impact does this have on the mechanical properties of 3D collagen matrices and on cancer cell migration through these special matrices?

11.6 The impact of fiber thickness, connection points and polymerization dynamics on cancer cell invasion

It has been shown that the fiber thickness can be modulated by polymerization of the collagen matrices at different temperatures, which influences the polymerization kinetics (Sapudom *et al* 2015). Indeed, it has been reported that the polymerization

process decreases with lowering temperature, leading to thicker collagen fibrils and larger pore sizes (Wolf *et al* 2013, Sapudom *et al* 2015). Recently, the impact of the fiber thickness on cancer cell migration was determined (Sapudom *et al* 2015). Indeed, the cellular invasiveness increased for two breast cancer cell types such as the highly invasive MDA-MB-231 and the weakly invasive MCF-7 cells, when the collagen fibril diameter is increased. However, the role played by the connection points within a 3D matrix is still elusive and need to be investigated in further studies. The connection points may contribute to the anisotropy of the 3D collagen matrices and may represent a greater confinement for cancer cell invasion than parallel collagen fibers and bundles.

Polymerization dynamics

The polymerization dynamics have a major impact on the structure and morphology and hence on the mechanical properties of the extracellular matrix. It is obvious that the temperature during the polymerization of collagen matrices is a critical factor for the regulation of the fiber thickness and the pore size or cross-sectional area. In addition, the pH value for the polymerization is additionally important for the structure and morphology and consequently the mechanical properties of the extracellular matrix. However, the pH values can only be varied over a small range, in which the buffer has its highest buffering capacity. There are still many points to investigate in terms of controlled collagen polymerization dynamics.

11.7 The role of a matrix stiffness gradient in cancer cell invasion

Tissue stiffness is precisely regulated under normal conditions, whereas it is altered in various diseases. In cancer, tumors are usually stiffer than the surrounding uninvolved tissue microenvironment, whereas the cancer cells themselves seem to be mostly softer and may in certain cases also be stiffer than normal or less invasive

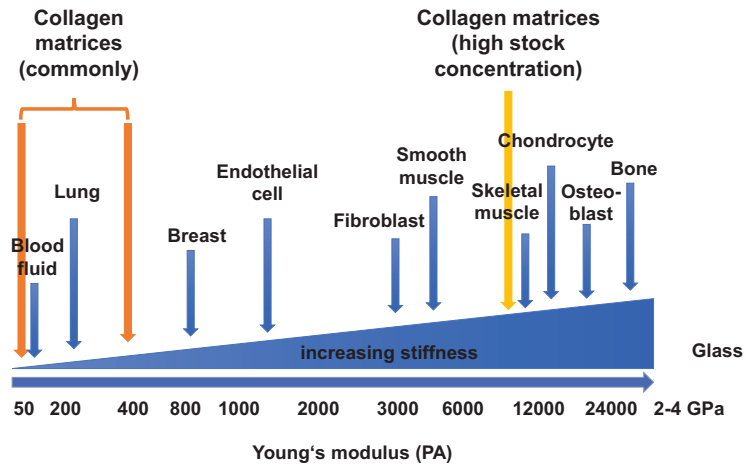

Figure 11.13. Mechanical properties such as stiffness (elastic modulus) of different tissue cell types. In breast tumors is the stiffness increased as indicated by the blue bar.

cancer cells. There is increasing and accumulating evidence that the stiffness of the extracellular matrix stroma of primary tumors affects the cancer and stromal cell mechanics and function, influencing hallmarks of cancer disease such as angiogenesis, proliferation, migration and metastasis. How do cancer cells and fibrosis-relevant stromal cells respond to extracellular matrix stiffness alterations? This question cannot yet be answered, as the possible sensing mechanisms and signaling mechanisms involved are not yet fully revealed, and the emergence of novel substrates such as those with scar-like fractal heterogeneity, which seem to mimic the *in vivo* mechanical environment of the cancer cell more accurately than isotropic and homogeneous substrates, need to be employed in future studies.

Cancer cells are tuned to the mechanical properties of the 3D microenvironment (for example, a 3D extracellular matrix). All cells—not only cancer cells, and including those in traditionally mechanically static tissues, such as those from the breast and brain—are exposed to isometric force or tension. The isometric force or tension is generated locally at the nanoscale level by cell–cell or cell–matrix interactions and it alters the cell's function through the regulation of the actomyosin contractility and actin dynamics (Álvarez-González *et al* 2015). In more detail, each cell type is specifically adapted to its specific 'home' tissue in which it is usually embedded. In particular, the brain is pronouncedly softer than bone tissue (figure 11.13). Thus, neural cell growth, survival and differentiation are supported by a highly compliant matrix. In contrast, osteoblast differentiation and survival are favored on stiffer extracellular matrices with mechanical properties that are more similar to newly formed bone tissue. Normal mammary epithelial cell growth, survival, differentiation and morphogenesis are regulated by the interaction of the cells with a relatively soft matrix. Upon transformation of healthy tissue to cancerous tissue, breast tissue becomes progressively stiffer and consequently cancer cells become significantly more contractile and hyper-responsive to matrix compliance alterations (Lopez *et al* 2011). In addition, it has been reported that normalizing the tensional homeostasis of cancer cells reverts them towards a non-malignant phenotype (Lühr *et al* 2012), thereby suggesting a functional link between the matrix's mechanical properties, cellular tension and normal tissue behavior. Although primary breast tumors are much stiffer than the normal healthy breast, the breast tumors are still pronouncedly softer than those of muscle or bone tissue tumors, highlighting the precise association between the tissue phenotype and the matrix's rigidity.

The finding that tumors are often stiffer than the surrounding uninvolved tissue has been known since cancer disease was identified (Chin *et al* 2016). The rigid nature of primary tumors forms the basis for using palpation as a diagnostic tool to identify and localize tumors in soft tissues such as breast and abdomen. More recently, it has served as the basis for the high-resolution detection of small lesions by MRI elastography (Streitberger *et al* 2014, Poterucha *et al* 2015, Weis *et al* 2015) or ultrasound (Zaleska-Dorobisz *et al* 2014). Based on these clinical observations and the *in vitro* experiments, it has been shown that stiffness-sensing by cancer and stromal cells affects their cell survival and proliferation efficiency, which enables us to envision many investigations employing novel biocompatible materials with

tunable viscoelastic properties in order to impair cancer progression such as primary tumor growth and metastasis. Indeed, these novel *in vitro* systems seem to possess the potential to elucidate the mechanical and molecular mechanisms through which cells detect alterations in their microenvironment and transfer physical signals to the biochemical signals regulating their function, biochemistry and subsequently gene expression.

The mechanotransduction of physical cues to induce intracellular signaling pathways has been found in many cancer types and a wide range of diverse effects have been detected, ranging from acute alterations such as the activation of ion channels or protein kinases to account for long-term changes in the cellular phenotype that need the promotion of gene transcription and protein production. How can the substrate stiffness affect some of the cancer hallmarks? What mechanisms seem to be involved? The usage of materials with tunable rigidity may help to identify the mechanosensing ability of cancer cells. Moreover, these new materials may enable the mimicking of the viscoelasticity of normal and cancerous tissues.

11.7.1 Stiffened matrices as a model for tumors

The alterations in mechanical properties during breast cancer are relatively well-studied, and hence the stiffening of the tumor surrounding tissue is correlated positively with the malignancy such as enhanced collagen deposition and the arrangement of linear patterns of collagen fibers within the surrounding matrix microenvironment (Acerbi *et al* 2015). Enhanced fibronectin expression such as a threefold increase, which in normal tissue is low (1%), is additionally related to a high-risk of breast cancer and moreover, in metastasis the expression of fibronectin is similarly increased by threefold (Zhou *et al* 2015, Fernandez-Garcia *et al* 2014, Suer *et al* 1998, Yao *et al* 2007). As a justifiable model for breast cancer, associated stromal cells, the 3T3-L1 preadipocytes, produce a more rigid fibronectin network with reduced porosity and enhanced fiber diameter, when these cells are preconditioned with soluble tumor factors compared to unstimulated (buffer) control cells (Wang *et al* 2015). These alterations in fibronectin are accompanied by reduced cellular adhesion and elevated vascular endothelial growth factor (VEGF) levels, suggesting a role for fibronectin in cell migration, tumor angiogenesis and proliferative growth of breast tumors.

A few studies have also shown that the extracellular matrix can be stiffened through crosslinking mechanisms, which are evoked by the non-enzymatic formation of advanced glycation end-products (AGE) and lysyl oxidase. In a prostate epithelial cell acini 3D model, the AGE-faciliated crosslinking of two major components, collagen IV and laminin, causes a stiffening of the basal lamina matrix and thereby induces the malignant transformation, which can be observed by loss of cell polarity, loss of cell–cell junctions, and luminal infiltration (Rodriguez-Teja *et al* 2015).

A novel and highly promising area of interest is the development and characterization of 3D matrices with tunable physical properties as models for cell biology and also as platforms for analyzing the effect of drugs and toxins. In particular, soft

and stiff alginate scaffolds with different RGD concentrations served as platforms to test the cytotoxic response of glioblastoma cells after addition of various compounds. Both substrate rigidity and cell–matrix adhesions can impact the cellular toxicity. It has been found that cells react more sensitively to toxins when seeded onto soft substrates with stiffnesses similar to brain tissue, whereas these effects are abolished when the integrin-binding is pharmacologically impaired (Zustiak *et al* 2016).

Polyethylene glycol diacrylate (PEGDA) hydrogels with compressive moduli between 2 and 70 kPa can be utilized to encapsulate cancer stem cells to determine their optimal matrix stiffness for growth without any confounding microenvironmental factors (Jabbari *et al* 2015). It has been reported that optimal stiffness for cell survival and proliferation as well as YAP/TAZ expression relies upon the stiffness of the original tissue, that is, 5 kPa for breast, 25 kPa for colorectal and gastric, and 50 kPa for bone.

In contrast to chemically inert and non-physiological polyacrylamide gels, hydrogels produced from crosslinked networks of biopolymers such as hyaluronic acid (HA) can be employed to investigate cancer cell behavior to stiffness. The levels of HA, a component of the extracellular matrix, are elevated in many cancers (Shen *et al* 2014, Toole 2004), such as ovarian (Ween *et al* 2011), non-small-cell lung adenocarcinomas (Pirinen *et al* 2001), prostate cancer (Posey *et al* 2003, Lipponen *et al* 2001), gastric and colorectal (Ropponen *et al* 1998, Setala *et al* 1999), bladder (Lokeshwar *et al* 2002), breast (Delpech *et al* 1990), and head and neck (Franzmann *et al* 2003). In many cancer cases, *in vitro* HA is methylated to enable crosslinking, which in turn abolishes the ability of HA to activate HA receptors such as CD44 that have been implicated in cancer (Kim *et al* 2014). However, HA hydrogels revealed cellular behaviors that are pronouncedly different from polyacrylamide or tissue-culture plastic. In particular, HT-1080 fibrosarcoma cells encapsulated in HA can even recover from hypoxic stress, whereas cells cultured on tissue-culture plastic cannot recover (Shen *et al* 2014). In more detail, networks of HA mimic more closely the native glycosaminoglycan, which can be produced when the chain is slightly modified with sulfhydryl groups and subsequently crosslinked by oxidation or PEGDA. When HA gels are connected to fibronectin, the proliferation of various cell types, such as neonatal ventricular rat myocytes, human mesenchymal stem cells (hMSC), 3T3 fibroblasts and human umbilical vein endothelial cells (HUVECs), is found to be pronouncedly increased even on soft substrates (200 Pa), which, when produced by purely polyacrylamide gels, cannot support proliferation (Chopra *et al* 2014).

Besides variations of 2D hydrogel substrates, numerous methods have been established for investigating how physical signals affect cancer cells *in vitro*. Among these methods are the production of pillar arrays that hamper cellular movement by trapping nuclei (Nagayama *et al* 2015), simplified microfluidic methods that exert pressure towards single cells (Lee and Liu 2015, Song *et al* 2014, Jeon *et al* 2015), patterned type I collagen microtracks that rebuild the migration paths by which cancer cells invade *in vivo* (Carey *et al* 2015), and optical tracking microrheology to characterize the mechanical properties of the very soft pericellular matrix which alters the tumor microenvironment (Nijenhuis *et al* 2012). All these more sophisticated techniques will enlighten how cancer cells sense their mechanical microenvironment.

Stiffening of the 3D collagen extracellular matrix induces epithelial dispersion

The requirement for epithelial attachment to the substrate such as a collagen matrix for survival and proliferation indicates that the invasive epithelial phenotype is mechanosensitive. What is the effect of an increasingly stiff stromal extracellular matrix microenvironment on the progression of cancer? Indeed, this has been revealed by using MCF-10A cells cultured in collagen matrix of increasing stiffness (Levental *et al* 2009). The collagen matrix can be stiffened via non-enzymatic glycation through the incubation of the acidified collagen solution with varying amounts of ribose prior to cell embedding, which leads to compressive moduli of 175 Pa (0 mM ribose), 340 Pa (50 mM ribose), and 515 Pa (100 mM ribose) (Madson *et al* 2013). When analyzing the epithelial collagen cultures after four days, a 3D epithelial phenotype has been seen that depends on the mechanical properties of the extracellular matrix (Carey *et al* 2017). Whereas all cells are assembled within multicellular organoids in floating collagen matrices, a stiffening of the attached collagen matrix resulted in an increase in the fraction of dispersed single epithelial cells (Carey *et al* 2017). Although increasing matrix stiffness slightly decreases the overall cell density, possible due to alterations in growth kinetics within stiffer matrices, this effect is not strong enough to account for the abundance of single cells in glycated collagen after four days, as the initial seeding density represents only approximately 5% of the final cell count in floating collagen matrices (Carey *et al* 2017). Indeed, both epithelial cells' ability to form multicellular structures and the nature of multicellular organoid structures itself are regulated by the 3D collagen matrix mechanics. Taken together, these data lead to the hypothesis that epithelial cells exposed to increasingly stiff collagen extracellular matrices transfer the cohesive invasive epithelial phenotype to a dispersive invasive phenotype.

The mammary epithelial cell phenotype relies on the mechanical properties of the extracellular matrix microenvironment (Levental *et al* 2009, Paszek *et al* 2005, Ng *et al* 2012, Provenzano *et al* 2009b). 3D collagen matrix mechanics have been shown to be crucial in determining whether epithelial cells can adapt an invasive epithelial phenotype and also in the determination of the nature of this invasive phenotype. Using floating collagen matrices, it has been revealed that the intra-matrix tension is needed for the invasive epithelial phenotype, which is not surprising as cell–matrix mechano-coupling represents a key determinant of the epithelial phenotype (Provenzano *et al* 2009a).

In particular, inhibition of cell contractility via the inhibition of ROCK overcomes this suppression and induces protrusion to rescue the invasive epithelial phenotype. This finding indicates that the potential for invasion is still present in floating matrices, whereas it is suppressed by ROCK activity when matrix tension is released. Indeed, MCF-10A cells can deposit fibronectin and not laminin in floating collagen matrices, which is highly similar to extracellular matrix deposition in attached collagen matrices, suggesting that the collagen extracellular matrix itself induces the mesenchymal gene expression independently of matrix anchorage. Indeed, it has been found that the relaxation of myosin-II contractility weakens cell–cell cohesion (Ng *et al* 2012) and increases the activity of Rac1 in mammary epithelial cells (Du *et al* 2012). These results suggest that epithelial cells can generate

increased contractility to stabilize cell–cell adhesions in the soft floating matrix impairing Rac1-dependent invasion. Hence, the inhibition of ROCK may provide the destabilization of cell–cell adhesions and reactivate Rac1 to induce protrusive invasion, which is consistent with a release of the cortical tension facilitating protrusive activity (Fischer *et al* 2009).

It has been found that enhanced 3D collagen matrix stiffness via non-enzymatic glycation induces an increasingly dispersive invasive phenotype in mammary epithelial cells (Carey *et al* 2017). Although the mechanisms by which increased 3D matrix stiffness downregulates cell–cell cohesion to induce the epithelial dispersion are not analyzed, it seems to be that Rho GTPases seem to fulfill a significant role in this behavior (Levental *et al* 2009, Provenzano *et al* 2009b, Godinho *et al* 2014, Lui *et al* 2012). Thus, the increasing 3D extracellular matrix stiffness alone seems to be sufficient for the induction of pronounced phenotypic alteration in epithelial cells, which is in contrast to a report in which both matrix stiffening and oncogene induction are needed for the invasive behavior (Levental *et al* 2009). However, the experimental differences including extracellular matrix composition may account for the observed differences, as well as the extent of collagen crosslinking (Levental *et al* 2009). However, these results suggest that additional events such as oncogene induction may be needed to fully drive the invasive epithelial behavior in response to smaller increases in stiffness of the extracellular matrix and/or in the presence a basement membrane. These results suggest that the extracellular matrix composition and mechanics co-regulate the mammary epithelial phenotype and hence further future studies are required to refine this relation.

11.7.2 Fibrosis models for cell culture: heterogeneous structure with homogeneous ligand

Although HA modifications for covalent crosslinking in extracellular matrices can inhibit the normal binding to cell–matrix receptors (figure 11.14) (Ropponen *et al* 1998), soft gels of crosslinked HA can also uniquely evoke the re-organization of some matrix macromolecules such as fibronectin into fiber-like regions (figure 11.14) (Kowal and Falk 2015). The detection of matrix heterogeneities has raised questions

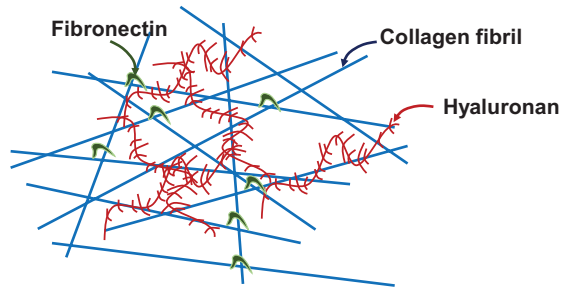

Figure 11.14. Hyaluronic acid and fibronectin crosslinked collagen networks.

about the effects of non-homogeneous gels on cellular functions. In order to decouple the effects of ligand density, which can be non-homogeneous and may lead to haptotaxis from the effects of non-homogeneous compliance, the mechano-sensing processes of adherent cells need be investigated. A heterogeneous matrix is regarded as a special feature of fibrosis, which is also frequently associated with solid tumors (Lee *et al* 2012). Fibrosis can also result from acute injury such as a heart attack (Berry *et al* 2006) or as chronic diseases such as liver cirrhosis or muscular dystrophy (Dingal *et al* 2015) and in addition it is often referred to as a scar. In particular, a scar can form locally in most or all tissues of higher animals and can be identified by a specific composition such as abundant crosslinked collagen I fibers that are heterogeneously distributed within a fibrotic tissue. Additionally, a scar is usually locally stiff and long-lasting (Berry *et al* 2006, Dingal *et al* 2015). With a focus on the cancer, cancer cells can respond to matrix stiffness, which leads to increased collagen deposition and crosslinking (fibrosis). A question still remains unanswered: where does the increased collagen come from?

A scar matrix is assembled largely *de novo* and a major role in the development of organ fibrosis is fulfilled by ubiquitous MSCs, which reside in perivascular niches of many organs such as heart, liver, kidney, lung and bone marrow (Kramann *et al* 2015). The MSCs proliferate and differentiate into multiple tissue lineages such as fat or bone. Moreover, genetic lineage tracing showed that tissue-resident MSCs (specifically the $Gli1^+$ MSCs), rather than circulating MSCs, proliferate after organ injury to produce myofibroblast-like cells that are typically found in scars. In mouse models, genetic ablation of these cells causes fibrosis and after induced heart failure, the heart also maintains an ejection fraction. Taken together, sophisticated, reductionist culture models with a scar-like heterogeneity are required to understand and regulate the sensitivity of human MSCs and other cell types towards matrix heterogeneity and fibrosis.

In order to identify the effects of non-homogeneous matrix stiffness on cells, a recently developed approach for the preparation of minimal matrix models of scars (MMMS), which are generated by mixing of soluble collagen I subunits with acrylamide monomers plus bis-acrylamide crosslinkers that are then polymerized to a gel (Dingal *et al* 2015). After the initiation of polymerization, the collagen I fibers phase separates from the pregelation clusters of polyacrylamide, which causes highly branched fractal fiber bundles that segregate as islands heterogeneously entrapped at

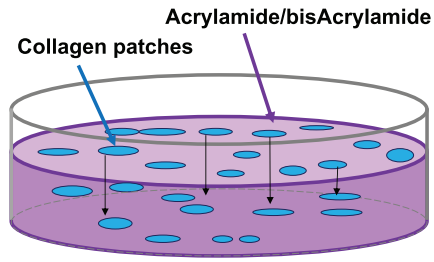

Figure 11.15. Scar-like collagen and PAA mixtures. Scar-like islands of the collagen type I are heterogeneously distributed with a soft PAA-hydrogel.

the subsurface of the hydrogel. It is important to notice that collagen in the subsurface fiber bundles is not accessible for the adhesion of cells. Moreover, a uniform overcoating of matrix ligand is required for cell adhesion. The preparation of this type of model scar could be seen as a diffusion limited cluster aggregation process, which reveals fractal sizes that could be easily regulated by varying the concentration of type I collagen. With the proper mixing ratios, a surface coverage of collagen fiber bundles of 30% approximates the extent of fibrosis, which has been detected in muscle cross-sections (Mann *et al* 2011, Smith and Barton 2014). Differences in the mechanoresponses have been found when MSCs are cultured in parallel on homogeneous gels and on MMMS. The stress-fiber-associated protein α-SMA has emerged as a key marker of fibrosis and scarring and although the expression of α-SMA is not unique to scarring, its expression levels are elevated with enhanced contractility (Wipff *et al* 2007). α-SMA is elevated *in vivo* in hepatic stellate cells such as liver MSCs and is associated with a paralleled stiffening of the toxin-injured liver, but it still precedes the occurrence of fibrotic collagen (Olsen *et al* 2011). Despite the soft-stiff heterogeneity of MMMS gels, MSCs have shown pronouncedly enhanced expression of α-SMA compared to the homogeneously low expression in MSCs on polyacrylamide gels still lacking the fiber islands (figure 11.15). However, the α-SMA expression has been observed to be more homogeneous between cells on MMMS than between cells on homogeneously stiff gels. This result can be explained by the increased presence of the transcription factor NKX2.5 that acts as a strongly cooperative repressor of α-SMA, which can exit the nucleus, when the cells are cultured on stiff substrates. As many applications may be interesting for investigating the scarring responses of cells, the effect of other cell types especially cancer cells, may be highly important in terms of the malignant progression of cancer due to stroma stiffening. As 80% of hepatocellular carcinomas are developed in the background of liver cirrhosis, it enhances the attention towards this co-occurrence (Davis *et al* 2008) and therefore MMMS seems to be a promising model to investigate the mechanotransduction of HCCs within the cirrhotic liver.

Since the biochemical signaling pathways involved in the malignant progression of cancer have been extensively analyzed, there is still much to reveal about the impact of the mechanical cues of extracellular matrix such as matrix stiffness, other microenvironmental factors, and their subsequent effects on cellular function and behavior. Tumors are commonly stiffer than normal healthy surrounding tissues, whereas they can even possess soft cores consisting of cells that are even softer than their normal counterparts. At first glance, these results seem to be contradictory, but it is hypothesized that cancer cells can generate traction forces on stiff substrates in order to migrate and at the same time they are required to be soft to squeeze through tight spaces to extravasate and subsequently metastasize.

The generalizations in cancer such as in the field of cell or tissue mechanics cannot be applied to every cancer cell type or to all the hallmarks of cancer. However, cancer cells often seem to possess a decreased mechanosensitivity and hence the stiffness of the substrate has solely a minor effect on spreading, cell–matrix adhesion, migration, proliferation and survival. In particular, apoptosis is preferentially detected on soft matrices, whereas the EMT and angiogenesis have been seen

identified on stiff matrices. Based on pharmacological and genetic manipulations, it has been shown that Ras, FAK and PI3K/ Akt signaling pathways are involved.

A variety of experimental approaches such as polyacrylamide or biopolymer gels with precisely tunable stiffness, have been employed frequently to investigate how cancer cells respond to the underlying stiffness of these substrates, which has been mostly determined by cell spread area, proliferation, migration, invasion, and apoptosis analysis. While the 2D substrates provide a simple method to analyze the effects of the substrate stiffness on cellular behavior, new approaches consisting of 3D scaffolds, micropillars, microfluidic devices, and heterogeneous matrices which mimic the tumor microenvironment will further unravel the role of mechano-transduction in the malignant progression of cancer.

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Claudia Tanja Mierke

Chapter 12

The impact of cells and substances within the extracellular matrix tissue on mechanical properties and cell invasion

Summary

The extracellular matrix of connective tissues represents the principal wires for mechanochemical interaction between tissues and cells. These matrix–cell interacting signals are communicated in a reciprocal way and play crucial roles in realizing stable tissue structure–function relationships and regulating cellular fate. In particular, the stiffness of the matrix is not solely a structural support for cells and tissues, it is also important in regulating a large number of cell activities, such as adhesion, proliferation, differentiation and migration. This chapter discusses the role of extracellular matrix embedded cells such as cancer-associated fibroblasts in providing the matrix's mechanical properties for cancer cells and their motility in 3D microenvironments. On the one hand, the effect of substances such as hyaluronan (which can associate with the extracellular matrix network) on cell functions is huge, including its interaction with the surface receptors of cancer cells (such as CD44) and its role as a key regulator of cancer cell migration and involvement in the malignant progression of cancer.

On the other hand, cells can sense the local forces exerted by surrounding extracellular matrix stroma and in turn they are able to alter the structure and composition of the local extracellular matrix. Many pathological conditions, such as tumorigenesis, metastasis, cardiovascular diseases and aging of connective tissues, initiate the occurrence of biochemical and mechanical changes within the extracellular matrix. However, the distinct mechanisms by which the mechanical properties of the extracellular matrix are affected and hence influence in turn cell and tissue functions remain to be determined, as the events associated with these processes span different length scales ranging from the tissue scale to molecular scale. Moreover, the extracellular matrix possesses extremely complex hierarchical 3D structures and

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there is a large interdependence of the extracellular matrix's composition, structure and mechanical properties. However, it is still not definitively known how mechanical forces can be transferred and transmitted within the extracellular matrix and how this is achieved from the tissue level to the cellular level. As the magnitude of the mechanical load at the tissue level is pronouncedly different from that experienced by a single cell and the differential mechanical load distribution is highly dependent on the architecture and mechanical properties of the extracellular matrix, precise knowledge of the transmission seems to be crucial in order to understand the functional regulation of cancer cell biochemical and mechanical properties by the extracellular matrix microenvironment.

12.1 The impact of tumor-associated fibroblasts on matrix mechanical properties

Syndecans are transmembrane receptors containing ectodomains modified by glycosaminoglycan chains. The ectodomains of syndecans are able to interact with a large number of molecules, including cytokines, growth factors, proteinases, adhesion receptors and extracellular matrix components. The four identified syndecans in mammals are found to be expressed in a development-, cell-type-, and tissue-specific manner and can act either as co-receptors with other cell surface membrane receptors or as independent cell–matrix adhesion receptors that facilitate cellular signal transduction pathways. Moreover, syndecans facilitate the regulation of cell proliferation and migration, angiogenesis, cell–cell and cell–extracellular matrix adhesion, and they also seem to be involved in the participation in several key tumorigenesis processes. In certain cancer types, the expression of syndecan controls the cancer cell proliferation, survival, adhesion, motility and other functions, and hence seems to be a reliable prognostic marker for tumor progression and patient survival. The short cytoplasmic tail of syndecans seems to be involved in these events by facilitating the recruitment of signaling partners. Moreover, the conserved carboxyl-terminal EFYA tetrapeptide sequence is present in all syndecans and can interact with some PDZ domain-containing proteins that are supposed to function as scaffold proteins, recruiting signaling and cytoskeletal proteins to the cell membrane. However, although there is growing knowledge of how these interactions are carried out at both the structural and biological levels, it has been shown that these interactions are highly complex. In particular, parameters altering the recruitment of PDZ domain proteins by syndecans, such as binding specificity and affinity, are the focus of active research in this field and are highly critical for revealing the underlying regulatory mechanisms. Indeed, it has been found that their binding may be affected by post-translational modifications altering the regulatory mechanisms, such as the phosphorylation of the syndecan cytoplasmic tail.

Stromal fibroblasts that locally surround breast carcinomas often express the cell surface proteoglycan syndecan-1 (figure 12.1). In human breast carcinoma samples, the stromal syndecan-1 expression correlates with an organized and parallel extracellular matrix fiber architecture. In order to reveal a possible link between stromal syndecan-1 and the fiber architecture of the extracellular matrix, bioactive

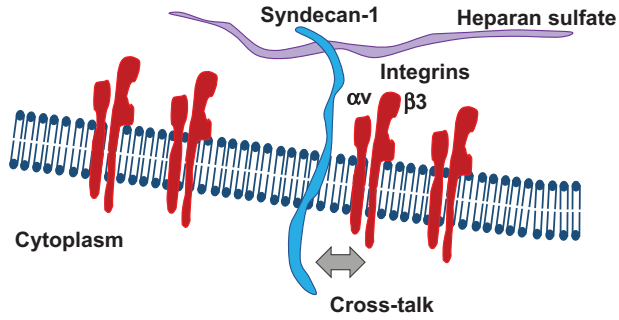

Figure 12.1. Structure of syndecan-1 and its association with integrins.

cell-free 3D extracellular matrices were prepared from cultures of syndecan-1-positive and syndecan-1-negative murine and human mammary fibroblasts (called extracellular matrix-syndecan-1 and extracellular matrix-mock, respectively). Indeed, extracellular matrix-syndecan-1 led to a parallel fiber architecture, whereas the extracellular matrix-mock provided a random fiber arrangement. When breast carcinoma cells were embedded into the fibroblast-free extracellular matrices, extracellular matrix-syndecan-1, but not extracellular matrix-mock, supported their adhesion, invasion and directional migration through the matrix. Moreover, the contribution of the structural or compositional alterations in extracellular matrix-syndecan-1 was investigated with respect to cancer cell behavior. By microcontact printing of cell culture surfaces, the syndecan-1-negative fibroblasts were forced to produce an extracellular matrix with parallel fiber organization, mimicking the architecture observed in extracellular matrix-syndecan-1. However, it was seen that the fiber topography governs the directionality of cancer cell migration. In contrast, an elevated fibronectin level in extracellular matrix-syndecan-1 was responsible for the enhanced adhesiveness of breast cancer cells. These findings suggest that syndecan-1 expression in breast-carcinoma-associated stromal fibroblasts supports the assembly of an architecturally abnormal extracellular matrix, which induces the breast carcinoma cell's directional migration and invasion.

Epithelial–stromal interactions seem to be crucial in directing mammary gland development and in maintaining normal tissue homeostasis. In contrast, during tumorigenesis, the stroma dramatically increases carcinoma growth and progression. In more detail, the predominant cell type within the stromal microenvironment is the fibroblast, which can synthesize, organize and maintain a 3D extracellular matrix network of glycoproteins and proteoglycans. It has been suggested that normal healthy stromal fibroblasts and their extracellular matrix provide an inhibitory constraint on tumor growth and progression (Bauer 1996, Kuperwasser *et al* 2004). In more detail, major alterations occur in the stromal fibroblasts and extracellular matrix during neoplastic transformation, indicating a permissive and supportive microenvironment for the development of carcinomas. In comparison with their quiescent normal fibroblast counterpart, carcinoma-associated fibroblasts exhibit an activated phenotype, which is characterized by the expression of smooth muscle markers, an enhanced proliferative and migratory capacity and altered gene

expression profiles. Carcinoma-associated fibroblasts produce and deposit elevated quantities and abnormal varieties of extracellular matrix components (Barsky *et al* 1984, Schor *et al* 2003, Tuxhorn *et al* 2002, Franco-Barrazza *et al* 2016). Recent evidence (Provenzano *et al* 2006, 2008b) indicates that the extracellular matrix composition and architecture are both altered in close proximity to carcinomas and that these alterations may lead to tumor progression. However, the contribution of these stromal modifications to the tumor development, the molecular mechanisms and the signal transduction events underlying these alterations is not yet well understood.

In general, syndecans are transmembrane proteoglycans that are frequently detected on the surface of many types of mammalian cells. In particular, the four identified syndecans in mammals are encoded by four genes, whereas invertebrates have just one syndecan. Based on chromosomal localization and exon organization studies, all syndecans arise from a single ancestral gene. Moreover, syndecans are found to be expressed in a development-, cell-type-, and tissue-specific manner and function either independently or co-receptor-dependently in order to facilitate cellular signal transduction processes (Bishop *et al* 2007, Multhaupt *et al* 2009).

In these type I transmembrane glycoproteins, the syndecan core proteins range in size from 20 to 45 kDa. In particular, syndecan core proteins contain an extracellular domain (ectodomain) that associates either with heparan sulfate only or with both heparan sulfate and chondroitin sulfate, a single transmembrane (TM) domain and a short cytoplasmic domain. The ectodomain can interact with a broad variety of molecules such as growth factors, cytokines, proteinases, adhesion receptors, and extracellular matrix components. Syndecan-1 has been found to be mainly expressed in mesenchymal and epithelial cells. Syndecan-2 has been detected to be highly expressed in endothelial and mesenchymal tissues as well as in liver, neural, and fibroblast cells. Syndecan-3, which is the longest of the four syndecans, is expressed in neural tissue and the developing musculoskeletal system, whereas it is not detectable in epithelial cells. Syndecan-4, which contains the shortest core protein, is widely expressed in multiple cell types. Moreover, syndecans regulate cell–cell, cell–pathogen and cell–matrix interactions through the assembly and recruitment of the actin cytoskeleton, cellular proliferation, differentiation and cellular migration. Syndecans can be detected in cell protrusions and focal adhesions, in which they colocalize with actin filaments (Granés *et al* 1999, Berndt *et al* 2004). An important role of syndecans is that they can serve as co-receptors for other cell surface receptors such as growth factor receptors and integrins (Morgan *et al* 2007, Couchman 2010, Rapraeger 2013). Moreover, syndecans can bind, immobilize, concentrate and evoke conformational alterations in growth factors, cell adhesion molecules and other molecules involved in signaling transduction processes through their heparan sulfate chains, which hence induces receptor interaction. In addition, syndecans can also impair the activation of ligands by protecting them or sequester them away from their membrane receptors (Zimmermann and David 1999, Alexopoulou *et al* 2007). In particular, syndecans can even undergo a controlled physiological shedding of their extracellular domain, which takes place increasingly under pathological conditions and thereby enables

them to function as soluble effectors and/or antagonists (Kim *et al* 1994, Manon-Jensen *et al* 2010). In addition, syntenins/syndecans, together with the syntenin-binding protein ALIX, seem to be involved in or increase the production of exosomes (Baietti *et al* 2012, Friand *et al* 2015).

Syndecans belong to a family of transmembrane heparan sulfate proteoglycans with four identified members, for example syndecans-1 to -4. Through their heparan sulfate glycosaminoglycan (HS-GAG) chains, syndecans can interact with a wide variety of proteins, such as growth factors and extracellular matrix constituents (Lopes *et al* 2006, Tkachenko *et al* 2005, Zimmermann and David 1999). Thus, they have a role in cell growth, adhesion, migration and morphogenesis. In more detail, it has been suggested that syndecan-2 is required for the assembly of laminin and fibronectin into a fibrillar matrix (Klass *et al* 2000). Moreover, syndecan-4 has also been shown to participate in fibronectin matrix assembly. In line with this, concomitant engagement of syndecan-4 and integrins triggers Rho GTPase and focal adhesion kinase (FAK) activity, which is crucial for efficient initiation of fibronectin matrix assembly (Saoncella *et al* 1999, Wilcox-Adelman *et al* 2002, Ilic *et al* 2004, Wierbicka-Patynowski *et al* 2002). However, syndecan-1 is expressed primarily by the epithelial and plasma cells of healthy adult tissue (Sanderson *et al* 1992). An induction of syndecan-1 expression in stromal fibroblasts of invasive breast carcinomas has been observed (Maeda *et al* 2004, Stanley *et al* 1999). Syndecan-1, which is aberrantly expressed by stromal fibroblasts in breast carcinomas, participates in reciprocal carcinoma growth, which means that it promotes the feedback loop, requiring proteolytic shedding of its ectodomain (Maeda *et al* 2004, 2006, Su *et al* 2007). Although the role of syndecan-1 in the assembly of the extracellular matrix has not yet been revealed, syndecan-1 has been shown to interact with several extracellular matrix components, such as fibronectin, fibrillar collagens, laminin, vitronectin, thrombospondin and tenascin (Lopes *et al* 2006, Tkachenko *et al* 2005, Zimmermann and David 1999).

Thus, the role of syndecan-1 expression by stromal fibroblasts has been explored and it has been suggested that syndecan-1 may be functionally involved in the altered matrix production present around tumors, the so-called tumor stroma. It has been observed that in mammary stromal fibroblasts, syndecan-1 facilitates extracellular matrix assembly and thus determines extracellular matrix fiber architecture (Yang *et al* 2011). Moreover, it has been shown that cell-free 3D extracellular matrices produced by syndecan-1 expressing fibroblasts provide the directional migration of mammary carcinoma cells and link this activity to the parallel fiber architecture (Yang *et al* 2011).

It has been observed that syndecan-1 is aberrantly expressed by stromal fibroblasts in most infiltrating breast carcinomas (Maeda *et al* 2004, Stanley *et al* 1999). It has been shown that syndecan-1 expression in stromal fibroblasts induces and promotes breast carcinoma growth and angiogenesis (Maeda *et al* 2004, 2006, Su *et al* 2007). How stromal syndecan-1 alters the extracellular matrix composition and architecture *in vivo* and *in vitro*, with the altered extracellular matrix fiber architecture subsequently supporting the directional migration of breast carcinoma cells, is discussed.

Syndecans have been associated with cancers, infectious diseases, obesity, wound healing and angiogenesis. Moreover, syndecans are widely considered as key regulators of tumor progression (Barbouri *et al* 2014, Couchman *et al* 2015, Theocharis *et al* 2015). In certain cancers, the syndecan expression seems to regulate the function of cancer cells and serves as a reliable prognostic marker for the progression of tumors and the survival of the patient. Syndecan-1 expression is dysregulated in a wide variety of cancers, including breast, ovarian, head and neck, and colorectal carcinomas (Teng *et al* 2012). Moreover, syndecan-1 functions as a tumor suppressor in human MDA-MB-231 breast cancer cells (Hassan *et al* 2013). When these cells are impaired in syndecan-1 expression through treatment with small interfering RNA, the β1 integrin and focal adhesion kinase activities are increased and cause elevated cellular adhesion and subsequently migration, whereas these cells show enhanced cellular resistance to irradiation. It has been reported that re-invasive breast cancer displayed an inverse correlation between the expression of syndecan-1 and the pro-metastatic microRNA miR-10b, which seems to be a promising novel type of post-transcriptional regulation of syndecan-1 (Hannafon *et al* 2011). In line with this, it has been demonstrated through the negative regulation of syndecan-1 by miR-10b and its invasion-promoting effect in human breast cancer cells, that syndecan-1 is a new regulatory target of miR-10b (Ibrahim *et al* 2012). Moreover, other studies showed that syndecan-1 reduces cell migration in lung epithelium through the activation of Rap1, which decreases the speed of the focal adhesion disassembly and hence its turnover (Altemeier *et al* 2012). In addition, syndecan-1 functions in squamous cell carcinoma collagen-facilitated motility and invasion by altering RhoA and Rac activity, which leads to the suggestion that reduced syndecan-1 expression during carcinoma progression can increase the invasive capacity of cancer cells (Ishikawa and Kramer 2010).

The presence of syndecan-1 is correlated with favorable outcomes in both lung cancer and mesothelioma (Kumar-Singh *et al* 1998, Anttonen *et al* 2001) and a loss of syndecan-1 represents a marker of hepatocellular carcinoma with high metastatic potential (Matsumoto *et al* 1997). In line with this, low syndecan-1 expression is associated with gastric carcinoma invasion and subsequently metastasis (Chu *et al* 2008). In contrast, it has also been found that high expression levels of syndecan-1 in breast carcinoma are correlated with high histological grade, high mitotic count, large tumor size, c-erbB-2 overexpression, and estrogen receptor-negative status. Moreover, these findings indicate that high syndecan-1 expression is related to most invasive breast carcinomas (Stanley *et al* 1999, Barbareschi *et al* 2003, Leivonen *et al* 2004, Lendorf *et al* 2011). By using an *in vitro* breast cancer model, it has been proposed that syndecan-1 is involved in the spreading and adhesion of cancer cells (Beauvais and Rapraeger 2003). Moreover, in prostate cancer, a high level of syndecan-1 expression is an indicator for the malignant and aggressive progression of cancer (Zellweger *et al* 2003). As reported in some review articles, the stromal expression of syndecan-1 seems to be a negative prognostic marker for cancer progression and hence elevated serum levels of the shed syndecan ectodomain may serve as a prognostic indicator (Gharbaran 2015, Szatmári *et al* 2015). In particular, a mechanism has been provided by which syndecan-1 and -4 ectodomains can

capture and promote the autophosphorylation of the tyrosine kinase receptors HER-2 and EGFR, respectively, which causes an integrin-dependent migration of cancer cells (Wang *et al* 2014a, 2015).

In addition to its cytoplasmic localization, syndecan-1 has been observed to localize in the nucleus, where it is supposed to fulfill the function of a transcription factor and hence may alter the gene transcription in order to regulate cancer pathogenesis (Brockstedt *et al* 2002). Moreover, heparanase and syndecan-1 can cooperate to facilitate growth factor signaling and cellular behavior such as providing increased tumor growth and cancer cell dissemination from the primary tumor (Ramani *et al* 2013, Palaiologou *et al* 2014, Roucourt *et al* 2015). It has been reported that syndecan-4 impairs the invasion of breast carcinoma cells (Beauvais and Rapraeger 2003) and hence its expression in human breast carcinoma has been found to be associated with good prognosis of cancer (Lendorf *et al* 2011).

In contrast, another study has found that the expression of syndecan-4 correlated significantly with a high histological grade of the tumor and a negative estrogen receptor status (Baba *et al* 2006) and hence has been regarded as a marker of poorer prognosis. In addition, in pancreatic cancer it has been revealed that syndecan-2 is associated with perineural invasion of pancreatic adenocarcinoma cells (De Oliveira *et al* 2012). The silencing of syndecan-2 expression within these cells significantly impaired the motility and invasiveness of cancer cells. In line with this, syndecan-2 has been detected to be increased expressed in breast tumors (Lim *et al* 2015) and in colon carcinomas (Park *et al* 2002, Ryu *et al* 2009, Choi *et al* 2010). Moreover, in highly metastatic colorectal cancer cells, the expression of syndecan-2 is increased by fibronectin, which is secreted by tumor surrounding stromal cells (Vicente *et al* 2013).

In colorectal carcinoma, the low epithelial expression of syndecan-1 is correlated with a higher histological grade, which is associated with a more advanced clinical stage of the patients and with possibly more unfavorable prognosis (Lundin *et al* 2005, Hashimoto *et al* 2008, Mitselou *et al* 2012). The results from a recent meta-analysis of colorectal cancer studies revealed that a loss of syndecan-1 expression in colorectal cancer is associated with the histological grade and tumor stage, but not with the involvement of the lymph nodes or with distant metastasis in targeted organs or tissues (Wei *et al* 2015). Moreover, it has been found that syndecan-1 expression does not possess prognostic value in colorectal carcinoma patients. Until now, syndecan-3 has not yet been associated with cancer. However, although the mechanisms are not yet clearly understood, several studies have determined the critical role of syndecans in malignant tumor progression and it has even been hypothesized that they are highly relevant and hence they represent suitable therapeutic targets (Ramani *et al* 2013, Barbouri *et al* 2014, Theocharis *et al* 2015). For instance, the anti-tumoral activity of zoledronic acid on human breast cancer cells has been shown to correlate with a differential alteration of syndecans (Dedes *et al* 2012). Synstatin peptides, which are based on HER-2 and EGFR interaction motifs on syndecan-1 and -4, respectively, are able to competitively interfere with the receptor tyrosine kinase interaction and thereby disrupt the activation of cellular motility (Wang *et al* 2015). Similar peptides have been generated to impair

IGF1R binding to the syndecan-1/$\alpha v\beta 3$ integrin complex and thereby diminish the integrin activity in endothelial and cancer cells (Rapraeger 2013).

The extracellular matrix provides a complex macromolecular network of glycoproteins and proteoglycans that is necessary for cell survival, proliferation, migration and differentiation. During cancer cell invasion, the extracellular matrix may be subject to extensive alterations due to the abnormal synthesis of extracellular matrix components and their proteolytic remodeling (Schor *et al* 2003, Wilhelm *et al* 1988). Extensive accumulation of the extracellular matrix protein fibronectin has been detected in the stroma of a variety of solid human tumors (Wilhelm *et al* 1988, Moro *et al* 1992) and a linear function between the fibronectin content and tumor stage or adverse outcome has been demonstrated (Yang *et al* 2011). Indeed, these results support the hypothesis that the fibronectin production in stromal fibroblasts seems to be regulated by syndecan-1. Fibronectin facilitates the attachment of breast carcinoma cells to the extracellular matrix, while it does not stimulate their migratory behavior. However, this observation in 3D matrices is in contrast to the biphasic relationship between migration velocity and adhesion molecule (such as fibronectin and laminin concentration observed under traditional 2D conditions) and may thus reflect the importance of the topographic presentation of adhesion ligands to the cell in the 3D microenvironment (DiMilla *et al* 1993, Goodman *et al* 1989, Palecek *et al* 1997).

In contrast to the intersecting meshwork of the extracellular matrix produced by syndecan-1-negative fibroblasts, fibronectin and collagen I fibers in extracellular matrix-syndecan-1 are organized in parallel patterns. Indeed, it has been observed that a parallel fiber arrangement is a characteristic feature of an extracellular matrix evoked by primary carcinoma-associated fibroblasts of the skin (Amatangelo *et al* 2005). However, the consequences of this parallel fiber arrangement for cancer cell behavior are elusive and require further investigation. In particular, the extracellular matrix architecture has been characterized as a functional determinant of the directional cell migration. The parallel fiber architecture produced by syndecan-1-positive fibroblasts tends to reflect on the collagen fiber signature identified in the transgenic Wnt-1 mouse mammary tumor model (Provenzano *et al* 2006). In this study, the parallel collagen fibers perpendicular to the advancing edge of the tumors were described, and these were spatially associated with carcinoma cells invading singly or collectively into the extracellular matrix of connective tissue. Because of the inherent limitations of the model system, it is not possible to judge whether the invasion is a consequence or the cause of the parallel fiber arrangement.

Thus, the molecular mechanism through which stromal syndecan-1 affects extracellular matrix assembly needs to be elucidated. An intact fibronectin matrix seems to be essential for the assembly and stability of a mature collagen-containing extracellular matrix (Sottile and Hocking 2002, Velling *et al* 2002). Thus, knowledge of the regulation of the fibronectin fibril assembly may lead to the precise understanding of the extracellular matrix organization. Fibronectin fibrillogenesis is a complex cell-facilitated process that engages the fibronectin-binding to cell surface receptors, fibronectin–fibronectin self-association and its interaction with the actin cytoskeleton (Wierzbicka-Patynowski and Schwarzbauer 2003, Mao *et al* 2005).

Fibronectin is secreted as tightly folded, disulfide-bonded dimers consisting of three types of repeating modules, such as types I, II and III. In particular, these fibronectin dimers are initially inactive until they interact with their specific integrins and other receptors displayed at the cell surface. In more detail, this binding interaction induces intracellular signal transduction pathways, supports rearrangements of the actin cytoskeleton and may cause conformational alterations in fibronectin that change the inactive fibronectin molecule into an extended and active form. Stromal-derived syndecan-1 seems to regulate the serial steps of the fibronectin fibrillogenesis, such as fibronectin fibril initiation and elongation. In more detail, the syndecan-1 regulates the activity of several integrins, including $\alpha v\beta 3$, $\alpha v\beta 5$ and $\beta 4$ (Beauvais *et al* 2004, 2009, McQuade *et al* 2006, Ogawa *et al* 2007). Although the fibronectin matrix assembly appears to be induced mainly by Arg–Gly–Asp-binding (RGD-peptide-binding), integrin $\alpha 5\beta 1$ (Fogerty *et al* 1990, Wu *et al* 1993) and alternative fibronectin-binding integrin receptors, such as $\alpha v\beta 3$, $\alpha 4\beta 1$ and $\alpha v\beta 1$, can also support this process when properly activated (Wu *et al* 1995, Yang and Hynes 1996, Wu *et al* 1996, Wennerberg *et al* 1996, Sechler *et al* 2000). However, it is possible that syndecan-1 induction activates integrins other than $\alpha 5\beta 1$ and hence initiates an alternative assembly pathway, thus resulting in an extracellular matrix that is structurally and compositionally different from an extracellular matrix assembled under the stringent control of $\alpha 5\beta 1$. Moreover, it seems to be possible that stromal syndecan-1 directly facilitates the fibronectin matrix assembly. The compact conformation of fibronectin dimers is provided by intramolecular interactions involving the type III12–14 repeats of fibronectin (Johnson *et al* 1999, Hynes 1999). In particular, the type III12–14 repeats (termed heparin II binding domain) are able to interact with the HS chains from various members of the syndecan family (Tumova *et al* 2000). In turn, the binding of syndecan-1 to heparin II can facilitate the unfolding of dimeric fibronectins and hence expose fibronectin self-assembly (fibronectin-binding) sites, consequently promoting fibronectin deposition and fibrillogenesis.

There is strong evidence supporting the importance of the mechanical properties of the extracellular matrix in the behavior of breast cancer cells. It has been shown that dense rigid mechanical properties suppress tubulogenesis and possibly stimulate invasion of well-differentiated breast carcinoma cells in collagen gels by inducing the activity of the small GTPase Rho (Wozniak *et al* 2003, Paszek *et al* 2005). In addition, the cell-derived extracellular matrices seem to be more suited than the basement membrane or collagen gels to mimic 3D matrix effects on the behavior of breast cancer cells (Green and Yamada 2007). Thus, a model is favored in which the fiber topography rather than the matrix rigidity governs the cell invasion in fibroblast-derived matrices. A dissection of the molecular mechanism of cell migration regulation by parallel extracellular matrix fibers and their orientation may involve Rac1, which is a member of the Rho family of GTPases. Indeed, the inhibition of Rac1 leads to a switch in the migration mode of fibroblasts and epithelial cells from a random to a more directionally persistent migration mode (Pankov *et al* 2005). The induction of cell migration is needed for cancer cell invasion and metastasis (Vicente-Manzanares *et al* 2005, Ridley *et al* 2003,

Zijlstra *et al* 2008) and thus it seems plausible that extracellular matrices with parallel fiber organization facilitate cancer cell spread and invasiveness. Taken together, a novel pathway has been discovered for how aberrant expression of syndecan-1 in stromal fibroblasts is able to increase cancer progression.

The components that comprise the extracellular matrix are integral to normal tissue homeostasis and the development and progression of breast tumors. In particular, the secretion, construction and remodeling of the extracellular matrix are regulated by a complex interplay between at least three different cell types: cancer cells, fibroblasts and macrophages. Transforming growth factor-β (TGF-β) is an essential molecule in facilitating the cellular production of extracellular matrix molecules and, moreover, providing the adhesive interactions of cells with the extracellular matrix. In more detail, hypoxic cell signals (caused by oxygen deprivation), the presence of additional metabolic factors and receptor activation are associated with extracellular matrix structural architecture and consequently the progression of breast cancer. However, it has been suggested that both TGF-β and hypoxic cell signals play a key role in the functional and morphological alterations of cancer-associated fibroblasts (CAFs) and tumor-associated macrophages. In line with this, the increased recruitment of tumor and stromal cells in response to hypoxia-induced chemokines leads to enhanced deposition and remodeling processes for the extracellular matrix, elevated formation of new blood vessels through the induction of neoangiogenesis within the endothelial cell lining, and increased migration of cancer cells. Thus, greater knowledge of the collaborative interactive networks between cancer and stromal cells in response to the combined signals of TGF-β and hypoxia may reveal insights into the treatment parameters for targeting both cancer and stromal cells.

The extracellular matrix consists of approximately 300 proteins that facilitate organogenesis, tissue homeostasis and the progression of inflammation and disease (Hynes and Naba 2012, Tlsty and Coussens 2006). In human cancers, tumor initiation, proliferation, migration and metastasis are associated with the composition of the matrix (Egeblad *et al* 2010, Tlsty and Coussens 2006). However, increased production and the deposition of extracellular matrix proteins within the extracellular microenvironment are identified risk factors in human breast cancers (Keely 2011). This dysregulated activity is caused by deviant tumor and/or stromal cell function and finally leads to the formation of an altered matrix displaying fibrotic, stiff and dense properties (Levental *et al* 2009).

The extracellular matrix in normal mammary development and breast cancer tumorigenesis is highly regulated by cytokine TGF-β (Moses and Barcellos-Hoff 2011, Silberstein *et al* 1992). In particular, TGF-β also facilitates mammary morphogenesis by inhibiting mammary lateral branching and ductal growth (Lanigan *et al* 2007) and increases tumor growth via dysregulated cell signal transduction pathways inhibiting then cell cycle arrest (Donovan and Slingerland 2000). However, the aberrant functions of TGF-β during breast cancer development seem to be linked to the disease progression into various metastatic sites, such as the brain (Dobolyi *et al* 2012), liver (Karkampouna *et al* 2012), lung (Bartram and Speer 2004) and skeletal bone (Janssens *et al* 2005), which additionally require TGF-β

during the formation of secondary tumors, the so-called metastases. However, site-specific organotropism is a complex process involving not only TGF-β, but also the tumor cell genotype and tumor–stroma interactions at the primary site, as well as at the targeted organ (Eckhardt *et al* 2012; Ganapathy *et al* 2012, Lu and Kang 2007). The various phenotypes of each metastatic site in combination with TGF-β have been investigated (Drabsch and ten Dijke 2011, Eckhardt *et al* 2012, Lu and Kang 2007, Nishizuka *et al* 2002).

In particular, TGF-β ligands are members of the TGF-β superfamily, consisting of more than 25 closely related proteins, such as growth differentiation factors (GDFs), bone morphogenetic proteins (BMPs), activins and inhibins (Kingsley 1994). The three isoforms of TGF-β—TGF-β1, TGF-β2 and TGF-β3—have been revealed in humans, and each of these molecules as well as their associated receptors have been characterized in human breast tissue cancer and stromal cells (Chakravarthy *et al* 1999). More precisely, TGF-β ligands are secreted from cells as an inactive homodimer bound non-covalently to a latency-associated peptide (LAP) that is facilitated by a disulfide bound to the latent TGF-β binding protein (LTBP) (Horiguchi *et al* 2012). After the release of this large latent complex, the tissue transglutaminase-2 (TG2) enzymatically crosslinks fibrillar proteins in the extracellular matrix (such as fibrillin and fibronectin) to LTBP and the associated LAP. The TGF-β complex then becomes bound to the extracellular matrix in a still-inactive form (Nurminskaya and Belkin 2012, Zilberberg *et al* 2012). The active form of TGF-β can be released through integrin-facilitated mechanical deformation of the extracellular matrix and/or through the degradation of LAP using cellular proteases such as MMPs, thrombospondin-1 and plasmin. However, the release of active TGF-β in turn induces adhesive interactions via the cell signals that regulate integrin expression and the production of additional extracellular matrix proteins and TGF-β molecules (Chandramouli *et al* 2011, Horiguchi *et al* 2012).

Cell surface integrins are transmembrane receptors that facilitate cell–extracellular matrix interactions in tissue homeostasis, disease formation and immunity (Luo *et al* 2007). Each integrin receptor consists of a heterodimer, composed of one α and one β subunit and the extracellular and intracellular microenvironments are connected by binding to special ligands outside the cell and cytoskeletal components underneath the integrins assembled in focal adhesions, which facilitate this link (such as vinculin and focal adhesion kinase) (Mierke *et al* 2008, 2010, Mierke 2013, Berman *et al* 2003, Luo *et al* 2007, Mierke *et al* 2017). Regarding the 24 known integrin heterodimers, it has been observed that the extracellular matrix ligands predominantly bind to the α subunit and thus activate intracellular signaling events via the β subunit (Hehlgans *et al* 2007). Indeed, subsequent conformational alterations and integrin clustering, which may even include cell surface TG2-to-integrin binding interactions, build a 3D matrix adhesion signaling complex that seems to be involved in tumor proliferation and migration, as well as matrix deposition and remodeling (Keely 2011, Nurminskaya and Belkin 2012, Provenzano *et al* 2009b, Wozniak *et al* 2003). The expression of the integrin subunits (such as α5, αv, β1, β3 and β5) that bind the extracellular matrix is also enhanced by TGF-β cell signals, and ligation of these integrins by their specific

ligands ($\alpha 2\beta 1$:collagen, $\alpha 5\beta 1$:fibronectin, $\alpha v\beta 3$ or $\alpha v\beta 5$:periostin) and in turn induces the production of TGF-β, leading to a feed-forward loop between cancer cells and the extracellular matrix (Bianchi-Smiraglia *et al* 2012, Garamszegi *et al* 2009, Kudo 2011, Margadant and Sonnenberg 2010, Soikkeli *et al* 2010).

The extracellular matrix in human primary breast cancers (such as ductal and non-ductal) includes higher protein levels of collagen I, III and IV, fibronectin, periostin, tenascin-C and vitronectin compared to normal healthy breast tissue (Aaboe *et al* 2003, al Adnani *et al* 1987, Gould *et al* 1990, Guttery *et al* 2010, Kadowaki *et al* 2011, Kharaishvili *et al* 2011, Vasaturo *et al* 2005, Zhang *et al* 2010). Indeed, these proteins are all regulated by TGF-β (Garamszegi *et al* 2009, Grande *et al* 1997, Guttery *et al* 2010, Koli *et al* 1991, Kudo 2011, Margadant and Sonnenberg 2010, Soikkeli *et al* 2010) and are produced by various cell types, such as breast cancer cells (collagen I and IV, fibronectin, periostin and tenascin-C), fibroblasts (collagen I and III, fibronectin, periostin and tenascin-C), endothelial cells (collagen IV), macrophages (fibronectin and tenascin-C) and hepatocytes (vitronectin) (Arancibia *et al* 2013, Goh *et al* 2010, Guttery *et al* 2010, Hielscher *et al* 2012, Kleinman *et al* 1981, Philippeaux *et al* 2009, Preissner 1991, Shao *et al* 2004, Taylor-Papadimitriou *et al* 1981). The adhesive interactions among these extracellular matrix proteins favor their co-localization (Kudo 2011).

The extracellular matrix is further altered by enzymatic crosslinking of collagen through molecules commonly expressed in breast cancer, such as TG2 and lysyl oxidase (LOX), which finally generates a stiff matrix (Barker *et al* 2012, Jiang *et al* 2003, Levental *et al* 2009, Nurminskaya and Belkin 2012, Taylor *et al* 2011). These molecules are additionally regulated by TGF-β, as well as hypoxic cell signals (Barker *et al* 2012, Nurminskaya and Belkin 2012). Interestingly, in a murine model of breast cancer metastases to the bone, small molecule inhibition of hypoxia (2-methoxyestradiol) or TGF-β (SD-208) reduced osteolytic lesions and increased the survival of mice compared to their healthy controls. The combined inhibition of both factors induced a synergistic response that was potentially regulated by cancer cell vascular endothelial growth factor (VEGF) production and the CXCR4 chemokine receptor expression, as these two end-points synergistically decreased in response to both inhibitors *in vitro* (Dunn *et al* 2009). Taken together, TG2, LOX and additional TGF-β cell signal end-points associated with the extracellular matrix and tumorigenesis may also be synergistically increased by hypoxia.

The reduction of oxygen/perfusion within the local tumor microenvironment produces hypoxia and consequently activates the hypoxia inducible factors (HIFs) (Porporato *et al* 2011). It has been suggested that these factors are also induced by receptor-facilitated cell signals, such as insulin, growth factors as well as cytokines, increased free radical production and cellular alterations in iron and/or metabolic homeostasis (Cascio *et al* 2008, Knowles and Harris 2001, Lopez-Lazaro 2009, Schulze and Downward 2011, Selak *et al* 2005, Spangenberg *et al* 2006, Thornton *et al* 2000). However, increased activation of HIF genes redirects the cellular metabolism away from oxidative phosphorylation to aerobic glycolysis and finally leads to the production of lactate (Porporato *et al* 2011). These metabolic alterations may help us to fully understand the increased breast cancer risks evoked by

premenopausal iron deficiency, postmenopausal obesity, hyperinsulinemia and even iron overload (Braun *et al* 2011, Jian *et al* 2011, Rose and Vona-Davis 2012).

HIFs are activated in metabolic disorders such as obesity and it has been revealed that obesity is a poor prognostic indicator in patients diagnosed with breast cancer (Braun *et al* 2011, von Drygalski *et al* 2011). As a characteristic feature of various solid tumors such as breast cancer, the expression of HIFs strongly increases metastasis, resists radiation/chemotherapeutic therapy and offers poor patient prognosis (Charpin *et al* 2012, Fokas *et al* 2012). The tumorigenic potential of HIFs involves the cancer and stromal cell production of hypoxia-induced growth factors (Krock *et al* 2011). The elevated production of growth factors and their associated receptor-facilitated cell signals alter cancer and stromal cell affinity and avidity for the extracellular matrix and provide immune tolerance, induce angiogenesis and finally support metastatic disease (Chouaib *et al* 2012, Hood and Cheresh 2002). Hence, tumor progression is a collaborative effort between cancer and stromal cells, such as fibroblasts and macrophages, which are located within a hypoxic microenvironment.

The effect of hypoxia on breast cancer cells

The hypoxia marker HIF-1α has been detected and characterized in primary human ductal carcinomas and elevated levels of HIF-1α correlate significantly with an unfavorable outcome for patients (Brito *et al* 2011, Charpin *et al* 2012). The additional identification of HIF-1α in circulating cancer cells isolated from the peripheral blood of metastatic breast cancer patients (Kallergi *et al* 2009) further supports the idea of an association between hypoxic marker concentration and cancer cell migration. Nonetheless, a migratory mechanism can involve the action of the fibronectin-associated integrin $\alpha5\beta1$. Indeed, the increased expression of this dimer pair has been observed to occur in response to oxygen deprivation and human epidermal growth factor receptor-2 (HER-2)-induced HIF activation (Spangenberg *et al* 2006). Increased expression of $\alpha5\beta1$ integrin compared to other integrins could thus fundamentally alter cancer cell-to-extracellular matrix interactions and finally alter the associated cancer cell migratory patterns (Mierke *et al* 2011, Mierke 2013).

In addition, the productions of TGF-β and fibronectin are also elevated by interleukin-19 (IL-19), which is a cytokine that can be induced by hypoxia in the 4T1 murine mammary tumor cell line (Hsing *et al* 2012). Moreover, similar to HIF-1α, the expression of IL-19 has been identified as a poor prognostic indicator in invasive ductal carcinoma patients (Hsing *et al* 2012). There is indeed evidence that hypoxia and TGF-β independently regulate $\alpha5\beta1$ expression (Bianchi-Smiraglia *et al* 2012, Margadant and Sonnenberg 2010, Spangenberg *et al* 2006) and this suggests that the HIF transcriptional factors may cooperate together synergistically with TGF-β (or other associated mediators, such as IL-19, HER-2 ligands and estrogen), which was also reported for the chemokine receptor, CXCR4 (Dunn *et al* 2009).

Although the activation of HER-2 has been shown to elevate the expression of $\alpha5\beta1$ (Spangenberg *et al* 2006), similar effects have not yet been observed for the expression of the $\alpha2$ component of the collagen/laminin receptor, $\alpha2\beta1$ (Ye *et al* 1996). Indeed, the $\alpha2$ integrin has been identified as a tumor metastasis suppressor in

a murine model of breast cancer (Ramirez *et al* 2011). *In vitro* examination of human breast cancer cell lines indicates that the expression of $\alpha 2$ seems to be dependent on estrogen and progesterone cell signals (Lanzafame *et al* 1996), suggesting that the phenotype may be common to HER-2^+, estrogen receptor $(ER)^-$ and progesterone receptor $(PR)^-$ cancers. Moreover, hypoxia-related breast cancer studies regarding $\alpha 2$ and other integrins of interest (αv, $\beta 3$ and $\beta 5$) have not changed significantly at the present time. However, it has been revealed that in human mesenchymal stem cells hypoxia induces the expression of various integrins ($\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 11$, αv, $\beta 1$ and $\beta 3$), but notably not the expression of the collagen/laminin integrin, $\alpha 2$ (Saller *et al* 2012). These data suggest that hypoxia may differentially direct migration, depending on the individual genotype and possibly via substrates other than collagen. Indeed, this is supported by a study that examined human breast cancer cells (triple-negative (HER-2^-, ER^-, PR^-) MDA-MB-231 cells) cultured in the mammary fat pad of severe combined immunodeficient mice (SCID mice, humanized), in which identified hypoxic regions displayed significantly fewer and less dense collagen type I fibers (Kakkad *et al* 2010).

In contrast, other studies noted increased collagen surrounding mammary tumors overall (Iacobuzio-Donahue *et al* 2002). Alterations to the tumor-associated matrix, including increased alignment of collagen fibers and matrix stiffness identified in murine models and human breast cancer tissue (Conklin *et al* 2011, Provenzano *et al* 2006), may be due to the hypoxic induction of MMPs, TG2 and LOX by cancer cells (Barker *et al* 2012, Choi *et al* 2011, Munoz-Najar *et al* 2006, Nurminskaya and Belkin 2012). Additionally, it should be noted that all these studies differ from a study in which the assessment of collagen deposition was performed in a model using immunocompetent animals (Kakkad *et al* 2010). Thus, the effect of hypoxia on collagen deposition seems to be facilitated by immune cells.

As the breast cancer cell triggered production of MMP-9 is additionally regulated by fibronectin adhesion and this interaction is then enhanced by LOX cell signals (Maity *et al* 2011, Zhao *et al* 2009), hypoxia supports forward processes initiated by oncogenes. Additional cancer cell facilitated production of chemokines such as endothelin-2 (ET2), chemokine C–C motif ligand 5 (CCL5) (Grimshaw *et al* 2002a, Lin *et al* 2012) and growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and connective tissue growth factor (CTGF) (Dunn *et al* 2009, Kondo *et al* 2002, Le and Corry 1999) in response to hypoxia alter the recruitment and activity of stromal cells that subsequently affect tumorigenesis.

The effect of hypoxia on fibroblasts

Fibroblasts are a pervasive and diverse population of cells that produce extracellular matrix proteins and maintain the extracellular matrix in normal tissue homeostasis, playing an active role in the wound-healing response and tumorigenesis (figure 12.2) (Sorrell and Caplan 2009). Comparison of fibroblasts isolated from normal tissue with human breast cancer tissue revealed that the CAFs contained a subpopulation of fibroblasts and myofibroblasts which could react to increase tumor cell growth, encourage tumor vascularization, exhibit increased collagen contractility and release higher levels of the chemokine stromal cell-derived factor-1 (SDF-1) compared to

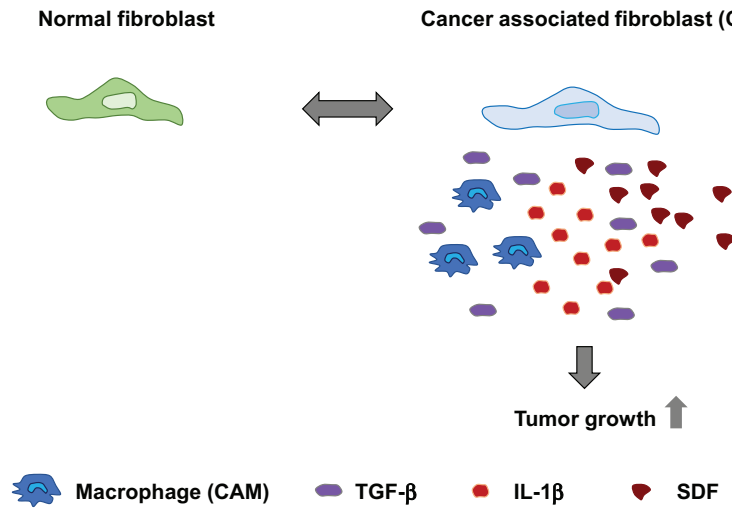

Figure 12.2. Effect of hypoxia in fibroblasts. Cancer associated fibroblasts (CAFs) encompass a subpopulation of fibroblasts and myofibroblasts, and they are exposed to hypoxia that induces the production of cytokines in cancer associated macrophages (CAMs).

normal fibroblasts (Orimo and Weinberg 2006, Shimoda *et al* 2010). Autocrine TGF-β and SDF-1, which are both cytokines found to increase in close proximity to tumors, evoked cell signals that support the differentiation of primary human breast normal fibroblasts to CAFs (Kojima *et al* 2010). In human synovial fibroblasts, hypoxic conditions can facilitate the release of SDF-1 and the cytokine interleukin-1β (IL-1β), which activates both the SDF-1 and HIF-1α genes in these same cells (Hitchon *et al* 2002, Thornton *et al* 2000). Indeed, the expression of IL-1β has been detected in human breast cancer tissue (Jin *et al* 1997, Kurtzman *et al* 1999) and linked to production by either cancer cells or macrophages located in the tumor microenvironment (Jin *et al* 1997). Thus, cytokines such as TGF-β and IL-1β that are secreted by cancer cells and cancer-associated macrophages may encourage normal fibroblasts to CAF differentiation, which is then further enhanced by hypoxia.

In a murine xenograft model involving human breast cancer cells (MDA-MB-231) and immortalized fibroblast (hTERT-BJ1) cell lines, the ectopic expression of HIF-1α in fibroblasts dramatically enhances the tumor growth through a mechanism that may support the transport of fibroblast metabolites such as lactate and pyruvate to the adjacent cancer cells (Chiavarina *et al* 2010). Similar results have been revealed in comparable studies involving MDA-MB-231 and the CL4 human foreskin mutant fibroblast cell line, which has previously been characterized as favoring aerobic glycolysis compared to the CL3 variant that favors the oxidative metabolism (Migneco *et al* 2010). However, utilizing the same murine model, the constitutive activation of HIF-2α in fibroblasts did not lead to a shift toward aerobic glycolysis or increased tumor growth (Chiavarina *et al* 2012). Furthermore, in the transgenic mouse mammary tumor virus model of breast cancer involving the polyoma virus middle T transgene (MMTV-pyMT), targeted fibroblast deletion of HIF-1α or VEGF increases tumor growth, whereas HIF-2α does not alter it (Kim *et al* 2012). These differences may possibly be explained by the varying genotype and/or mutations within the cancer

or fibroblast cells used in these research studies. In addition, there may be an interplay between hypoxic targets such as HIF-1α, HIF-2α and HIF-3α, differences between primary murine cells and human cell lines, and/or differences in the mediators or cell–cell contacts in the local tumor microenvironment. Thus, the presence of paradoxical and convergent findings suggests there remain aspects of the regulation of hypoxic responses that are not yet fully understood.

It has been reported that human dermal fibroblast production of collagen, fibronectin, MMP-1, MMP-2 and MMP-3 is induced in co-cultures involving the estrogen receptor positive human breast cancer cell MCF-7 cell line where cell-to-cell contact is required and dependent solely on the fibroblast population for maximal stimulation (Ito *et al* 1995, Noel *et al* 1992a, 1992b). An additional study demonstrated that suspension of human dermal fibroblasts and MCF-7 cells in Matrigel™ and subsequent implantation of the cell/matrix mix into nude mice induces tumor growth that can be inhibited by MMP inhibitors (TIMPs) (Noel *et al* 1998). Thus, this tumor growth mechanism seems to involve paracrine interactions between MMP-2 bound to fibroblasts and cancer cell surface membrane-type 1 MMP (MT1-MMP), which is able to activate MMP-2 (Saad *et al* 2002). Subsequently, MMP-2 and MT1-MMP have also been implicated in the proteolytic degradation of the matrix crosslinker tissue transglutaminase (TG2), which additionally facilitates crosslinks between integrins (Nurminskaya and Belkin 2012). Thus, the interactions between cancer cells and fibroblasts may affect the expression of TG2 at the cell surface or within the extracellular matrix and facilitate cell adhesion and migration. Moreover, these interactions may then be further supported by hypoxia, which increases the expression of MT-MMP-1 in MDA-MB-231 breast cancer cells and in addition enhances the production of an additional factor, the so-called connective tissue growth factor (CTGF) (Kondo *et al* 2002).

The mediator CTGF is produced by both cancer cells and fibroblasts and is thus implicated in fibrosis, metastatic disease and chemotherapeutic resistance (Chien *et al* 2011, Shi-Wen *et al* 2008, Wang *et al* 2009). Fibroblast production of CTGF starts in response to TGF-β, MMP-2 and collagen ligation (Grotendorst *et al* 2004, Tall *et al* 2010). Fibroblast treatment with CTGF may then facilitate fibroblast-to-myofibroblast trans-differentiation, LOX activity and collagen deposition (Droppelmann *et al* 2009, Grotendorst *et al* 2004, Hong *et al* 1999). Interestingly, in a xenograft model overexpression of CTGF in hTERT-BJ1 immortalized fibroblasts supported the growth of co-injected MDA-MB-231 cells through a mechanism that included the induction of autophagy and HIF-1α-dependent metabolic alterations, independent of the matrix deposition (Capparelli *et al* 2012). Thus, the deposition of a stiff matrix may be induced in response to TGF-β, CGTF and/or hypoxia stimulation of either cancer cells or CAFs that then starts to produce collagen, LOX and TG2.

The effect of hypoxia on macrophages

Macrophages are sentinel cells in innate and adaptive immunity that fulfill additional roles in tissue homeostasis and the wound-healing response (Murray and Wynn 2011). In particular, macrophages are found during normal mammary gland development, the process of involution and breast cancer progression (Laoui *et al* 2011,

Schwertfeger *et al* 2006). The spatial localization of macrophages within the stromal tissue tightly surrounding human breast tumors, but not directly within the growing primary solid tumors, serves as a prognostic indicator of a poor patient outcome (Medrek *et al* 2012). As a result, macrophages at the tumor invasive front are thought to increase tumorigenesis, angiogenesis and the promotion of cancer metastasis (Hao *et al* 2012). However, this may occur partly in a paracrine-fashion interaction, where cancer cells secrete colony-stimulating factor-1 (CSF-1) and sense epidermal growth factor (EGF), whereas macrophages secrete EGF and sense CSF-1 (Patsialou *et al* 2009). Additional chemotactic signals that facilitate the recruitment of macrophages are substances released from hypoxic endothelial cells (SDF-1), fibroblasts (SDF-1) and/or breast cancer cells (ET2, CCL5) (Grimshaw *et al* 2002a, Grimshaw *et al* 2002b, Jin *et al* 2012, Lin *et al* 2012, Schmid *et al* 2011, Soria and Ben-Baruch 2008). There is some evidence that leads to the suggestion that hypoxia inhibits macrophage recruitment and decreases CSF-1 production, which would explain why macrophages are located around the peripheral edge of tumors rather than directly within the primary tumor complex where hypoxia is most pronouncedly identified (Green *et al* 2009; Hockel and Vaupel 2001, Turner *et al* 1999).

The directed migration of macrophages toward the tumor is facilitated by the binding of the blood monocyte integrin $\alpha 4\beta 1$ to the endothelium via the vascular cell adhesion molecule-1 (VCAM-1), diapedesis through the blood vessel wall, monocyte-to-macrophage differentiation within the extracellular matrix microenvironment and chemokine-facilitated migration into mammary tissue (Jin *et al* 2006, Schmid *et al* 2011, Stewart *et al* 2012). The increased deposition of type I collagen in human breast cancer tissue (Guo *et al* 2001, Ramaswamy *et al* 2003) suggests that macrophages may migrate through the $\alpha 2\beta 1$ integrin, as it has been demonstrated previously with mouse peritoneal macrophages (Philippeaux *et al* 2009). Collagen-binding interactions with murine macrophages also trigger the production of fibronectin, which indeed increases the cellular adhesion to collagen that is partially inhibited by the addition of competitive RGD peptides known to block integrin binding sites for fibronectin by binding to them (termed competitive inhibition) (Philippeaux *et al* 2009). In more detail, macrophages bind to fibronectin via $\alpha 4\beta 1$ and $\alpha 5\beta 1$, and it has been proposed that $\alpha 5\beta 1$ is the primary integrin involved in fibronectin-induced human or murine macrophage MMP-9 production (Xie *et al* 1998).

Increased production of MMP-9 and vascular endothelial growth factor (VEGF) has been detected in human breast cancers (Vinothini *et al* 2011), where the release of VEGF is triggered by the hypoxic-induced activation of both cancer cells and tumor-associated macrophages (Harmey *et al* 1998). Additional growth factors such as platelet derived growth factor (PDGF) and bFGF are produced at higher levels in breast cancer and are additionally produced and secreted by hypoxic human macrophages in order to induce and enhance endothelial cell migration and proliferation (Kuwabara *et al* 1995, Rykala *et al* 2011). Thus, breast-cancer-associated macrophages seem to increase matrix deposition as well as remodeling and support the formation/recruitment of new blood vessels (neoangiogenesis).

Moreover, macrophages regulate the innate and adaptive immune responses via various mechanisms, such as phagocytosis, free radical production and the

professional presentation of antigen to T cell subsets (Laskin 2009). The cytokine TGF-β, which is produced by cancer cells and fibroblasts (Kojima *et al* 2010, Margadant and Sonnenberg 2010), down-regulates the free radical production in macrophages and reduces the expression of co-stimulatory molecules required for antigen presentation (Li *et al* 2006). These immuno-suppressive functions of TGF-β are supposed to be enhanced by breast tumor hypoxic induction of the anti-inflammatory cytokine IL-19 (Azuma *et al* 2011, Hoffman *et al* 2011, Hsing *et al* 2012). The identified expression of IL-19 in murine macrophages may also suggest that macrophages produce this cytokine in response to hypoxia, as has been identified for 4T1 cancer cells (Azuma *et al* 2011, Hsing *et al* 2012). Moreover, it has also been indicated that hypoxia increases phagocytosis and enhances antigen presentation in the RAW 264.7 cell line and murine peritoneal macrophages in a HIF-1α-dependent fashion (Acosta-Iborra *et al* 2009, Anand *et al* 2007). The expression of HIF-1α has been detected in the differentiation of murine myeloid-derived suppressor cells, such as macrophages (Corzo *et al* 2010). Conditional deletion of HIF-1α in these cells within MMTV-pyMT mice showed a loss of T cell suppression and hence decreased tumor growth that was dependent on a lack of inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1) and independent of the VEGF production (Doedens *et al* 2010). However, it has been reported that TGF-β cell signals in murine macrophages antagonize the expression of iNOS (Sugiyama *et al* 2012), but induce the expression of Arg1 (Li *et al* 2012), suggesting a possible cell signal synergy between TGF-β and HIF-1α in regulating Arg1 expression.

Interestingly, macrophage TGF-β production is induced through a process called efferocytosis, which involves the phagocytosis of apoptotic cells (Fadok *et al* 1998, Korns *et al* 2011). The process of efferocytosis occurs via various mechanisms, such as macrophage integrin $\alpha v\beta 3$ and integrin $\alpha v\beta 5$-facilitated interactions with apoptotic cells (Korns *et al* 2011). Molecules that bind these integrins and confine them, such as blocking antibodies or the high mobility group box 1, have been shown to eliminate efferocytosis (Friggeri *et al* 2010, Stern *et al* 1996), suggesting that macrophage $\alpha v\beta 3$ and $\alpha v\beta 5$ interactions with known extracellular matrix ligands such as vitronectin, fibronectin and periostin (Kudo 2011, Nemeth *et al* 2007) may indeed impede efferocytosis and thereby foster chronic inflammation. In addition, the expression of TG2 on the cell surface of murine macrophages induces the phagocytosis of apoptotic cells by creating a bridge between the $\beta 3$ integrin and milk fat globulin epidermal growth factor (EGF) factor 8 (MFG-E8). In particular, the MFG-E8 contains the MGF-E8 RGD motif that can bind to $\alpha v\beta 3$ and additionally the MGF-E8 factor VIII-homologous domain can bind to the apoptotic cell exposed phosphatidylserine (Toda *et al* 2012, Toth *et al* 2009). In particular, TG2 is produced in response to hypoxia and the bacterial cell wall component, lipopolysaccharide (LPS) (Ghanta *et al* 2011, Nurminskaya and Belkin 2012), indicating that a hypoxic microenvironment and/or infection may lead to macrophage phagocytosis or TG2 matrix deposition.

What is the future direction of cancer research?

The etiology of human breast cancer is still elusive but it may involve endogenous (hereditary genes, hormonal/reproductive alterations, breast density, obesity) and/or

exogenous (carcinogens, radiation, hormone replacement therapy, alcohol consumption) risk factors, which may even act synergistically in promoting the formation of tumors (Mansfield 1993). Genetic and epigenetic alterations involved in oncogenesis also trigger the conversion of TGF-β cell signals from cytostatic to tumorigenic in an uncharacterized process known as the TGF-β paradox (Schiemann 2007). The tumor-promoting properties of TGF-β not only affect the cancer cell, but also the stromal cell functions associated with angiogenesis and immunosuppression. Thus, the blockade of TGF-β cell signals via various inhibitors, such as neutralizing antibodies, decoy receptors, antisense oligonucleotides, small molecule receptor kinase inhibitors and peptide aptamers, has been assessed as a therapeutic target (Connolly *et al* 2012). Indeed, the function of MMPs in the formation of active TGF-β and tumor progression suggest that the inhibition of MMPs may also reduce tumor viability and cancer cell migration. Thus, previous studies in murine models using MMP inhibitors were found to pronouncedly reduce tumor growth, however, clinical trials have not yet been similarly successful (Coussens *et al* 2002). This may be due to MMP production coming from primarily stromal cells in human cancers or the lack of drug specificity to a particular MMP expressed at a certain tumor stage or within certain patient subsets (Coussens *et al* 2002, Zucker and Cao 2009). However, perhaps a more refined therapy directed against specific MMPs will lead to more specificity and success. In line with this, this type of drug selectivity has been described in studies that require HER-2 (Herceptin) or estrogen (Tamoxifen) receptor expression for efficacy (Khasraw and Bell 2012, Pearson *et al* 1982). In particular, studies *in vitro* and in an *in vivo* murine xenograft model of breast cancer showed that a soluble synthetic cytoplasmic tail peptide of MT1-MMP significantly inhibits HIF-1α, lactate production and tumor growth (Sakamoto *et al* 2011). HIF-1c is activated by an unanticipated function of MT1-MMP causing the stimulation of ATP production through glycolysis. The cytoplasmic tail of MT1-MMP can bind to hydroxylase FIH-1, which inhibits the function of HIF-1α. Moreover, FIH-1 can be inhibited by the identified inhibitor, Mint3 (synonymously known as APBA3). Similar studies were performed in murine macrophages that revealed a mechanism involving MT1-MMP cytoplasmic tail induced localization of the factor inhibiting HIF-1α (FIH-1) with an inhibitor of FIH-1 (Mint3), which then provided the transcriptional activity of HIF-1α (Sakamoto and Seiki 2010).

Moreover, it has been suggested that these associated functions also affect fibroblast MMP-2 production, which relies on MT1-MMP function for activation, in breast tumorigenesis (Saad *et al* 2002). As MT1-MMP and additional MMPs are induced by hypoxia (Harmey *et al* 1998, Munoz-Najar *et al* 2006), mechanisms to abrogate the hypoxic response may also be therapeutically useful.

However, receptor activation by HER-2 ligands and estrogen seems to be implicated in the regulation of the hypoxic response. In more detail, HER-2 ligands induce the activation of HIF-1α, suggesting that in HER-2^+ tumors Herceptin may be able to decrease the expression of the poor prognostic indicator HIF-1α (Charpin *et al* 2012, Spangenberg *et al* 2006). With respect to the estrogen receptor, the functions of HIF-1α appear to be more complex. In more detail, it has been

indicated that hypoxia acts synergistically with estrogen in the activation of estrogen response elements (ERE) and the transcription of genes supporting tumorigenesis, angiogenesis and metabolism (Seifeddine *et al* 2007, Yi *et al* 2009). This synergy has led to increased clinical research in this field indicating that HIF-1α expression is a poor response predictor for chemoendocrine (Tamoxifen, Epirubicin) therapy (Generali *et al* 2006), suggesting that the examination of additional treatment approaches for hypoxia is necessary.

Alternative approaches for blocking hypoxic cell signals may include 2-deoxy-D-glucose (2-DG), antioxidants and aryl hydrocarbon receptor (AHR) ligands. In more detail, 2-DG is a stable glucose analog, which can be actively taken up by hexose transporters and is phosphorylated by a hexokinase. This phosphorylated molecule (2-DG6P) then becomes a non-competitive inhibitor to hexokinase and a competitive inhibitor to glucose phosphoisomerase in the glycolytic pathway (Chen and Gueron 1992, Sols and Crane 1954, Wick *et al* 1975). Typical use of 2-DG involves F-18 labeling and non-invasive detection and staging of human tumors via positron emission tomography (PET) (Dwarakanath and Jain 2009). Renewed interest in 2-DG as a therapeutic agent has been reported in a combined treatment of 2-DG and a mitochondrial inhibitor (Mito-CP), which significantly decreased tumor weight without detriment to vital organs in a breast cancer xenograft model (Cheng *et al* 2012). In addition, it has also been reported that the cytotoxic effects of 2-DG increase under hypoxic conditions, thus enhancing the sensitivity of HIF-1 positive tumors to radiation and drug therapies (Aghaee *et al* 2012). Moreover, the functional mechanisms of 2-DG are not limited to the reduction of energy (ATP), because the molecule blocks protein glycosylation (Kurtoglu *et al* 2007). As the formation of collagen relies on glycosylation (Gelse *et al* 2003), fibroblast extracellular matrix deposition and remodeling seem to be altered by 2-DG. There is evidence that integrins also rely on glycosylation in establishing conformational structures and activation (Janik *et al* 2010), thus linking the migratory functions of cancer and stromal cells to potential inhibition by 2-DG.

In addition, it has also been suggested that antioxidants regulate integrin expression, particularly in subsets of immune cells, in which the molecule *N*-acetyl-L-cysteine (NAC) dramatically decreases the expression of the integrin α4 associated with macrophage tissue invasion (Curran and Bertics 2012, Jin *et al* 2006, Laragione *et al* 2003, Puig-Kroger *et al* 2000). In a variety of cancer cell lines and murine models, NAC has been seen to decrease tumor growth and reduce HIF-1α levels by stimulating the HIF-1α degradation in a prolyl hydroxylase- and von Hippel–Lindau-dependent fashion (Gao *et al* 2007).

The activity of HIF-1α may also be inhibited by the cross-talk with the AHR, which shares a dimerization partner, HIF-1β (also called the AHR nuclear translocator, or ARNT), with HIF-1α for transcriptional activation of responsive genes (Chan *et al* 1999, Zhang and Walker 2007). In particular, the AHR is also able to exhibit positive and negative cross-talk with the estrogen receptors after activation by xenobiotic ligands, such as dioxin, or endogenous ligands, such as tryptophan metabolites (Denison and Nagy 2003). However, increased AHR cell signaling downstream of CXCR4 has been characterized as a biomarker of Tamoxifen-resistant MCF-7 cell

lines, suggesting that AHR antagonists may offer therapeutic advantages for Tamoxifen-resistant patient subsets (Dubrovska *et al* 2012, Ohtake *et al* 2011). Alternatively, in a murine model involving mitoxantrone-treated MDA-MB-231 cells injected intravenously into NOD SCID gamma mice, a gavage treatment with the non-toxic AHR ligand, named Tranilast, significantly reduced tumor growth and lung metastases (Prud'homme *et al* 2010). This result seems to be a response to direct tumor cytotoxicity, Tranilast-induced TGF-β inhibition, (Prud'homme *et al* 2010), reduced differentiation of macrophages (van Grevenynghe *et al* 2003), an absence of AHR-induced T regulatory cells (Schulz *et al* 2012) and/or altered fibroblast extracellular matrix production (Lehmann *et al* 2011).

As TGF-β and hypoxia cell signals lead to elevated extracellular matrix deposition, the cellular responses to the extracellular matrix may also be of interest as therapeutic targets. In particular, reduced breast tumor growth and metastases *in vitro* and *in vivo* has been found to involve small peptides (ATN-161, Cilengitide) or blocking antibodies (Abegrin) that antagonize integrin ($\alpha5\beta1$, $\alpha v\beta3$, $\alpha v\beta5$) activation (Bauerle *et al* 2011, Khalili *et al* 2006, Mulgrew *et al* 2006). The force associated with breast density and tumorigenesis also increases integrin expression and activity (Paszek and Weaver 2004, Sawada *et al* 2006). Crosslinker molecules such as TG2 and LOX ensure the formation of a stiff matrix and the generation of forces (Nurminskaya and Belkin 2012, Paszek and Weaver 2004). In a murine model of breast cancer, tumorigenesis is connected to the increased expression of stromal cell LOX, and inhibition of LOX with antibodies or β-aminopropionitrile (BAPN) increased tumor latency and subsequently decreased tumor incidence (Levental *et al* 2009). Additional studies with cancer cell TG2 expression also revealed reduced tumor growth in the absence of TG2 (Oh *et al* 2011), suggesting that continued research regarding the particular intracellular and extracellular functions of extracellular matrix crosslinkers, which are supposed to play a role in both cancer and stromal cell function, is warranted.

The downstream targets of the integrin activation are enhanced in response to a dense collagen matrix such as focal adhesion kinase (FAK) and RhoA (Heck *et al* 2012, Keely 2011, Provenzano *et al* 2009b). Interestingly, the expression of FAK in the mammary epithelium is essential for murine MMTV-PyMT breast tumor progression, suggesting that FAK inhibitors may act as potential inhibitors of breast cancer (Provenzano *et al* 2008a, Schultze and Fiedler 2010). The molecule RhoA not only coordinates cell migration and survival, it has also been reported to be an inhibitor of macrophage efferocytosis (Korns *et al* 2011, Ridley 2004). The identification of RhoA in human tumors is linearly correlated with lymph node metastasis and tumor invasion and it may be mediated through a mechanism involving hypoxia-induced RhoA, as has been demonstrated in the human MCF-7 cell line (Ma *et al* 2012). In the orthotopic breast cancer model involving MDA-MB-231 cells, the tumor burden was drastically reduced upon treatment with an oral inhibitor of Rho kinases, Fasudil (Ying *et al* 2006). Thus, FAK and RhoA, as well as additional downstream signals of the integrin receptor (such as extracellular matrix interactions) may serve as effectual therapeutic targets for breast cancer treatment. However, understanding how these pathways agree or differ in cancer

and stromal cells would provide therapeutic advantages for certain tumors or patient subsets.

Taken together, breast cancer is a heterogeneous disease disparately characterized by receptor status (HER-2, ER, PR), regulated by the cytokine TGF-β and the properties of the extracellular matrix that locally surrounds the primary tumor. Cell-facilitated adhesive interactions induce the production and mediate the release of TGF-β, which in turn supports cell signals associated with the expression of integrins, the deposition and remodeling of the extracellular matrix. Moreover, the formation of an extracellular matrix that serves as a bridge to blood vessels and metastatic sites involves contributions from both cancer and stromal cells. The introduction of hypoxia to the microenvironment differentially affects each particular cell type and may even depend additionally on cell-specific mutations, cell–cell contact, extracellular matrix interactions and the production of effectors. Moreover, cells not only reveal specific signs of hypoxia in response to oxygen deprivation, but also from additional metabolic factors, cytokines and growth factors. The subsequent activation of HIFs induces metabolic shifts and evokes the production of chemokines and adhesive factors involved in tumor survival, recruitment and migration. However, these transcription factors are also implicated in fibroblast and macrophage differentiation and in the production of proteins that remodel the extracellular matrix, such as the enzyme crosslinking molecules of LOX and TG2. Due to their complexity, LOX and TG2 are also pleiotropic proteins involved in cell surface receptor-facilitated functions and intracellular cell signaling. These various interactions display and illuminate the diverse nature of the hypoxic tumor microenvironment and the complex highly integrated networks between cancer and stromal cells in tumor development and the immune response. However, the elucidation of these networks may lead to improved treatments that target both cancer and stromal cells in breast cancer.

12.2 Cancer-associated fibroblasts align fibronectin in the matrix to enhance cancer cell migration

Cancer-associated fibroblasts (CAFs) represent a major component of the carcinoma microenvironment promoting malignant tumor progression. However, the mechanisms by which CAFs provide the regulation of the cancer cell migration are not yet precisely understood. CAFs represent a highly abundant cell type in the tumor microenvironment and possess the ability to support tumor growth (Olumi *et al* 1999, Orimo *et al* 2005). The most prominent function of normal fibroblasts is to provide the maintenance of homeostasis within the extracellular matrix (Kalluri and Zeisberg 2006). Contrarily, CAFs and other activated fibroblasts evoke alterations in this critical process. In particular, CAFs secrete high levels of extracellular matrix proteins, including fibronectin, type I and type II collagen, and they even express oncofetal isoforms of fibronectin (Barsky *et al* 1984, Tuxhorn *et al* 2002, Schor *et al* 2003, Clarke *et al* 2016, Gopal *et al* 2017). Moreover, CAFs can affect the architecture and physical properties of the extracellular matrix, altering cell migration, invasion and tumor growth (Jolly *et al* 2016, Kaukonen *et al* 2016).

Through force-facilitated extracellular matrix remodeling, CAFs structurally remodel the 3D collagen type I matrices in order to generate tracks along which cancer cells can migrate and invade (Gaggioli *et al* 2007). In addition, CAFs also have been found to generate aligned matrix fibers *in vitro* (Amatangelo *et al* 2005, Lee *et al* 2011, Franco-Barraza *et al* 2017). Indeed, the alignment of extracellular matrix fibers has also been detected in primary tumors and additionally revealed to be correlated with poor patient prognosis (Conklin *et al* 2011, Franco-Barraza *et al* 2017). However, the mechanisms of the fibroblast-driven extracellular matrix alignment and its functional role in the CAF–cancer cell interactions remain to be clearly revealed.

In particular, fibronectin is among the most abundant extracellular matrix proteins and facilitates a broad variety of cellular activities, such as cell adhesion, migration, tumor growth and differentiation (Pankov and Yamada 2002). Fibronectin binds to extracellular matrix proteins, such as collagen, periostin, fibrillin and tenascin-C, and regulates their assembly and organization (Kadler *et al* 2008, Kii *et al* 2010). Moreover, aberrant fibronectin expression has also been correlated with the malignant progression of cancer (Insua-Rodríguez and Oskarsson 2016, Topalovski and Brekken 2016, Wang and Hielscher 2017). Thus, substantial knowledge is required in understanding the specific function of fibronectin in the tumor microenvironment.

It is known that fibronectin can assemble into fibers through its binding as a ligand to transmembrane integrin adhesion receptors (Mao and Schwarzbauer 2005, Campbell and Humphries 2011). In particular, integrin $\alpha5\beta1$ is the major fibronectin receptor and mediates the fibronectin fibrillogenesis through the activation of cellular contractility and the application of traction forces towards fibronectin (Hinz 2006, Lemmon *et al* 2009, Schwarzbauer and DeSimone 2011). However, the precise function of $\alpha5\beta1$ integrin in the assembly of the fibronectin matrix is yet to be clearly understood, but it has been unraveled how the inside–out signaling is regulated within activated fibroblasts and how this signaling causes the reorganization of the 3D extracellular matrix.

The signal transduction through growth factors is critical for facilitating the cancer cell–tumor stroma interactions to support the malignant progression of cancer. The platelet derived growth factor (PDGF) represents a key growth factor, which is able to provide an interaction between cancer cells and stromal cells. PDGF has been shown to be a potent activator of fibroblasts, as it can bind to cell surface PDGF receptors (PDGFRs). These PDGFRs belong to the group of tyrosine kinase receptors, which consist of homo- or heterodimers of two PDGFR chains, termed PDGFRα and PDGFRβ (Donovan *et al* 2013). Most cancer cell types such as prostate carcinoma, express and secrete PDGF ligands but not possess PDGFRs (Sariban *et al* 1988, Sitaras *et al* 1988). In contrast to normal fibroblasts, CAFs even overexpress PDGFRα and PDGFRβ (Augsten 2014). Moreover, PDGF ligands secreted by cancer cells can promote proliferation, migration, and recruitment of stromal fibroblasts (Östman 2004). It has been revealed that the inactivation of PDGFRα in fibroblasts diminished the remodeling of connective tissue (Horikawa *et al* 2015). However, its functional role in restructuring of other tissues and during disease states is not yet clearly known.

Hence it has been shown that fibronectin fibrillogenesis by CAFs guides CAF–cancer cell interactions and facilitates the directional migration of cancer cells using co-culture assays. Fibronectin-rich cell-derived matrices (CDMs), which have been isolated from CAF cultures, but not from normal fibroblasts cultures, display aligned fiber organization and facilitate the directional migration of cancer cells. Compared with normal fibroblasts, it has been revealed that the matrix organization by CAFs is driven by increased myosin II-based contractility and enhanced traction forces, which are transduced to the extracellular matrix through $\alpha 5\beta 1$ integrins. Moreover, there is some evidence that increased PDGFRα activity in CAFs provides contractility and the organization of parallel fibronectin structures. Moreover, the αv integrin acts as a regulator of the migration of cancer cell on CAF containing matrices. In particular, a new mechanism has been provided for facilitating the CAF–cancer cell interaction and the directional migration of cancer cells.

There is indeed some evidence that alterations in the tumor microenvironment regulate the progression of cancer (Miles and Sikes 2014). CAFs seem to represent a key component of the tumor microenvironment that fulfill tumor-supportive roles (Mezawa and Orimo 2016). Cancer cell migration and invasion are crucial initial steps in the metastatic cascade. However, the precise mechanisms by which tumor–stroma interactions facilitate those processes are not yet clearly revealed. Indeed, a new mechanism by which CAFs promote the migration and invasion of cancer cells has been characterized. Using an *in vitro* co-culture system, it has been found that cancer cells can interact with primary human prostate CAFs and hence migrate persistently along them in one direction. There is some evidence that the interaction between CAFs and cancer cells is guided by the fibronectin fibrils, which are assembled by CAFs, and in turn cancer cells probe CAF-assembled fibronectin fibrils to migrate along them. In more detail, the CAF–cancer cell interaction can be inhibited by knock-down of fibronectin in CAFs. In particular, prostate CAFs can induce an enhanced association with HNSCC cells and support their directional migration. This finding leads to the suggestion that there is a ubiquitous mechanism by which CAFs originating from different tumor microenvironments are able to alter the migration of cancer cells, which has been reported for the first time.

Moreover, apart from the CAF-based influence on the CAF–cancer cell association, fibronectin seems to be critical for extracellular matrix production and organization by CAFs. Indeed, fibronectin seems to be a key player of the CDMs that are produced by CAFs and normal fibroblasts, and the knock-down of the CAF-based fibronectin expression entirely abolishes the extracellular matrix synthesis and reorganization. In addition, CAFs facilitate the alignment of fibronectin into parallel fibers, whereas normal fibroblasts lead to the assembly of a mesh-like fibronectin matrix scaffold. Moreover, the extracellular matrix architecture is able to govern the directional migration of cells, which is based on physical cues, as the migrating cells utilize the extracellular matrix as cell–matrix attachments during their migration (Petrie *et al* 2009). CAF-facilitated parallel organization of CAF CDMs induces the directional migration of both prostate cancer and HNSCC cells, where the cell migration is in the direction of the fiber orientation. In clinical

prostate cancer, it has been found that the aligned fibronectin fibers at the sites of invasion are indeed adjacent to invading cancer cells. Moreover, parallel-organized fibronectin is also present in pancreatic ductal adenocarcinomas, which leads to the hypothesis that the alignment of fibronectin fibers represents a clinical feature of carcinomas, as has been shown for at least two carcinomas, and hence may also contribute to the dissemination of cancer cells.

Although the overexpression of fibronectin and its EDA isoform have been identified to be features of CAFs (Kalluri and Zeisberg 2006), the effects of these alterations in the fibronectin matrix scaffold on the migration and invasion of cancer cells are just emerging as a clear picture. Indeed, in the CAF matrisome, fibronectin–EDA has been identified as a biomarker of poor survival in patients with HNSCC. Intriguingly, the directional migration of HNSCC cells on CAF CDMs have been characterized, in a collective manner (Gopal *et al* 2017). In contrast, in another study the collective migration of either prostate cancer cells or HNSCC cells on CAF CDMs has not been observed (Erdogan *et al* 2017). However, a main reason of the differences between the two studies may be based on the differences in epithelial properties of the cancer cell lines that have been selected or differences in the cell density used in them. Nevertheless, both studies pointed out that fibronectin is a critical component of CAF CDMs facilitating cell migration.

Several factors have identified that can cause extracellular matrix alignment, among them are the serine proteinase fibroblast activation protein (Lee *et al* 2011), and the two transcription factors Snail1 and Twist1 that seem to function downstream of TGF-β to provide the CAF phenotype (Stanisavljevic *et al* 2015, García-Palmero *et al* 2016). However, the mechanism through which CAFs restructure the extracellular matrix remains largely elusive. It has been found that mechanical force is an important key parameter that enables CAFs to produce and generate an aligned extracellular matrix. Myosin II-driven contractility is a specific feature of CAFs (Calvo *et al* 2013). In line with this, it has been seen that prostate CAFs possess increased myosin II activity, are highly contractile, and exert high-traction stresses on fibronectin fibrils (Erdogan *et al* 2017). Moreover, the addition of low doses of a myosin II inhibitor perturbed the alignment of fibronectin fibrils by CAFs, which led to a more-random network of fibers that is similar to the fibronectin scaffold assembled by normal fibroblasts. As DU145 prostate cancer cells did not display directional migration when plated onto CAF CDMs, which have been produced during blebbistatin treatment of CAFs. These findings indicate that the matrix organization is a driving factor for the directional cancer cell migration and is facilitated by myosin II-based contractility and the high traction force exerted by CAFs.

The $\alpha5\beta1$ integrin fulfills a prominent role in fibronectin fibrillogenesis and they can be activated intracellularly by mechanical forces caused by actomyosin contractility (Friedland *et al* 2009). In line with the finding that $\alpha5\beta1$ integrins are identified as a mechanotransducer (Schwartz and DeSimone 2008, Roca-Cusachs *et al* 2012), it has been shown that high traction forces evoked by CAFs are transduced through $\alpha5\beta1$ integrin towards fibronectin (Erdogan *et al* 2017). The enhanced activation of $\alpha5$ and $\beta1$ integrins in CAFs compared to normal fibroblasts

cannot be explained by different expression levels of $\alpha 5$ and $\beta 1$ integrin subunits on the two cell types, as they are nearly the same (Erdogan *et al* 2017). The increased expression of fibronectin and the enhanced contractility of CAFs may provide an explanation why the $\alpha 5\beta 1$ integrin activation induces an elevation in cancer cell migration and directional persistent movement (Lin *et al* 2013). Moreover, the overexpression and activation of PDGFRα can additionally increase the $\alpha 5\beta 1$ integrin activity, as revealed by using inhibitory antibodies directed against $\alpha 5\beta 1$ integrins. As many signals accumulate on integrins to facilitate inside–out signaling, it may be possible that there are additional mechanisms leading to elevated activation of $\alpha 5\beta 1$ integrin in CAFs. These include deregulations in the cell's metabolism sensor AMP-activated protein kinase, which has been shown to act as a negative regulator of the activity of $\beta 1$ integrins and fibronectin fibrillogenesis in fibroblasts (Georgiadou *et al* 2017) or the deregulation of the integrin inhibitor, the Sharpin protein that has been found to regulate the remodeling of collagen fiber networks and subsequently the exertion of traction forces (Peuhu *et al* 2017). However, other integrins can also provide to alterations in CDM organization, such as $\alpha v\beta 5$ integrins (Franco-Barraza *et al* 2017).

The mechanism by which CAFs induce extracellular matrix organization involves the overexpression of PDGFRα in prostate CAFs and hence the elevated phosphorylation of Y762 on PDGFRα. In turn the inhibition of PDGFRα significantly abrogated the contraction of collagen gels by impaired traction stresses generated by CAFs, decreased $\alpha 5\beta 1$ integrin activity and impaired fibronectin organization. These results are in line with previous data demonstrating cross-talk between $\alpha 5\beta 1$ integrins and PDGFRα in mesenchymal stem cells (Veevers-Lowe *et al* 2011). However, the $\alpha 5\beta 1$ integrin and PDGFRs have been found to be in a complex with the tissue transglutaminase, regulating the activity of both receptors, and hence potentially accumulates and hence amplifies their downstream signal transduction processes (Akimov and Belkin 2001, Zemskov *et al* 2009). Moreover, it seems to be possible that PDGFRα signaling causes a direct activation of the contractility, which subsequently causes an indirect activation of $\alpha 5\beta 1$ integrins. For instance, the PDGFRα signal transduction activates the RhoA–ROCK signal transduction pathway in mesenchymal stem cells, which enhances the polymerization of αSMA in actin filaments (Ball *et al* 2007) that is a characteristic attribute of CAFs. Therefore, the integrin $\alpha 5\beta 1$ and PDGFRα signal transduction pathways seem to compile on the activation of RhoA-mediated contractility (Danen *et al* 2002). In particular, PDGFR signal transduction is a promising target in various cancer types (Heldin 2013), therefore, knowledge of the regulation of CAFs and the stromal extracellular matrix by PDGFRs seems to be crucial for targeting the tumor stroma in carcinomas and fully discovering their cancer progression supporting potential.

Due to the dramatic alterations of the tumor microenvironment, cancer cells express and activate different integrins on their membrane surface to facilitate processes, such as cell adhesion and migration. It has been found reported that HNSCC cells increase the expression of the $\alpha 5\beta 1$, $\alpha v\beta 5$ and $\alpha v\beta 6$ integrins on the cell surface, when cultured on fibronectin–EDA-rich CAF CDMs (Gopal *et al* 2017). It has been revealed that the collective migration of HNSCC cells on CAF

CDMs is based on $\alpha v\beta 6$ integrins and fibronectin–EDA-binding to $\alpha 9\beta 1$ integrins (Gopal *et al* 2017). Based on these findings, integrins have been identified that are functionally associated with the directional migration of prostate cancer cells on CAF CDMs (Gopal *et al* 2017). Similarly, the inhibition of the $\alpha 5\beta 1$ integrin in prostate cancer cells evoked a faster migration with reduced directionality and also the inhibition of the αv integrin subunit activity reduced both directionality and the migration speed. These findings indicate that $\alpha 5\beta 1$ integrins seem to be responsible for the formation of stronger attachments to the matrix (Roca-Cusachs *et al* 2009), whereas the αv integrins seem to be crucial for cell migration. The aberrant expression of RGD-binding integrins $\alpha 5$ and αv have been revealed in prostate cancers and hence identified as potential targets for therapy (Goel *et al* 2008, Sutherland *et al* 2012). It has been found that EDA-binding of the integrin $\alpha 9\beta 1$ regulates cellular migration on CAF CDMs (Gopal *et al* 2017). Although CAFs have been reported to express high levels of EDA–fibronectin, it has not yet been reported that the $\alpha 9$ integrin is expressed in prostate cancers. In particular, ITGA9 expression has not been found in 11 prostate cancer tissues using the Human Protein Atlas, and hence this integrin was not further included in these studies. Moreover, other EDA-binding integrins, such as $\alpha 4\beta 1$ and the $\alpha 4\beta 7$ integrins, have not been investigated, as there exist multiple studies revealing that the $\alpha 4$ integrin is not expressed on the plasma membrane of prostate cancer and DU145 prostate cancer cells (Rokhlin and Cohen 1995, Barthel *et al* 2013, Chen *et al* 2015). Collectively, these results indicate vastly different roles for integrins in CAFs and cancer cells in controlling matrix assembly and cell migration and, thus, point out the complexity of integrin signal transduction processes in primary tumors.

Taken together, CAFs have been reported to arrange the organization of the fibronectin matrix through enhanced contractility and traction forces, which are facilitated by myosin II, the $\alpha 5\beta 1$ integrin and the PDGFRα (Erdogan *et al* 2017). This matrix organization enables cancer cells to migrate directionally using αv integrins. The alignment of the fibronectin fibers represent a prominent feature of both prostatic and pancreatic cancer stromata under *in vivo* and *in vitro* conditions and is hence well suited to govern the migration and invasion of cancer cells. These data suggest that CAFs in the tumor stromal microenvironment are not tissue-type-specific in their capacity to guide the migration of cancer cells through the stroma. For instance, CAFs from the prostate can regulate the migration of HNSCC cells. These results suggest a commonly employed mechanism for altering the migration of cancer cells that possess a broad and wide impact on the development of tumor metastases. Moreover, the biochemical targeting of this pathway can prove beneficial in limiting the stromal support during the process of metastasis of cancer cells.

12.3 The role of substances and growth factors within the extracellular matrix for cancer cell mechanical properties

Low molecular weight hyaluronan (LMW-HA), which is generated by degradation of the extracellular matrix component hyaluronan (HA), has been identified as being

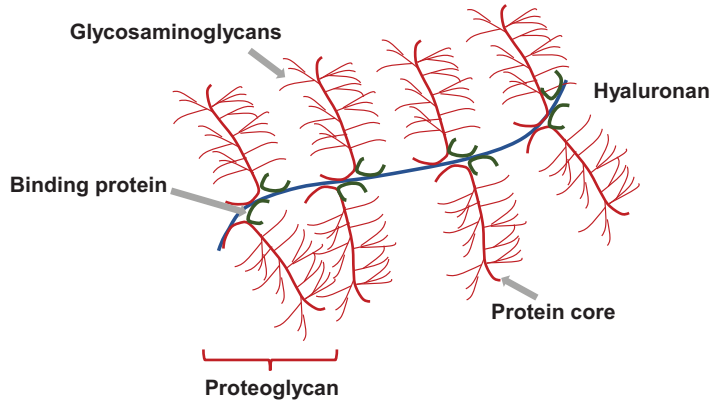

Figure 12.3. Structure of hyaluronan.

crucial to cancer progression (figure 12.3). However, no systematic clinical study of breast cancer has been performed in order to reveal a correlation between LMW-HA levels and cancer metastasis. It has been found that in 176 serum specimens the serum LMW-HA (but not total HA) level correlated significantly with lymph node metastasis, suggesting that serum LMW-HA represents a better prognostic indicator of breast cancer progression than HA (Wu *et al* 2015). Similarly, breast cancer cells which display a higher invasive potential, had a higher LMW-HA concentration than less-invasive cancer cells. Indeed, this higher LMW-HA level was accompanied by the overexpression of hyaluronan synthase (HAS2) and hyaluronidase (both HYAL1 and HYAL2) (Wu *et al* 2015). Importantly, decreasing LMW-HA production significantly inhibited breast cancer cell migration and invasion (Wu *et al* 2015). Taken together, these results suggest that during cancer progression cancer cells may actively remodel their microenvironment through an autocrine/paracrine-like process, which results in elevated LMW-HA levels that in turn promote cancer progression by supporting the migration and invasion of cancer cells into the extracellular matrix of connective tissue. Thus, cancer-associated LMW-HA seems to be a more promising molecular biomarker than total HA for detecting metastasis and it may hence provide further applications in the treatment of breast cancer.

Hyaluronan is known to be a prominent component of the local microenvironment in most malignant tumors and can be used as a prognostic factor for tumor progression. Extensive experimental evidence in animal models indicates that hyaluronan interactions in tumor growth and metastasis are essential, but it is also known that a balance of synthesis and turnover by hyaluronidases is critical. CD44 is a major hyaluronan receptor, and is commonly but not uniformly associated with malignancy and hence frequently serves as a marker for cancer stem cells in human carcinomas. Multivalent interactions of hyaluronan with CD44 collaborate in inducing numerous tumor-promoting signaling pathways and transporter activities. It is widely accepted that hyaluronan–CD44 interactions are

crucial in both malignancy and resistance to therapy, while investigating the mechanism for the activation of hyaluronan–CD44 signaling in cancer cells, the relative importance of variant forms of CD44 and other hyaluronan receptors (such as Rhamm) in different tumor contexts and the role of stromal versus cancer cell driven hyaluronan and its turnover are some of the major challenges for future research. Despite these, it is clear that hyaluronan–CD44 interactions are an important target for translation into the clinic. Among the approaches that seem to be promising are antibodies and vaccines for specific variants of CD44 that are uniquely expressed at critical progression stages of a particular cancer, hyaluronidase-mediated reduction of barriers to drug access and small hyaluronan oligosaccharides, which attenuate constitutive hyaluronan-receptor signaling and enhance chemosensitivity. In addition, as a novel development in drug delivery, hyaluronan is being used to tag drugs and delivery vehicles for targeting of anti-cancer agents toward CD44-expressing cancer cells.

The importance of the microenvironment in cancer progression has been demonstrated in numerous studies (Nelson and Bissell 2006, Polyak *et al* 2009, Joyce and Pollard 2009, Kalluri and Weinberg 2009). Hyaluronan is a prominent and well-known constituent of the local microenvironment in most malignant tumors. In particular, hyaluronan immediately surrounds the pericellular milieu around cancer cells and is located in the tumor stroma, but its association with either compartment can be prognostic for tumor progression (Knudson *et al* 1989, Tammi *et al* 2008). A major receptor for hyaluronan, CD44, is currently much investigated, as it is a common marker for 'tumor-initiating cells/cancer stem cells' (CSCs) in human carcinomas. Although the nature of these CSCs is highly controversial in the literature, there is a reasonable consensus that CD44-expressing sub-fractions of many human carcinomas are highly malignant and resistant to therapy, which are all properties that are also associated with CSCs (Visvader and Lindeman 2008, Polyak and Weinberg 2009). However, the functions of CD44 and its hyaluronan ligand for the properties of these particular cells are still elusive and need further investigation. The functional dynamics of hyaluronan and its receptors such as CD44 were recently investigated in more detail with respect to cancer (Tammi *et al* 2008, Stern 2008, 2009, Maxwell *et al* 2008, Bourguignon 2008, Lokeshwar and Selzer 2008, Itano and Kimata 2008, Naor *et al* 2008, Toole *et al* 2008, Simpson and Lokeshwar 2008). The current state of knowledge for the functions of hyaluronan–CD44 interactions in cancer and the mechanisms through which these interactions influence a large number of signaling pathways and cellular behaviors was summarized (Toole 2009). Some of these activities are summarized in figure 12.4.

Hyaluronan synthases produce and extrude hyaluronan, which may be retained by the synthase or released into the pericellular milieu. The extruded hyaluronan has been shown to interact multivalently with CD44 to induce and/or stabilize signaling domains within the cell membrane. These signaling domains contain receptor tyrosine kinases such as ErbB2 and EGFR, other signaling receptors (TGFβR1) and non-receptor kinases (Src family) that are able to drive oncogenic pathways, for instance, the MAP kinase and PI3 kinase/Akt cell proliferation and survival pathways, as well as various transporters that provide drug resistance and induce

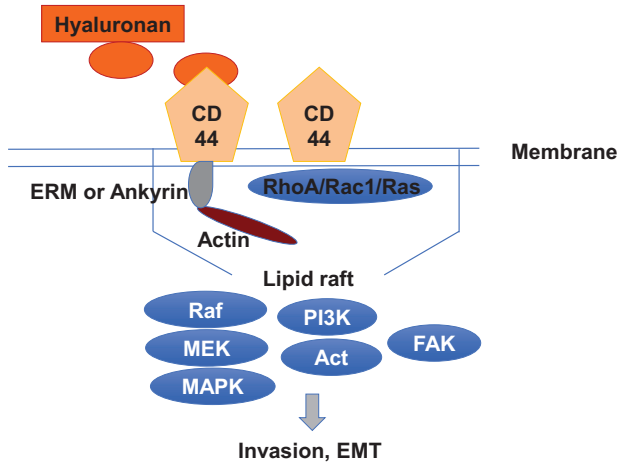

Figure 12.4. Regulation of signal transduction cascades by hyaluronan–CD44 interaction.

malignant cell properties (Toole *et al* 2008, Bourguignon 2009). Various adaptor proteins, such as Vav2, Grb2 and Gab-1, facilitate the interaction of CD44 with upstream effectors, such as RhoA, Rac1 and Ras, which can drive these pathways (Bourguignon 2008, 2009). In other cases, carbohydrate side groups on variant regions of CD44, such as heparan sulfate chains, bind additional regulatory factors and co-activate receptor tyrosine kinases, such as the c-Met receptor (Ponta *et al* 2003). Hyaluronan–CD44 interactions also facilitate cytoskeletal alterations that induce cell motility and invasion. In this case, actin filaments are recruited to the cytoplasmic tail of CD44 through members of the ezrin–radixin–moiesin (ERM) family or ankyrin (Bourguignon 2008, Ponta *et al* 2003). Proteoglycans and associated factors attached to pericellular hyaluronan are also able to influence these activities (Itano and Kiata 2008, Evanko *et al* 2007) (figure 12.4). However, hyaluronan produced by stromal cells may have overlapping or different activities than that produced by cancer cells, while the relative contributions of stromal and tumor-derived hyaluronan are still elusive (Tammi *et al* 2008).

Hyaluronan

Hyaluronan (synonymously termed hyaluronic acid or hyaluronate) is a very large, linear, negatively charged polysaccharide, which consists of repeating disaccharides of glucuronate and *N*-acetylglucosamine. Hyaluronan is produced by three hyaluronan synthases (Has1/Has2/Has3), which are integral cell membrane proteins whose active sites are located at the intracellular site of the cell membrane (Weigel and DeAngelis 2007). In particular, newly synthesized hyaluronan is extruded as it is elongated and then targeted to the cell surface or to pericellular extracellular matrices. However, hyaluronan is widely distributed in vertebrate tissues, but it is especially concentrated in regions of cell division and cell invasion (Toole 2001). In adult tissues such as synovial fluid, cartilage and dermis, hyaluronan plays a pronounced structural role, based on its unique hydrodynamic properties and its

interactions with other extracellular matrix components. On the other hand, hyaluronan has an instructive cell signaling role during dynamic restructuring of cellular processes such as morphogenesis, inflammation, wound repair and cancer, in which hyaluronan–receptor interactions are activated and hence induce numerous signaling pathways (Bourguignon 2008, Turley *et al* 2002, Toole 2001). In addition to the signal transduction, the hyaluronan–receptor interactions act in at least two other important physiological processes: endocytosis of hyaluronan and assembly of pericellular matrices (Toole 2001, Knudson *et al* 2002, Evanko *et al* 2007).

Hyaluronan receptors

Hyaluronan interacts with several cell surface receptors, such as CD44, Rhamm, LYVE-1, HARE/stabilin-2 and Toll-like receptors-2 and 4 (Turley *et al* 2002, Jiang *et al* 2007, Jackson 2009). CD44 is widely distributed, but particularly important in the immune system and inflammatory processes (Jiang *et al* 2007, Johnson and Ruffell 2009), and in diseases such as atherosclerosis and cancer (Toole 2001, 2002). In contrast to CD44, LYVE-1 and HARE, Rhamm does not belong to the linkage module family of hyaluronan-binding proteins. Instead, Rhamm is located either in the cytoplasm or on the cell surface and is an important factor in cell motility in wound-healing processes and diseases such as cancer (Maxwell *et al* 2008). LYVE-1 is closely related to CD44 and is mainly restricted to lymphatic vessel and lymph node endothelia, whereas its function is not well understood (Jackson 2009). In addition, HARE/stabilin-2 is a scavenging receptor that is able to clear hyaluronan and other glycosaminoglycans from the blood vessel circulation (Pandey *et al* 2008). Moreover, the Toll-like receptors recognize hyaluronan fragments during inflammatory reactions (Jiang *et al* 2007).

The major receptors implicated in cancer are CD44 and Rhamm. Thus, the focus is on CD44 during the investigation of cancer progression and metastasis, while it is still important to note that CD44 and Rhamm can exhibit both cooperative and interchangeable signaling functions. Moreover, it has been revealed that interactions at the cell membrane between CD44 and Rhamm activate CD44 signaling through ERK1/2 and subsequently promote cancer cell motility (Maxwell *et al* 2008). In some cases, for instance, in animal models with autoimmune diseases, Rhamm is able to compensate for CD44, which is a very important consideration when interpreting experiments in CD44-null mice (Naor *et al* 2007).

CD44 is a single-chain, single-pass and transmembrane glycoprotein, which is widely expressed in physiological and pathological systems. In more detail, CD44 was first characterized as playing a role in hyaluronan–cell interactions, lymphocyte homing and cell adhesion (Toole 1990) and its role in these phenomena seems to be well established (Johnson and Ruffell 2009, Ponta *et al* 2003). Although CD44 arises from a single gene, numerous transcripts are provided by alternative splicing, in which different (alternative) splice sites are used in order to splice out the introns. The standard and normal CD44 is comprised of the constant, non-variant exon products, whereas the so-called variant isoforms are generated by alternative splicing of numerous additional exon products into a single site within the membrane-proximal region of the ectodomain (Ponta *et al* 2003). In more detail,

cancer cells typically produce several variant forms of CD44 and the standard CD44, whereas some cancer types such as gliomas, mainly produce the standard form of CD44 (Heider *et al* 2004). All forms of CD44 include an N-terminal, membrane-distal, hyaluronan-binding domain, which has significant homology with the hyaluronan-binding region, such as the link module of several other proteins and proteoglycans. Hyaluronan is the most widely studied ligand for CD44, but other ligands are similarly important. The best characterized among these are osteopontin and factors such as FGF and selectin ligands, which are able to recognize carbohydrate side chains covalently bound to CD44 (Ponta *et al* 2003, Sackstein 2009). One of the most interesting aspects of CD44 is its activation upon hyaluronan binding and subsequent signaling. Possible factors contributing to the activation include post-translational modifications of CD44 such as glycosylation, CD44-cytoskeletal interactions, localization of CD44 within specialized domains such as lipid raft domains in the cell membrane, as well as the mode of pericellular organization and presentation of hyaluronan. However, which of these activation factors or steps is the most important remains elusive and will have to be worked out in more detail. Nevertheless, it is common knowledge that numerous cytokines, growth factors and alterations in the cell context can induce the events that result in CD44 activation (Ponta *et al* 2003).

Hyaluronan–CD44 signal transduction processes

In several types of cancer cells, binding of hyaluronan to CD44 leads to a direct or indirect interaction of CD44 with signaling receptors such as ErbB2, EGFR and TGF-β receptor type I, and thus impacts on the activity of these receptors (Toole *et al* 2008, Toole 2004, Bourguignon 2009). However, hyaluronan can subsequently promote the interaction with and the altered activity of non-receptor kinases of the Src family or Ras family GTPases (Bourguignon 2008, Bourguignon 2009). The complex formation with adaptor proteins such as Vav2, Grb2 and Gab-1 facilitates the interaction of CD44 with upstream effectors such as RhoA, Rac1 and Ras, which all induce intracellular signaling pathways (Bourguignon 2008, Bourguignon 2009). Thus, hyaluronan–CD44 binding influences the activity of a variety of downstream signaling pathways, in particular MAP kinase and PI3 kinase/Akt pathways, and consequently supports cancer cell proliferation, survival, motility, invasiveness and chemoresistance (Bourguignon 2008, Toole *et al* 2004, 2008, Turley *et al* 2002, Ponta *et al* 2003, Bourguignon 2009). In addition, binding of hyaluronan to CD44 activates multidrug and metabolic transporters that are important in providing therapy resistance (Toole *et al* 2008, Bourguignon *et al* 2004, Miletti-Gonzalez *et al* 2005, Colone *et al* 2008, Slomiany *et al* 2009a, 2009b) and induces the presentation of proteases that promote cancer cell invasion (Ponta *et al* 2003, Stamenkovic and Yu 2009). Most of the current evidence indicates that these interactions involve specific variants of CD44, while the particular variant almost certainly depends on the type of cancer cell as well as on the stage of malignant progression and in some cases even standard CD44 rather than variant CD44 is critically involved. However, the regulatory mechanisms of these various interactions in different cancer cell types and stages are not yet well understood and need

further investigation, while the widespread deregulation of many normal pathways in cancer cells also contributes to the induction of anomalous involvement of hyaluronan–CD44 interactions that operate normally in other contexts, such as embryonic development (Toole 2001) and inflammation (Jiang *et al* 2007). A related possibility is that deregulated splicing in cancer cells (Skothein and Nees 2007) causes the different CD44 variants that support oncogenic events such as inappropriate Ras signaling (Cheng *et al* 2006) and binding of osteopontin, stromal growth factors and proteases (Ponta *et al* 2003).

In addition to its function as a co-receptor or co-activator of membrane-associated signaling molecules, CD44 may regulate other cellular, disease-associated events, such as cancer cell proliferation and motility through crosslinking to the actin cytoskeleton via ankyrin or members of the ezrin–radixin–moiesin family (Bourguignon 2008, Ponta *et al* 2003, Stamenkovic and Yu 2009). The tumor suppressor merlin has been shown to act by blocking the hyaluronan–CD44 interaction and in addition dissociating the ezrin–radixin–moiesin proteins from the cytoplasmic tail of CD44. In turn, the release of merlin suppression may trigger the activation of hyaluronan–CD44 binding, which then supports the formation of signaling complexes in order to facilitate tumor progression and cancer cell invasiveness (Stamenkovic and Yu 2009). Another regulatory mechanism whereby hyaluronan–CD44 interactions induce intracellular signal transduction pathways functions through the intracellular cleavage of CD44, its translocation of the cytoplasmic part into the nucleus and subsequently its activation of gene transcription (Nagano and Saya 2004).

Many studies indicate that CD44 is localized at least in part to the lipid microdomains with the properties of so-called lipid rafts and that it associates indirectly or directly therein with signaling proteins and transporters. Moreover, most of these studies also demonstrate that CD44 is recruited into these lipid raft domains in response to ligand interactions (Bourguignon 2008, Bourguignon *et al* 2004, Ghatak *et al* 2005, Lee *et al* 2008). In particular, endocytosis of hyaluronan and CD44 occurs from these lipid raft domains (Thankamony and Knudson 2006). Because of the large variety of signal transduction pathways regulated by CD44, there is a strong possibility that indirect and direct interactions with a wide variety of effectors occur within such lipid rafts domains and, consequently, that these domains are induced and/or stabilized by the multivalent interactions of hyaluronan with CD44. Finally, this offers an interesting hypothesis to guide current and future investigations.

A puzzling aspect of many studies investigating hyaluronan-induced oncogenic signal transduction is that they are based on exogenous hyaluronan, which is simply added to cultured cancer cells. Although these studies have led to apparently solid data, which indicate strong effects on the mechanical signal transduction pathways. However, based on these findings, they are difficult to investigate further, while there is a long history of safe utilization of hyaluronan in numerous reconstructive or regenerative experiments performed on human patients. For instance, hyaluronan is used successfully widely in eye and knee surgeries and in the prevention of adhesions (Balazs and Denlinger 1989, Prestwich and Kuo 2008). Moreover, hyaluronan-based

hydrogels have also been developed for a variety of purposes, such as drug delivery, encapsulation of progenitor cells and tissue engineering (Allison and Grande-Allen 2006, Prestwich 2008). Can hyaluronan still be used or is the risk of developing cancer indeed too high? However, such studies suggest that the oncogenic effects of hyaluronan only occur in the context of the tumor microenvironment and that stromal hyaluronan, as well as tumor-cell-produced hyaluronan, play important roles in tumorigenesis, which is supported by correlative studies of numerous human tumor types (Tammi *et al* 2008). Strong evidence for the tumor-promoting effects of hyaluronan have been derived from studies in which tumor hyaluronan levels and interactions with receptors have been manipulated *in vivo*.

Hyaluronan–CD44 interactions

Strong experimental evidence for the involvement of hyaluronan in tumor growth and cancer metastasis has been gained from animal models of several tumor types. The approaches performed include the manipulation of the hyaluronan levels and the perturbation of endogenous hyaluronan–receptor interactions using a number of methods (Itano and Kimata 2008, Toole 2004). In line with this, it has become evident that the turnover of hyaluronan by hyaluronidases is indeed an essential aspect of the promotion of cancer progression by hyaluronan and that the balance of synthesis and degradation is critical (Lokeshwar and Selzer 2008, Simpson and Lokeshwar 2008). Hyaluronan synthesis has been reported to be conditionally increased in mammary tumors that arise spontaneously in MMTV-Neu mice, which highlights the importance of hyaluronan in cancer promotion, especially through the enhanced recruitment of stromal cells and *de novo* induced angiogenesis (Itano and Kimata 2008). As expected, numerous studies have demonstrated that hyaluronan–CD44 interactions have an important role in the recruitment or homing of various cell types, including circulating immune cells and precursor cells (Johnson and Ruffell 2009, Haylock and Nilsson 2006). The MMTV-Neu studies (Itano and Kimata 2008) also revealed the importance of hyaluronan in epithelial-to-mesenchymal transition (EMT). It was found that there are cells that are not able to undergo EMT. In particular, a major defect in the Has2-null mouse is its inability to undergo EMT during early cardiac development (Camenisch *et al* 2000). In turn, the increased expression of Has2 in phenotypically normal epithelium induces EMT characteristics, including anchorage-independent growth and enhanced invasiveness (Zoltan-Jones *et al* 2003, Preca et al 2017), which are two of the major capabilities of malignant cells.

The evidence for the involvement of CD44 in cancer progression is strong, but seems to be very complex. Studies of tumorigenesis in CD44-null mice and manipulation of CD44 levels in various tumor systems have produced contradictory results, while treatments with CD44 antibodies and vaccines have demonstrated the key regulatory role of CD44 in tumor growth and metastasis in mouse models of leukemias and carcinomas (Naor *et al* 2008, Jin *et al* 2006, Krause *et al* 2006, Wallach-Dayan 2008). Several studies have implicated variants of CD44 rather than standard CD44 in cancer progression, however, this is not a unique effect of the CD44 variants for all cancer types, rather it depends on the stage of progression and

type of the tumor (Naor *et al* 2008, Ponta *et al* 2003). A special feature of CD44 is its emergence as a marker for sub-populations of several types of human carcinomas, termed CSCs, which exhibit highly malignant and chemoresistant properties (Visvader and Lindeman 2008, Polyak and Weinberg 2009). In line with this, the characteristics of EMT have recently been connected to the properties of these cell sub-populations. For instance, a $CD44^{+}/CD24^{-}$ subpopulation exhibiting CSC properties was induced through the up-regulation of EMT-associated transcription factors in the primary human breast epithelium and a similar subpopulation exhibiting both EMT and CSC properties was isolated from transformed epithelial cells (Polyak and Weinberg 2009, Hollier *et al* 2009). These cells exhibited anchorage-independent growth of colonies in soft agar, which is a property that usually reflects resistance to apoptosis and in turn seems to be linked to chemoresistance. Numerous studies have revealed that the CSC subpopulation of carcinomas and other tumor types is indeed resistant to chemotherapeutic agents, which is most likely attributable to enhanced anti-apoptotic pathway activity and enrichment of multidrug transporters (Polyak and Weinberg 2009, Toole *et al* 2008, Hollier *et al* 2009). Another important feature of EMT is invasiveness (Kalluri and Weinberg 2009, Turley *et al* 2008) and thus CSCs have been connected to invasiveness and metastasis (Visvader and Lindeman 2008, Polyak and Weinberg 2009, Sleeman *et al* 2007). As noted previously, hyaluronan is closely associated with EMT and in addition these same properties of anchorage-independent growth, resistance to apoptosis, drug resistance and invasiveness are induced or enhanced by up-regulation of hyaluronan synthesis and can be even reversed by antagonists of hyaluronan–CD44 interactions (Toole *et al* 2008, Toole 2004). In more detail, there is strong evidence that hyaluronan-dependent interaction of CD44 with receptor kinases (Toole 2004, Ponta *et al* 2003, Bourguignon 2009) and transporters (Toole *et al* 2008, Slomiany *et al* 2009a, 2009b, Bourguignon *et al* 2004, Milletti-Gonzalez *et al* 2005, Colone *et al* 2008) plays a prominent role in drug resistance and malignancy. The question of whether there are hyaluronan–CD44 interactions in a CSC-like subpopulation of cells isolated from a human patient's ovarian carcinoma ascites has been investigated. It has been demonstrated that CSCs are enriched in receptor tyrosine kinases and ABC-family drug transporters and in addition that these proteins are present in close proximity to CD44 in the cell membrane of the CSCs, while this association depends on constitutive hyaluronan interactions.

Hyaluronan–CD44 interactions in cancer

Although the studies on hyaluronan–CD44 interactions in cancer disease are contradictory and contain paradoxes, agreement has been reached in the field that these interactions are an important target for translation into the clinic. A frequently expressed concern regarding these studies is the widespread expression and the broad variety of cellular functions of hyaluronan and CD44 under normal physiological conditions. However, two observations seem to be promising that therapeutic interventions can be developed targeting oncogenic events with some degree of specificity or differential sensitivity. First, there is the finding that many of the interactions involve variants of CD44, which are amplified greatly in many

tumor types in comparison to normal processes (Naor *et al* 2008, Heider *et al* 2004, Skotheim and Nees 2007). Second, there is the nature of the activation process that leads to hyaluronan–CD44 interactions in malignant cancer cells. Although these processes may have some overlapping features with immune and inflammatory pathways, there are also clear differences, and it may be possible to selectively target them. Some of the studies have utilized antagonists that may ultimately have therapeutic value, but these have not yet reached the clinic.

CD44 antibodies and vaccines

Several studies have revealed that the administration of antibodies against CD44 reduces tumor growth and restricts tumor progression. For instance, injection of monoclonal antibodies directed against CD44 that inhibit the binding of hyaluronan eliminated the invasion of mouse lymphoma cells into local lymph nodes (Naor *et al* 2008). Moreover, blocking antibodies directed against CD44 have been shown to inhibit homing and promote differentiation of acute myeloid leukemic stem cells, consequently eliminating tumor-initiating cells (Jin *et al* 2006). In line with this, prolonged survival also occurred in mice with leukemic stem cells expressing BCR-ABL after treatment with an inhibitory CD44 antibody (Krause *et al* 2006). Indeed, a CD44 variant-based vaccine was designed to decrease mouse mammary carcinoma tumor growth and metastases (Wallach-Dayan *et al* 2008). Moreover, it is recognized in this medical field that these approaches will be greatly improved by tailoring antibodies and vaccines to specific variants of CD44 that are then uniquely expressed during the critical stages of cancer progression (Naor *et al* 2008, Ponta *et al* 2003). However, phase I trials in breast as well as in head and neck carcinoma patients with an antibody directed against CD44v6 have been discontinued due to toxicity (Rupp *et al* 2007, Riechelmann *et al* 2008).

Hyaluronidases

Although constitutive hyaluronidase may support the pro-oncogenic functions of hyaluronan, overexpression or exogenous administration of large amounts of hyaluronidase is usually inhibitory to malignant cancer progression (Lokeshwar and Selzer 2008, Simpson and Lokeshwar 2008, Stern 2008). Hence, hyaluronidase has been used in the clinic for several years as an adjunct to chemotherapy, because it has been supposed to improve the access of drugs to cancer cells through the interference with cancer cell adhesion and their capacity to break through matrix barriers (Lokeshwar and Selzer 2008, Baumgartner *et al* 1998). Indeed, highly purified recombinant hyaluronidase (Frost 2007) is currently being used in a phase I trial for patients with advanced solid tumors. Interestingly, it was also demonstrated that hyaluronidase sensitizes mouse mammary carcinoma cells to chemotherapeutic drugs, if the cells are cultured as drug-resistant spheroids (St Croix *et al* 1998), which is a technique reported to enrich for CSCs. Although hyaluronidase may indeed function in part by reducing barriers to drug diffusion, it is also able to act through its oligosaccharide products, which have been found to inhibit constitutive hyaluronan–CD44 signal transduction processes, resulting in reduced cell survival and induced chemoresistance (Toole *et al* 2008).

Small hyaluronan oligosaccharides

Small oligomers of hyaluronan can suppress anti-apoptotic signaling pathways in cancer cells and thus inhibit the activity of transporters that increase resistance to therapeutic agents (Toole *et al* 2008). Initially, the use of these was based on certain findings that oligomers consisting of three to nine disaccharides bind CD44 monovalently (Lesley *et al* 2000) and displace the hyaluronan polymer from membrane-bound receptors (Underhill and Toole 1979), while another study showed that these oligomers even reduce hyaluronan synthesis (Slomiany *et al* 2009b). Indeed, the treatment of cancer cells with these oligomers leads to disassembly of CD44-transporter and CD44-receptor tyrosine kinase complexes, internalization of the disassembled components and attenuation of CD44 function (Slomiany *et al* 2009a, 2009b, Ghatak *et al* 2005). In addition, treatment *in vivo* with small hyaluronan oligomers suppresses tumor growth and/or even triggers tumor regression in experiments using xenografts of various tumor types, such as melanoma, carcinomas, glioma, osteosarcoma and malignant peripheral nerve sheath tumors (Toole *et al* 2008, Toole 2001, Slomiany *et al* 2009a, Hosono *et al* 2007, Gilg *et al* 2008). One of these studies reported significant effects on cancer metastasis (Hosono *et al* 2007). In addition, significant effects on tumor growth and invasion were demonstrated when CSC-like sub-populations were obtained from a glioma cell line (Gilg *et al* 2008) or from human-patient ovarian carcinoma ascites and used for co-culture or supernatant experiments. Moreover, systemic administration of sub-optimal doses of hyaluronan oligomers were shown to sensitize highly resistant, malignant peripheral nerve sheath tumors to doxorubicin treatment *in vivo* (Slomiany *et al* 2009b). Although this approach might be expected to interfere with all activated hyaluronan–CD44 interactions, malignant tumors appear to be far more sensitive than normal physiological processes.

Targeting drugs to cancer cell CD44

In addition to targeting hyaluronan–CD44 interactions directly, the interactions can be used for the targeted delivery of chemotherapeutic drugs and other anti-cancer agents to cancer cells. It has been reported that increased efficacy in cell and animal tumor models has been obtained through conjugating drugs to hyaluronan, a CD44 antibody, incorporating drugs or siRNAs (gene knockdown) into vehicles such as liposomes, hydrogels and nanoparticles, which are masked with hyaluronan or antibodies against CD44 in order to target them to cancer cells and primary tumors (Platt and Szoka 2008). Indeed, the first human-patient trials with drugs conjugated to CD44 antibody have shown some promising results, although there were complications regarding various toxicities (Platt and Szoka 2008). However, specific targeting to the relevant variants of CD44 is a crucial point of this drug approach. In particular, it has also been revealed that the enormous hydrodynamic domain encompassed by hyaluronan is well suited to entrap drugs, without any need for chemical conjugation, and finally to target them to CD44-expressing tumors (Brown 2008). In line with this, when connected to hyaluronan, irinotecan has demonstrated increased safety and efficacy, and this has been shown to be a promising approach for colorectal carcinoma patients in a pilot trial (Gibbs *et al* 2009).

12.4 Mechanical properties of extracellular matrix fiber networks using magnetic twisting cytometry

The underlying mechanisms through which the mechanical properties of the extracellular matrix affect cell and tissue function are still elusive, as the events regulating this process span size scales from tissue scale to molecular length scales. In addition, the extracellular matrix is composed of an extremely complex hierarchical 3D structure and hence the load distribution relies mainly on the architecture and mechanical properties of extracellular matrix. What are the mechanical properties on the macro- and microscale of 3D collagen fiber matrices? The dynamic rheological probing has been used to investigate the macroscale mechanical properties of 3D collagen matrices. Instead, the microscale mechanical properties of 3D collagen matrices can be determined by using magnetic twisting cytometry (MTC). In more detail, ferromagnetic beads are embedded in the matrix and served as mechanical probes for the exertion of distinct spatially defined regions of the cell. A study on the multiscale mechanical properties of 3D collagen fiber matrices revealed that several of the interesting features between macroscale and microscale 3D mechanical properties measurements originated from the different length scales of measurements.

In particular, at the macroscopic scale, the storage and loss modulus are elevated with the collagen concentrations used for 3D collagen fiber matrix preparation. A nonaffine collagen fibril structural network deformation is important in revealing the macroscopic mechanical properties of 3D collagen fiber matrices. At the microscopic scale, however, the local mechanical properties have been found to be less sensitive to alteration in collagen concentration, as the more immediate/direct deformation of the 3D collagen fibrils in the MTC measurements by the forces exerted through locally membrane receptor adhered ferromagnetic beads. In contrast, the loss modulus is more sensitive to being affected by the local interstitial fluid microenvironment, which causes a rather dramatic elevation in viscosity with frequency, especially at higher frequencies (>10 Hz). Hence, a finite element model has been developed to investigate the geometric factors in the MTC measurements when the 3D collagen matrix was verified to be hyperelastic. In particular, it has been found that the geometric factors are dependent on the collagen concentration or on the stiffness of the matrix, when nonlinear material properties of the 3D matrix are considered, and hence the interpretation of the apparent modulus from MTC measurements need to be performed carefully.

Collagen is a major extracellular matrix component, hence has a prominent role in altering cellular functions and additionally delivers structural and mechanical support for cells and tissues. The type I collagen gel has been largely analyzed in cell–extracellular matrix interaction and cell mediated extracellular matrix remodeling, as it maintains essential parameters of the cell–matrix 3D interactions, although the structural and molecular complexities, which are present *in situ*, are largely not fully covered. By controlling environmental factors, such as temperature, pH and ionic strength, in a physiological manner, the triple helical type I collagen molecules first self-assemble to build collagen fibrils and then form bundles of collagen fibrils,

which further aggregate to finally form collagen fibers (Motte and Kaufman 2013, Yang and Kaufman 2009). Moreover, together with the interstitial fluid, a 3D collagen matrix displaying a hierarchical structure is built. The macroscopic mechanical properties of 3D collagen matrix have been extensively studied using the rheological probing method. The storage and loss modulus have been determined by characterizing the elastic and viscous mechanical properties of 3D collagen fiber matrices, respectively, by varying several parameters such as the collagen concentration (Julias *et al* 2008, Xu *et al* 2011, Raub *et al* 2007).

Indeed, the local mechanical properties of 3D collagen matrices have also been studied by utilizing several microscopic measurement methods. The laser microrheometry technique has been invented to probe the local mechanical properties of the 3D collagen fiber matrix by oscillating a trapped particle (Velegol and Lanni 2001). Using this technique, the force is applied to one bead at a time, so the results can indeed depend on the local heterogeneity (Leung *et al* 2007) and it seems to be highly time-consuming to capture the behavior of a population of beads (Parekh and Velegol 2007). Hence, the MTC seems to be suitable solely for measuring the collective behavior of a population of beads (for high-throughput measurements) and has been utilized to extensively to analyze the viscoelastic microrheology of living cells (Valberg and Butler 1987, Wang *et al* 1993, Fabry *et al* 1999, Maksym *et al* 2000, Laurent *et al* 2002). As cell surface receptors are physically coupled to the cytoskeleton, mechanical properties of the cytoskeleton can be directly determined. The MTC method has been adapted to the measurement of the mechanical properties of 3D type I collagen matrices and indeed it has been shown to be suitable in mimicking the cellular probing and thereby modulation of the mechanical properties of the matrices, such as the stiffness (Leung *et al* 2007).

The MTC method is based on measurements of alterations in a remnant magnetic field. It has been shown that this method includes mostly weakly bound beads, which leads to an overestimated mean angular bead rotation and underestimated mean shear modulus (Fabry *et al* 1999), as the binding strength cannot not be determined in parallel to the measurement. In order to overcome this major limitation, the so-called optical magnetic twisting cytometry (OMTC) method has been developed, in which the bead rotation can be addressed by optically tracking the lateral displacements of the magnetic bead centroids (Fabry *et al* 2001, 2003, Smith *et al* 2003, Deng *et al* 2004, Trepat *et al* 2004, Fredberg and Fabry 2006). In particular, an advantage of the OMTC method is that loose or weakly bound beads or bead clusters can be identified and excluded from further analysis and subsequently the method is more reliable (Fabry *et al* 2001). Moreover, the OMTC method can be employed to determine the local mechanical properties of type I collagen matrix. In more detail, streptavidin and poly L-lysine (PLL) coated ferromagnetic beads are used to obtain a strong bead–matrix binding. The macroscopic mechanical properties of the collagen gel can be determined by using a parallel plate rheometer. Specifically, a 3D finite element model was established to simulate the OMTC measurements, and to investigate the effects of the nonlinear material properties of the extracellular matrix such as matrix geometry and the degree of bead embedding and hence possible endocytosis of the beads on the measurements.

The knowledge of the differences between global and local mechanical properties of the hierarchical 3D extracellular matrix scaffold is quite useful to transfer the mapping of mechanical forces from tissue level to cellular level. Using a micro-rheometer and OMTC measurements, it has been revealed that there are specific alterations between the macro and microscopic mechanical properties of 3D collagen matrices, which leads to the suggestion that microscopic and macroscopic scales of measurements are highly important for characterizing the properties of 3D collagen matrices. In particular, the collagen gel contains a collagen fiber network and interstitial fluid, which corresponds to the solid and fluid phases, respectively, and both phases provide viscoelastic mechanical behavior (Knapp *et al* 1997). Moreover, its frequency- and strain-dependent properties have been extensively investigated using rheological analysis. Within a physiological range of bovine collagen concentrations (such as 2–4.8 mg ml^{-1} collagen type I), the collagen gel is revealed to be a more solid-like viscoelastic material, as the storage modulus is about 10 times that of the loss modulus (Li *et al* 2017), which is consistent with other studies (Yang and Kaufman 2013, Velegol and Lanni 2001, Hsu *et al* 1994). Thus, the collagen gel displays a strain-stiffening behavior under shear (Motte and Kaufman 2013, Stein *et al* 2011, Arevalo *et al* 2011, Kurniawan *et al* 2012). The strain-stiffening behavior is supposed to emerge from the nonaffine deformation and rearrangement of the collagen fibrils within the fibrous collagen network, which has been shown by numerical simulations (Stein *et al* 2011, Onck *et al* 2005, Kang *et al* 2009) and imaging studies (Arevalo *et al* 2011, Vader *et al* 2009, Tower *et al* 2002). In the undeformed state of the 3D collagen matrix, the wavy collagen fibrils are distributed randomly. When exposed to strain, the wavy collagen fibrils start to disentangle, and rearrange in the loading direction, which evokes a transition from a bending-based response at small strains to a stretching-based response at large strains, and hence displays the strain-stiffening behavior of the 3D collagen fiber matrices (Onck *et al* 2005).

As no cross-linking agent has been used, the interaction between collagen fibrils seems to be mostly transient and originate purely from entanglements and weak noncovalent interactions between fibrils (Shayegan and Forde 2013). Macroscopic measurements display the combined response from both elastic and viscous phases (Knapp *et al* 1997). In more detail, the elastic contributions are related to the solid phase in the 3D collagen network (Kurniawan *et al* 2012, Xu and Craig 2011). In particular, the viscous contributions arise from two major parts. First, as the formation of 3D collagen gel deals with weak noncovalent interactions and entanglements, the rearrangement and disentanglement of collagen fibrils contributes mainly to the relaxation of the fibril network under deformation. Second, the viscous contribution is evoked by the movement of interstitial fluid, which is linearly proportional to the rate of deformation, and is small except at high frequencies (Fabry *et al* 2003). In particular, under oscillatory shear at low frequency levels, there is sufficient time for the collagen fibrils to rearrange and disentangle and hence the measured properties are dominated by the equilibrium elastic deformation of the entire network. Physical disentanglements between collagen fibrils appear rather quickly compared to the low oscillatory shear frequency and hence they cannot

contribute much to elastic energy (Grillet *et al* 2012). However, as the frequency increases, there is not enough time for a microstructural rearrangement and the collagen fibrils stay entangled and create so-called 'pseudo-crosslinks' (Wu *et al* 2005), which causes a pronounced increase in storage and loss modulus (Grillet *et al* 2012). At higher frequencies (>10 Hz), the contribution of viscous fluid becomes dominant and causes a faster increase in loss modulus, whereas the storage modulus is nearly unchanged. Indeed, within the range of probing frequency, the local frequency-dependent behavior measured by employing OMTC revealed a similar trend as that measured on a 0.5 mg ml^{-1} collagen gel using optical tweezers (Shayegan and Forde 2013). The finding that the apparent storage and loss modulus from OMTC measurements are not altered by the collagen concentration leads to the hypothesis that the measurements depend on the length scale. In local mechanical property measurements, microscale ferromagnetic beads of an averaged diameter of 4.7 μm have been utilized to locally deform the bound collagen fibrils. The diameter of the bead can be compared to the averaged pore size of the 3D collagen matrix, which was reported to be in the range of 2.3–3.2 μm for the used collagen concentration (Yang *et al* 2009). As the averaged pore size characterizes the averaged distance between the neighboring collagen fibrils, the number of collagen fibrils attached to a single bead is nearly the same among the three collagen concentrations, such as 2.0, 3.0 and 4.8 mg ml^{-1} at the microscale and hence the dependency on collagen concentration is nearly abolished when switching the measurement scales from macro- to microscale.

Indeed, the finding that local properties of the 3D collagen fiber matrices do not match the global properties has been predicted numerically using discrete network models (Stein *et al* 2011, Chandran and Barocas 2006, D'Amore *et al* 2014, Huisman *et al* 2007). These studies revealed that the deformation of the interconnected fibrils in a 3D fibrous network is dominated by nonaffine deformation and the fibrils tend to gradually reorient commonly to the loading direction. It has been demonstrated that fibril strains are evoked by extensive reorientation and also a large portion of fibrils have nearly zero fibril strains, as they are reoriented without fibril stretching during the deformation (Chandran and Barocas 2006, D'Amore *et al* 2014). Hence, only a small fraction of fibrils contributes to all the mechanical properties of a fibrous network. In line with this, the remodeling of the 3D fibrous extracellular matrix by exerted contractile cell forces, alignment of fibers and long force transmission has been shown (Rowe *et al* 2015, Baker *et al* 2015, Babaei *et al* 2016, Alhilash *et al* 2014). The aggregated thick bundles of aligned fibers reach out over very long distances to increase the 3D extracellular matrix networks and affect the entire viscoelasticity of the 3D matrices (Marquez *et al* 2006, Wang *et al* 2014b, Aghvami *et al* 2013). Taken together, the length and time scales of measurements are important when analyzing the 3D extracellular matrix properties, however, the relevance of the length scales for regulating cellular behavior is still not well known. The nondimensional quantity, the phase angle, enables us to directly compare between the measurements at macroscopic and microscopic length scales. Indeed, the phase angle allows us to determine the dissipated energy relative to the stored energy. In particular, an alteration in phase angle in the frequency domain is

commonly associated with alterations of the elastic and viscous parts as the frequency is varied. Within probing a frequency range, the phase angle from the macroscopic rheometer measurements display a decreasing trend, when the collagen concentration is increased. Using the confocal reflectance method, it has been found that the fibril density is elevated at higher collagen concentrations (Motte and Kaufman 2013, Yang and Kaufman 2013, Iordan *et al* 2010, Arevalo *et al* 2010).

A decrease in the phase angle with increasing collagen concentration obtained from rheometer measurements leads to the suggestion that there is even more energy stored relative to the energy that is dissipated, which is caused by an elevated fibril density. However, the phase angle from OMTC measurements revealed independence from the collagen concentration. Thus, this result further supports that the dependence on collagen concentration is nearly abolished in the microscopic mechanical property measurements. Interestingly, when the twisting frequency is increased above 10 Hz, the viscous contribution becomes more pronounced and thereby results in a rapid increase in the phase angle using OMTC measurements. Thus, it can be proposed that the contribution of the viscous effect is more pronounced in the OMTC measurements, as the beads connected to collagen fibrils are surrounded by the 3D matrix interstitial fluid and are even more sensitive to the high frequency dependence at the microscopic local fluid environment.

Indeed, the local measurements seem to have a smaller phase angle compared to the phase angle at macroscales at low frequencies, in particular for the 2.0 and 3.0 mg ml^{-1} 3D collagen matrices. This seems to be based on the more immediate/direct deformation of collagen fibrils in OMTC measurements through the forces exerted by locally bound ferromagnetic beads, as opposed to the nonaffine fiber network deformation in macroscopic measurements, which usually evoke a reorientation of the collagen fibers ruling out the development of the fibril strain (Chandran and Barocas 2006, D'Amore *et al* 2014, Huisman *et al* 2007). The discrepancy in phase angle between macro- and microscopic measurements at low frequencies is reduced when the collagen concentration increases to 4.8 mg ml^{-1}, as the nonaffinity is also reduced due to higher collagen concentration (Onck *et al* 2005), as the assembly of a denser network restricts the free space available for collagen fiber reorientation.

The geometric factor fulfills a critical role in translating the apparent modulus to the shear modulus in OMTC measurement. In particular, the geometric factor has been found to be affected by the degree of bead embedding, matrix thickness (Mijailovich *et al* 2002) and bead radius (Ohayon *et al* 2005). The measurement of the degree of bead embedding is not easily performed and hence requires some effort. Indeed, the degree of bead embedding has been experimentally quantified by using spatial reconstruction of the matrix using confocal microscopy (Laurent *et al* 2002). When using the FEM model, the degree of bead embedding can be estimated, when both the bead rotation and bead lateral displacement are determined (Ohayon *et al* 2005). In previous finite element modeling, only linear elastic material properties have been used (Mijailovich *et al* 2002). The 3D collagen matrix has also been modeled as a nonlinear hyperelastic material (Li *et al* 2017). When comparing the results from Li, the geometric factor curves deviate from

Mijailovich's results (Mijailovich *et al* 2002), which is even more pronounced at smaller degrees of bead embedding. It is known that for the same magnetic torque, the strain in the matrix associated with the bead twisting is higher with a smaller degree of bead embedding. Hence, the effect of the nonlinear hyperelastic material consideration becomes even more significant, as the discrepancies between a hyperelastic and linear elastic model are more pronounced at larger strains. The effective strain is about 10%, for a 20% degree of bead embedding. Hence, this strain is large enough to access the nonlinear stress–strain behavior, as it will deviate significantly from the linear regime. At higher degrees of bead embedding or much smaller strain, the discrepancy between the two regimes vanishes, as the differences in the results using linear and nonlinear material properties are much smaller. Moreover, it has been revealed that the geometric factors are dependent on collagen concentration, or the stiffness of the matrix, when nonlinear hyperelasticity is taken into account. The dependency on material property is omitted when the matrix is modeled as a purely linear elastic material (Mijailovich *et al* 2002). Thus, these results show that the nonlinear material properties of the matrix have an effect on determining the geometric factor, thus the interpretation of the apparent modulus from OMTC measurements is not easy and must be performed carefully.

Several limitations of the current microrheological measurements are that the 3D collagen matrix in the FEM model is assumed to be purely elastic. However, the results of some studies need to be extended to viscoelastic 3D collagen matrices, as supported by Ohayon *et al* (2005) and Kamgoue *et al* (2007), under the assumption that the viscoelasticity of the 3D collagen matrix is indeed strain-independent. In particular, the FEM model allows us to estimate the geometric factors as the degree of embedding of the bead varies. With the geometric factor, the shear modulus can be calculated based on the apparent modulus measurements from OMTC. As the degree of bead embedding is unknown, it is not possible to quantitatively compare between rheological and OMTC measurements. Moreover, the 3D collagen matrix has been assumed to be a homogeneous continuum in the FEM model, which means that based on the FEM model, the local and global mechanical properties should be the same (Mijailovich *et al* 2002, Ohayon *et al* 2005). Indeed, discrete network models have been established to analyze the mechanical and structural behaviors of the 3D fibrous network (Stein *et al* 2011, Onck *et al* 2005, Chandran and Barocas 2006, D'Amore *et al* 2014, Huisman *et al* 2007, Aghvami *et al* 2013, Abhilash *et al* 2014, Hatami-Marbini *et al* 2013). In order to understand the nonaffine matrix reorganization and fiber alignment in response to a local mechanical excitation, a discrete model for the collagen fiber network is necessary. When these limitations are addressed, a better understanding of the connection between the local and global extracellular matrix properties can be provided.

When investigating the multiscale mechanical properties of the 3D collagen matrix, it can be suggested that the many interesting differences between the macro- and microscopic mechanical properties originate from the length scales of measurements (Li *et al* 2017). Macroscale rheometer measurements revealed that the storage and loss modulus are increased with elevated collagen concentrations. Indeed, the nonaffine deformation of the fibril structural network is supposed to fulfill an

important role. At the microscopic scale, the local properties turned out to be less sensitive to alterations of the collagen concentration. However, the loss modulus seems to be more affected by the local interstitial fluid microenvironment, which causes an increase in viscosity at very high frequencies. Nonlinear hyperelastic material properties can be assumed when transforming the apparent modulus, which has been analyzed by OMTC, to the shear modulus. In summary, light has been shed on the multiscale mechanical properties of the 3D extracellular matrix, which seems to be critical when analyzing the transmission of forces from tissue scales to cellular scales (Li *et al* 2017).

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Physics of Cancer, Volume 2 (Second Edition)
Cellular and microenvironmental effects
Claudia Tanja Mierke

Chapter 13

The active role of the tumor stroma in regulating cell invasion

Summary

The genetic, morphological and biochemical alterations in cells undergoing malignant transformation have been the focus of cancer research for several decades. However, a key feature of the malignant progression of cancer, the tumor stroma, is still understudied, but seems to be crucial for the induction of cancer cell motility and subsequently for the emergence of metastases in distinct targeted tissues or organs. Based on the biophysically driven approaches of oncology, our knowledge of cancer progression has become substantially wider and hence it is becoming evident that mechanical aberrations seem to be major contributors to the malignant progression of cancer. In particular, the combined analysis of the biochemical, structural and mechanical properties of the stroma emerges as a key issue for future cancer research, including the advancement of the mechanical probing of the stroma. The impact of the stroma on cellular motility, and hence on the metastatic cascade leading to the malignant progression of cancer, is still an evolving issue and therefore is still controversial, as there are two different and opposing effects within the stroma. On the one hand, the stroma can promote and enhance the proliferation, survival and migration of cancer cells through stroma-facilitated mechanotransduction processes that in turn cause alignment or crosslinking of fibers within cells as a result of increased stroma rigidity. These mechanotransduction-induced alterations of the cytoplasm of cells enables various types of cancer to overcome restrictive biological capabilities. On the other hand, as a result of its structural constraints, the stroma acts as a steric obstacle or physical barrier for cancer cell motility in relatively dense three-dimensional (3D) extracellular matrices, when the pore size is much smaller than the cell's nucleus. In particular, if the structural confinement requires the deformability of the nucleus to be over the limit of nucleus ruptures and subsequently cell death, the migration of the cell is impaired and hence the

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movement is stalled. The mechanical properties of the stroma including the tissue matrix stiffness and the entire architectural network are the major players in yielding the optimal microenvironment for the migration and invasion of cancer cells. Finally, the analysis of the mechanical properties of the stroma and the development or improvement of biophysical methods determining the mechanical properties of the stroma, such as atomic force microscopy or magnetic resonance elastography, are critical for understanding the underlying mechanisms of the stroma–cancer cell interaction, diagnosis and prediction of early cancer stages. Indeed, there is a close connection between fibrogenesis and cancer, as there is an elevated risk of cancer and its malignant procession such as metastasis on cystic fibrosis or, subsequently cirrhosis.

13.1 The stroma enhances malignant cancer progression

The cellular mechanical properties are emerging as a quantitative measure to distinguish between physiological and various pathological states of single cells including cancer disease. In this novel research field, termed the physics of cancer, a lot of research involves the comparison of normal and cancer cell mechanical properties. A clear result has not yet been revealed, as the biophysical methods applied to measure the mechanical properties of cells differ in the dimensionality, adhesiveness, polarization, morphology and the cytoskeletal organization of cells. However, several studies have supported the hypothesis that cancer cells are softer than their normal healthy counterparts and, moreover, these softer cells display increased migratory capacity into 3D extracellular matrix invasion assays. However, there are also various studies stating that cancer cells or cells with increased migratory capacity need to be stiffer. At the first glance this view seems to be contradictory, but it may depend on the mechanical microstate of the cell during the measurement, which may not reflect the mechanical state during the aggressive and invasive migration of the cell through the tissue matrix.

In situ, the primary tumor is usually stiffer compared to the surrounding normal tissue, termed the tumor stroma. The adaption of an increased stiffness feature by tumors is one of the key hallmarks of cancer. There exists a lot of evidence for an altered mechanical phenotype that is not solely an effect of the malignant progression itself, but it rather seems to directly facilitate cancer initiation and cancer progression such as the formation of metastases (Suresh 2007, Lekka *et al* 2012a, Lekka and Laidler 2009, Lekka *et al* 2012b, Goetz *et al* 2011, Butcher *et al* 2009, Cross *et al* 2007, DuFort *et al* 2011, Hayashi and Iwata 2015, Katira *et al* 2013, Wirtz *et al* 2011, Friedl and Alexander 2011, Ciasca *et al* 2016). The cells become pronouncedly softer after increasing time after cell harvesting and cells of confluent cell cultures are softer than cells of subconfluent cultures (Lange *et al* 2017). The cellular stiffness (the Young's modulus) increases when the adhesion of the cells is reduced by adding the non-ionic surfactant pluronic F-68, which impairs cell adhesion to glass (Lange *et al* 2017). Moreover, previous reports have established that cell mechanical properties can be stress- and strain-sensitive (Levental *et al* 2007, Gardel *et al* 2006, Wang *et al* 1993, Bursac *et al* 2007, Mierke *et al* 2010, Mierke *et al* 2011a).

Why are there obvious discrepancies between the stiff primary tumor and the soft malignant cancer cells? As not every cancer cell or collection of cancer cells needs to be able to migrate out of the primary tumor and invade the surrounding stroma, there is no reason why there might not be a subgroup of aggressive and highly invasive cancer cells within the tumor mass that exhibit decreased mechanical stiffness compared to the other major tumor cell mass or the healthy cells of the same cell type. Moreover, it has been demonstrated that there exist different mechanical subgroups in cancer cell lines such as breast, lung or kidney cancers. This substantiates the hypothesis that the extracellular matrix plays a crucial role in the regulation of cancer cell properties and function. In particular, rearrangements in the extracellular matrix evoke alterations in the mechanical properties of cancer cells and even under specific conditions the general hypothesis about the softening of cancer cells can even be disproved. The contribution of the extracellular matrix in proving the mechanical phenotype of cancer cells and the finding that cancer cells are softer than normal healthy cells needs to be analyzed together with the mechanical properties of the cellular microenvironment such as the stroma and related to the specific stage or grade of the cancer cell progression. The mechanical phenotype of cancer cells depends on the stroma's mechanical phenotype, topography such as pore size and chemical properties.

Cancer is a complex disease that is composed of multistep processes facilitating the malignant progression of cancer, such as the initial transformation of normal cells into malignant cancer cells. It has been revealed that primary tumors do not consist of only uncontrolled proliferating cells, they can in fact be treated as heterogeneous tissues which contain various distinct cell types, such as endothelial cells, pericytes, inflammatory immune cells or cancer-associated fibroblasts that have been recruited during tumorigenesis through biochemical signals to the tumor mass and hence fulfill their role in the progression of cancer (figure 13.1) (Egeblad *et al* 2010).

During cancer progression, the cells adopt specific properties, which are identified as the hallmarks of cancer and hence strongly affect their state and functions. In more detail, they promote their own growth by secreting growth factors, possess insensitivity to growth reducing signals, are resistant to the programmed cell death (termed apoptosis), can replicate unlimitedly, facilitate angiogenesis by endothelial growth signal secretion, and promote cell invasion and cancer metastasis in distant targeted organs or tissues (Hanahan and Weinberg 2000). Indeed, the supportive role of the extracellular matrix in the control of multiple cellular responses has been revealed (Hanahan and Weinberg 2011, Weigelt *et al* 2014, Pickup *et al* 2014). Based on these findings it seems to be clear that tumorigenic processes and their therapeutic approaches need to model cancer as a disease of collections of cells and not just as a single cell and hence requires the integration of the contribution of the surrounding microenvironment including their biochemical and biophysical properties (Lu *et al* 2012). In particular, the cells are affected continuously by the extracellular matrix and the reverse, the microenvironment is continuously altered by the cells. Alterations in the extracellular matrix subsequently cause disorders in cell behavior and from the inverse viewpoint of the cells, they rearrange, secrete and degrade

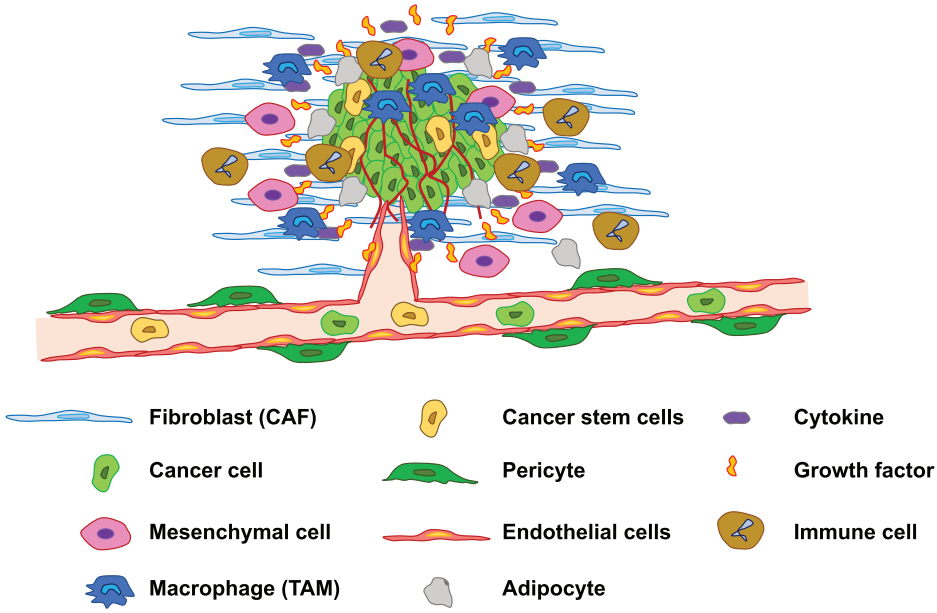

Figure 13.1. Interaction of immune cells, fibroblasts, mesenchymal cells, pericytes and endothelial cells in a primary tumor.

extracellular matrix components during cellular physiological and pathological processes (Wells 2008, Discher *et al* 2005, Bonnans *et al* 2014). During cancer initiation and progression, the extracellular matrix dynamically changes its properties, which in turn affect or promote tumor progression, such as cancer cell invasion and the formation of metastases in distant organs (Stewart *et al* 2004, Lu *et al* 2012, Provenzano *et al* 2006, Nelson *et al* 2014, Giussani *et al* 2015, Seewaldt 2014). In line with this model, many different tools such as hydrogels or patterned structures have been employed to mirror the properties of the extracellular connective tissues and determine their precise effect on the cancer cell's functions, such as mechanical properties, cell migration, transmigration or invasion (Kraning-Rush and Reinhart-King 2012, Rianna and Radmacher 2016a, Pathak and Kumar 2012, Alvarez-Elizondo and Weihs 2017, Fischer *et al* 2017).

The cell's mechanical properties represent the most important feature among the diverse biophysical properties of a cell and have emerged as a promising quantitative tool to unravel the distinct features of cellular processes in the field of regenerative medicine and tissue engineering (Shieh and Athanasiou 2003) and in the diagnosis of numerous pathological conditions such as cancer (Suresh *et al* 2005, Suresh 2007). In cancer biology, cell mechanical properties can be utilized to distinguish between healthy and cancerous states, as malignant cancer cells undergo a dramatic challenge in stiffness compared to their normal healthy counterparts. Several biophysical approaches are available to determine the cellular mechanics under suspended or adherent culture conditions, such as micropipette aspiration (Lee and Liu 2014, Pachenari *et al* 2014) as well as the optical cell stretcher (Guck *et al* 2005)

for measuring the mechanics of whole cells in suspensions, and atomic force microscopy (AFM) for the investigation of single adherent cells (Radmacher *et al* 1992, Lekka *et al* 2012a) as well as modified AFM with a flat cantilever for the measurement of suspended cells (Fischer *et al* 2017). In contrast to optical cell stretchers and micropipette aspiration, AFM is suitable for studying the cellular mechanics under physiological conditions, which enables us to decipher the impact of matrix properties on cellular behavior (Rianna and Radmacher 2016a, Acerbi *et al* 2015, Raczkowska *et al* 2017).

The general finding that cancer cells are softer than their normal counterparts has initially emerged from many AFM measurements on normal and cancer cells. However, the majority of experiments have been performed using conventional cell cultures such as Petri-dishes, which are stiff, unstructured and flat compared to natural tissue microenvironments. Thus, the cells experience a microenvironment which is from a mechanical point-of-view vastly different from the physiological condition in tissues, additionally, the chemical and topographical aspects are also neglected in this experimental set-up. Hence, the mechanical properties measured depend largely on the mechanical properties of the culture substrate and the stiffness measurement difference may differ from matrices with tunable mechanical and/or topographical properties. Is the analysis of the mechanical properties of cancer cells optimally suited for AFM compared to other biophysical approaches? In order to determine the effect of the extracellular matrix properties on cancer cell behavior, an adherent biophysical method needs to be utilized for determining the cell's mechanical properties, as the cells need to be undisturbed and attached to the underlying natural or synthetic matrices, which mimic the natural extracellular matrix of tissues in the mechanobiological field of cancer research. Why is it important to investigate the mechanical properties of normal and cancer cells together with the microenvironmental properties?

13.2 Biomechanical alterations in cancer cells

A close connection between the mechanical properties of cells as well as entire tissues with human diseases has been established. Moreover, in many diseases alterations in cellular mechanical properties have been found, which leads to the suggestion of cellular mechanical properties as a marker for the identification of pathological states (Rianna and Radmacher 2016a). In cancer disease, the aggressive and invasive cells possess biophysical and biomechanical properties providing their mechanical phenotype, which seems to be required for their migratory capacity. The results on the stiffness of cancer cells are dependent on the biophysical approach and are contradictory, as some studies state that highly invasive cancer cells are stiffer than normal or less invasive cells such as human breast cancer cells (Mierke *et al* 2011a and 2011b), hepatocellular cancer cells (Zhang *et al* 2002), cervical carcinoma cells (Ding *et al* 2015), and myeloid and lymphoid leukemia cells (Rosenbluth *et al* 2006), whereas multiple other studies state that highly invasive cancer cells are associated with higher deformability and decreased stiffness, such as bladder carcinoma, breast carcinoma, chondrosarcoma cells, ovary carcinoma, thyroid carcinoma, cervix

carcinoma and prostate carcinoma cells (Lekka 2016). In comparative measurements of adherent and non-adherent breast cancer carcinoma cell measurements using AFM, it has been shown that the highly invasive cells are softer compared to the normal control cells (Fischer *et al* 2017), which indicates that the adherence of cells does not cause the different results, it may rather be the specific manner of the mechanical probing of the cells, such as the applied force (force regime) and the force application region (entire cell versus coupling to certain receptors or local regions of the cells). However, the reason why cancer cells are softer than their normal counterparts is yet not fully understood and needs further investigation. Alterations in the structure of the cytoskeleton are proposed to fulfill a key regulatory role (Hall 2009, Wirtz *et al* 2011). Nevertheless, during various processes in cancer disease, such as invasion and formation of metastases, the cancer cells invade into the surrounding stroma, rupture their cell–cell adherence junctions, remodel their cell–matrix focal adhesion sites and possibly move along a chemoattractive gradient path through the extracellular matrix network of the stroma (Yilmaz and Christofori 2009). During all these consecutive steps cancer cells alter their cell shape, which is facilitated by the remodeling of their cytoskeleton and is hence correlated with alterations in their mechanical properties. Moreover, in the process of tissue indentation, the metastatic cancer cells exert higher forces towards the surrounding matrix stroma in order to rupture extracellular matrix components or their connections and migrate to new targeted locations with a novel tissue, which in turn impacts the mechanical properties of the matrix as well as the mechanical properties of the cells strongly.

What forces are applied by metastatic cancer cells towards the local microenvironment during their migration and invasion? What are the time and length scales of the interaction between cancer cells and soft or stiff matrices? Indeed, the interaction of metastatic cancer cells with soft gel matrices have been analyzed, which mirrors the interaction with the compliant extracellular matrix during the invasion of cancer cells (Kristal-Muscal *et al* 2015). Using traction force microscopy, it has been revealed that highly metastatic cells can exert higher forces, which indent the gel or displace fiducial markers inside the gels that indicates a correlation with the metastatic potential, depth of indentation or marker displacement, and cellular forces (Fischer *et al* 2017, Alvarez-Elizondo and Weihs 2017, Kristal-Muscal *et al* 2013, Dvir *et al* 2015, Mierke *et al* 2008, Mierke *et al* 2011a, 2011b, Mierke 2013). In line with these results, the invasion of cancer cells into collagen I matrices evoked a stiffening of the fully embedded cells compared to their non-invasive counterparts, that adhered on top of the gels but did not invade (Staunton *et al* 2016). In particular, the exertion of strong forces by invading cancer cells to indent tenaciously soft matrices is correlated to their own stiffness.

Using AFM, the mechanical properties of normal and cancer thyroidal cells are analyzed on polyacrylamide gels with diverging stiffness compared to standard cell culture dishes and it has been found that cancer cells are softer compared to normal cells cultured on plastic cell culture dishes (Rianna and Radmacher 2016a). In contrast to plastic surfaces, on soft gels with Young's moduli in the rage of 3–5 kPa, cancer cells are stiffer than their normal counterparts. This observation leads to the

following questions: Are cancer cells really softer than normal cells or does the stiffness depend on the microenvironmental stiffness? Do cancer cells, during all steps of cancer disease, exhibit a constant stiffness value? Is the stiffness of cancer cells adaptable to the individual step of cancer progression? What are the underlying mechanisms causing a softening of cancer cells? There are questions raised on which intracellular factors or components are causing the softening of cancer cells (Alibert *et al* 2017). However, there exists still the basic hypothesis that cancer cells need to soften and be highly deformable to migrate even through small spaces during the process of cancer metastasis. The hypothesis seems to be useful to explain the behavior of cancer cells during all the steps and barriers that they need to overcome to move forward from their primary tumor mass location to the secondary metastatic sites in targeted organs or tissues. However, the criticisms leveled at the hypothesis are based on two main aspects. First, cancerous tissues are usually stiffer than their healthy counterparts and the stiffening of the stroma promotes the induction of tumor rigidity and malignant progression (Lu *et al* 2012). As cells are mechanosensitive, they sense and respond to the microenvironmental mechanical properties (Discher *et al* 2005) and thereby an elevated stiffness of cancer cells seems to be expected when the tumor stroma stiffens.

Second, during the epithelial–mesenchymal transition (EMT), cancer cells pronouncedly reorganize their actin cytoskeleton (Yilmaz and Christofori 2009), which alters their cellular mechanical properties accordingly. In addition, the mechanical properties of cancer cells can be altered due to different environmental properties during their trafficking through the tissues and organs. For each step of the metastatic cascade and the entire tumor progression, many different mechanical properties are needed to fulfill each function successfully. In particular, in the first step of the metastasis formation in cancer, the invasion of cancer cells into the surrounding tumor stroma network, the cells need to be stiff and strong in order to transmigrate through the basement membrane, to degrade extracellular matrix components and withstand the pressure produced by the rigid tumor mass (Friedl and Alexander 2011, Clark and Vignjevic 2015). Thus, the mechanical properties of cancer cells are assumed to dynamically adapt to the special requirements of a certain microenvironment to survive them during their migration track through the tumor stroma, blood or lymph vessels and their targeted normal organ or tissue. All these observations lead to the hypothesis that the softening of cancer cells compared to normal counterparts depends on the specific tumorigenic step and the specific microenvironments, and may therefore also be reverted to a stiffening, if necessary for the further progression of cancer. Thus, the main question whether cancer cells are really softer than their healthy counterparts is not yet answered.

13.3 Extracellular matrix evoked alterations in cancer cell functions

A fibrillary network can be created with fibronectin, which is known to regulate the behavior of cells under both physiological and diseased conditions such as cancer. Indeed, stromal cells, which are associated with breast cancer, increase the amount of secreted fibronectin and they even modify the conformation of fibronectin.

However, whether there exists a functional association between the mechanical and conformational properties of these early primary tumor-associated fibronectin networks and its overall effect on the neovascularization of primary tumors is not yet well characterized. Using a surface force apparatus, it has been shown that 3T3-L1 pre-adipocytes stimulated with tumor-secreted factors produce a stiffer fibronectin matrix than control cells not treated with these factors. As determined by Förster resonance energy transfer, the initial extracellular matrix stiffening is associated with enhanced molecular unfolding of fibronectin fibers, which hence can no longer be easily elastically deformed. In summary, the resulting alterations in cell–matrix adhesion and the secretion of proangiogenic factors such as VEGF of newly seeded 3T3-L1 fibroblasts are determined and combined with the disturbed integrin specificity, which seems to be a potential mechanism of altered cell–matrix interactions through integrin inhibitors. In particular, tumor-modified fibronectin impairs the cell–matrix adhesion during increased VEGF secretion by pre-adipocytes, which leads to an integrin switch that evokes the alterations in adhesiveness and adhesion strength (Wang *et al* 2015). Both the simultaneous stiffening of the stroma microenvironment and unfolding of initially deposited tumor-modified fibronectin reduce first cell–matrix adhesion and then the proangiogenic behavior of the surrounding stromal cells, which induces neovascularization and hence the growth of the primary breast tumor. These results increase our knowledge of the cell–fibronectin interactions with the extracellular matrix stroma, which can be used to produce and develop biomaterials-based applications for advanced tissue engineering approaches. Finally, the cellular behavior is driven by various physicochemical properties of the extracellular matrix, dynamical remodeling and the structural architecture of the collagen fiber complex.

In primary solid tumors, the extracellular matrix proteins are produced mostly by cancer-associated cells such as fibroblasts and adipogenic precursors and provides the continuous growth and survival of the primary tumor (Spaeth *et al* 2009, Mishra *et al* 2008, Karnoub *et al* 2007, Chandler *et al* 2012, Butcher *et al* 2009, Kumar and Weaver 2009, Lu *et al* 2012, Paszek *et al* 2005). The tumor-surrounding extracellular matrix stroma possesses various altered material properties relative to the physiological normal extracellular matrix scaffold, which displays variations in the overall protein composition, their structure and their individual or network rigidity. The analysis of tumorous extracellular matrix networks identified alterations in collagen I deposition compared to normal extracellular matrix networks that have been proposed to contain elevated levels of certain extracellular matrix proteins, display a restructured organization, possess additional crosslinking proteins and subsequently altered stiffness of these collagen fiber networks (Chandler *et al* 2012, Fischbach *et al* 2009, Calvo *et al* 2013, Levental *et al* 2009, Provenzano *et al* 2006, 2009). In particular, fibronectin seems to be the driving factor in secondary structural modifications of the extracellular matrix, since highly stretched and unfolded fibronectin fibers have been detected in tumor-associated stromal matrices (Mammoto *et al* 2009, Chandler *et al* 2011). Tumor-associated fibronectin and collagen modifications are functionally connected, as fibronectin is required for the deposition of collagen I within the extracellular stroma (Chandler *et al* 2012, Midwood *et al* 2004, Sottile and

Hocking 2002, Anderson 2001) and it is also a marker for enhanced tumor aggressiveness (Zhang *et al* 2004). Taken together, a correlation between fibronectin in this process and the structural, conformational and mechanical properties of the tumor stroma network has been revealed. This correlation is impaired to a certain degree by the intrinsic composition of the extracellular matrix assembled complex and by the inability to analyze the extracellular matrix as well as cellular properties *in vivo* and compare the results to the molecular scale. However, both collagen and fibronectin fibers are found to be connected in the mature extracellular matrix and seem to synergize in the regulation of bulk properties of the tumor extracellular stroma (Sottile and Hocking 2002, Curran and Keely 2013). In particular, it is currently not possible to separately analyze the morphology and mechanical properties of native and hence non-crosslinked extracellular matrix at different length-scales such as the matrix-, cellular- and molecular-scales under normal physiological conditions.

Challenges in the material properties of the tumor extracellular matrix micro-environment are clinically highly important as they may promote the malignant progression of cancer through induction of direct effects in cancer cells (Paszek *et al* 2005) and indirect effect through the increased neo-angiogenesis driven formation of new blood vessels (Chandler *et al* 2012, Fischbach *et al* 2009, Calvo *et al* 2013, Levental *et al* 2009, Provenzano *et al* 2006, 2009). In particular, the altered extracellular matrix increases the angiogenesis through enhanced proliferative activity of surrounding endothelial cells (Mammoto *et al* 2009, Chandler *et al* 2011) or through the induction of the secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF) by cancer-associated fibroblasts (CAFs) (Chandler *et al* 2012, Midwood *et al* 2004, Sottile and Hocking 2002, Anderson 2001). The specific requirements of the extracellular matrix properties and associated mechanisms responsible for this altered functional process increasing the proangiogenic capability of tumor-associated cells are not yet fully understood and require more investigation. However, several biophysical approaches can be used in cancer biology to determine the mechanical properties, conformation and the of tumor-surrounding fibronectin matrices at the matrix and molecular length-scales, and the material properties associated with cell–matrix adhesion and the secretion of proangiogenic factors such as VEGF by adipose stromal cells. Indeed, tumor-modified fibronectin matrices are stiffer and more unfolded compared to control matrices and in turn these disordered matrices evoked the increased VEGF secretion by stromal cells. In line with these results, it can be proposed that breast cancer cell-secreted factors alter the early fibronectin matrix assembly driven by stromal cells. Indeed, enhanced amounts of fibronectin are observed in primary tumors (Stenman and Vaheri 1981), and additionally plasma fibronectin seems to play a role in tumor growth (von Au *et al* 2013). Moreover, the mechanical and the structural or conformational properties of fibronectin seem to be altered in tumor stroma compared to control stroma, as matrices modulated by stromal cells stimulated by tumor-secreted factors displayed enhanced overall stiffness, increased fiber stretching and enhanced molecular unfolding. These alterations of fibronectin are related to the increased proangiogenic capability of the stromal cells that in turn affect the angiogenesis of the primary tumor. As assumed, tumor-modified matrices are

mechanically stiffer than normal counterparts. Hence, tumor-modified matrices seem to exhibit slower alterations in indentation depth when probed with a cantilever, indicating elastic and time-dependent viscous changes. These matrices are structurally and conformationally modified across multiple length-scales, since they are thicker, denser and assembled of thicker fibers, which are formed by more unfolded fibronectin molecules. By combining the surface force apparatus (SFA) approach with FRET-dependent mapping, a detailed picture of the early deposited fibronectin matrix from the matrix/cellular level to the molecular level can be revealed (Rianna and Radmacher 2016a). These results show direct involvement of fibronectin-driven tumor stroma progression. In line with these results are FRET analyses on a single-cell level, where the strain application to manually extruded fibronectin fibers caused a stiffening and unfolding occurring above 150% strain of these fibronectin fibers (Smith *et al* 2007). Another FRET analysis of the entire matrix calculated the average strain in cell-derived fibronectin fibers based on a FRET versus strain calibration measured on individual single manually extruded fibers, but the matrix stiffening was solely estimated, as the matrix stiffness was not measured directly (Chandler *et al* 2011). However, these results show a quantitative and direct correlation between overall fibronectin matrix stiffness and the topology at the matrix/cellular scale with the configuration of fibronectin at the molecular scale (Chandler *et al* 2011). The fixation of tissue or matrix samples has a large effect on the mechanical and structural properties of compliant (of the 0.1 kPa range) and porous biomaterials and should be avoided. Indeed, although the general stiffening and unfolding behavior is still maintained for tumor-modified matrices compared to control matrices, the fixative formalin elevates the rigidity and reduces the strain (indicated by a FRET increase). In control fibronectin matrices it can particularly be observed that the random crosslinking of lysine residues frequently leads to the pinning of relaxed fibronectin fibers within the matrix network. Indeed, the stiffness of tumor-modified fibronectin matrices is lower than for macroscopic tumors *in vivo* (Samani *et al* 2007) and single fibronectin fibers (Klotzsch *et al* 2009). These differences can be caused by: the higher porosity of cell-depleted fibronectin networks (with increased fluid transport) than the denser tumor tissue; the presence of both fibronectin and collagen in mature tumor tissues; and the different deformation regimes of individual fibers relative to entangled fibers in the extracellular matrix networks. In the latter, the deformation (or compression) is distributed over a broad scaffold of disordered and connected fibers that respond in a collective manner to stress by initially aligning/ordering along the compressive surface before they are compressed by indentation. In addition, creep results reveal a trend of slower responses of tumor-modified matrices than their control counterparts, with characteristic slow decay times, which are 10–100 times slower that the values calculated using measured diffusion constants for cell and tissues (Moeendarbary *et al* 2013, Rosenbluth *et al* 2008). Although poro-elastic processes affect the biphasic system relaxation at shorter timescales, the longer decay times seem to be related to viscoelasticity properties, such as the conformational/structural alterations of the entire fibronectin network, rather than to the redistribution of the solvent through the matrix pores.

Moreover, to generate cell-derived fibronectin matrices, a well-established protocol can be utilized (Castello-Cros and Cukierman 2009) comprising a mild detergent and multiple washing steps. The decellularization of matrices relaxes these matrix networks by approximately 20% (Kubow *et al* 2009) and changes the ratio of soluble (nascent) to insoluble (crosslinked) fibronectin in these 3D matrices (McKeown-Longo and Mosher 1983), as the soluble fibronectin has been washed out. These effects caused by the decellularization of the matrices are not expected to alter the results of the relative differences between control and tumor-modified decellularized matrices. However, the contribution of both relaxation and loss of fibrillar heterogeneity of these matrices on mechanical properties need to be determined, which may require the development of novel methods for the decellularization procedure.

Moreover, the topology of the matrix, such as a denser network and thicker fibers, and the molecular unfolding of the initial fibronectin matrix may both indirectly affect the overall mechanical properties of the tumor-modified matrix. The fibronectin unfolding may evoke the deposition of an increased unfolded fibronectin matrix (Antia *et al* 2008) or in contrast, altered fibronectin characteristics may alter tumor stiffness by disrupting the collagen I binding site or exposing cryptic sites with enzymatic activity such as fibronectin type IV collagenase, which belongs to the matrix metalloproteases and degrades collagen (Schnepel and Tschesche 2000). Thus, the unfolding of fibronectin can also indirectly affect the mechano-signaling of tumor-associated collagen I that finally facilitates the tumorigenesis (Levental *et al* 2009, Provenzano *et al* 2006, 2008). When untreated stromal cells are seeded onto tumor-associated matrices, they showed reduced adhesion and increased VEGF secretion. As tumor-modified fibronectin matrices displayed topological, conformational, and mechanical alterations, which can in turn alter the cell–matrix interactions, the fibronectin changes seem to function as a mechano-sensor that provides a so-called integrin switch.

Tumor-modified fibronectin extracellular matrices have been revealed to be dense matrices containing thick fibers. These thicker fibers can affect the ligand density, which then changes the stability of the focal adhesion formation and subsequently the downstream cellular behavior (Cavalcanti-Adam *et al* 2007, Arnold *et al* 2009, Geiger *et al* 2009, Bradshaw and Smith 2013). The enhanced ligand density seems to represent another mechanism behind altered cell–fibronectin matrix interactions within tumors, and thus, it needs to be figured out how antagonizing RGD binding sites are affecting (possible in a dose-dependent manner) the subsequent cell behavior in tumors. Cells with enhanced VEGF secretion on tumor-associated matrices, which contain mainly stretched/unfolded fibronectin fibers, prefer $\alpha v\beta 3$ integrins, instead of the classical fibronectin receptor, the $\alpha 5\beta 1$ integrin, to bind to fibronectin. This behavior may depend on the distinct engagement of either strain-sensitive integrins, such $\alpha 5\beta 1$, or strain-insensitive integrins, such as $\alpha v\beta 3$, with the surrounding matrix. Indeed, the integrin binding site FnIII9–III10 of fibronectin is highly sensitive to conformational alterations due to increased tension exerted by cells. The distance between the synergy binding site to fibronectin PHSRN site in the FnIII9 domain and the RGD site to fibronectin in the FnIII10 domain is crucial for

the engagement and activation of $\alpha 5\beta 1$ integrins (Obara *et al* 1988, Aota *et al* 1991), where it has only a negligible effect on engagement of $\alpha v\beta 3$ integrins (figure 13.2). Thus, the so-called integrin switch observed on tumor-modified matrices can be explained to a certain degree by the strain-induced increased separation between the FnIII9 and FnIII10 domain (Krammer *et al* 2002), which abolishes the binding of $\alpha 5\beta 1$ to both fibronectin binding sites simultaneously (Pierschbacher and Ruoslahti 1984) and hence forces the cells to bind fibronectin more through $\alpha v\beta 3$ as a compensatory effect (Petrie *et al* 2006). Indeed, a higher engagement of $\alpha v\beta 3$ integrins elevates the VEGF secretion (Wan *et al* 2013, De *et al* 2005). In line with this result, the $\beta 1$ inhibition of cells seeded on control (relaxed) fibronectin affected the secretion of VEGF to a larger extent than the αv inhibition of cells on tumor-modified (stretched) fibronectin. This result may be explained through the availability of the VEGF receptor, as VEGF is immobilized to fibronectin through an $\alpha 5\beta 1$ integrin–VEGF-receptor interaction (Wijelath *et al* 2002). Thus, the immobilization of VEGF inhibits the accessibility of $\alpha 5\beta 1$ integrins that causes in turn a VEGF release. A second explanation for the functional difference may be that $\alpha 5\beta 1$ possesses a higher binding affinity (4 nM) to fibronectin (Takagi *et al* 2003) than $\alpha v\beta 3$ (1.3 mM) (Liu *et al* 2010). The increase in the secretion of VEGF can even be further affected by the physicochemical complexity of the tumor-modified extracellular matrix. This complexity is not solely based on the alterations in fibronectin stiffness and conformation, which encompasses the different spatial distribution of ligands at the fiber surface, but is also based on variations in quantity and composition, such as proteoglycans, which can additionally also facilitate the switching between the integrins. The difference in VEGF secretion is small, but still significant, and comparable to small differences in

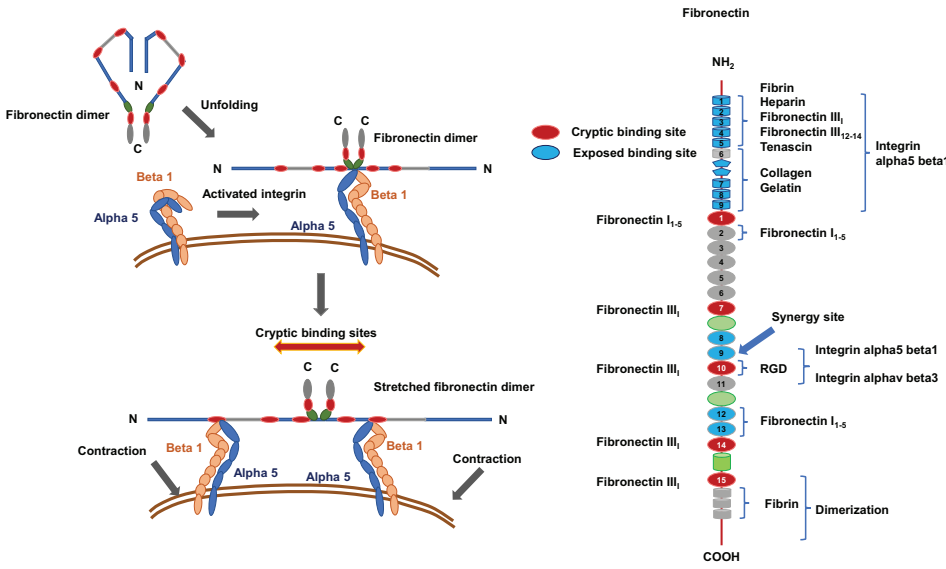

Figure 13.2. A fibronectin dimer binds to the $\alpha 5\beta 1$ integrin, which leads to the exposure of cryptic binding sites in the fibronectin dimer. Hence the integrins can be crosslinked (left panel). Structure of fibronectin (right panel).

VEGF secretion on dense mature extracellular matrix networks, which altered pronouncedly the function of endothelial cells (Wan *et al* 2013).

Finally, the results on the early fibronectin matrix assembly in regulating the proangiogenic factor secretion of breast tumor-modified stroma have revealed more insights on the involved mechanisms. However, the role of fibronectin in 3D tissue microenvironments needs to be investigated in more detail, as all the studies described have been performed on cell-derived fibronectin-matrix coated mica surfaces, where both substrates belong to extracellular matrix components, as the fibronectin fibers additionally act as templates for the collagen deposition (Sottile *et al* 2007). In more detail, the specific binding of the collagen I a1 chain to the gelatin-binding domain of fibronectin, which is located on FnI6, FnII1–2, and Fn III7–9, is required for the initial co-deposition of collagen (Schnepel and Tschesche 2000). As the interaction of fibronectin with collagen seems to be dependent on its conformation, the unfolded and hence highly strained fibronectin fibers, which are initially generated by 3T3-L1 cells, may pronouncedly alter the fibrillogenesis of collagen through the rigidity and culture dimensionality and subsequently facilitate alterations in the behavior of cells. Moreover, the mica substrates are usually stiffer than the tumor tissue, which can alter the mechanical properties and the conformation of the deposited fibronectin matrix. Similarly, the assembly of focal adhesions differs in 2D and 3D cell cultures (Fraley *et al* 2010), which additionally alters the mechanical and structural properties of the fibronectin matrix. Finally, it needs to be determined whether the observed results on the integrin-dependent VEGF secretion using the artificial mica substrates are transferable to a physiologically relevant 3D matrix exhibiting controlled stiffness and topology, which may then provide discrimination between the roles of stiffness and conformation (Janmey and Weitz 2004) and subsequently reveal new insights for refining the anti-VEGF therapies (Cao and Langer 2010).

13.4 Biomechanical alterations in multicellular spheroids

Multicellular spheroids can be utilized as a reliable platform to investigate the behavior of tissues and the growth of tumors in a precisely controlled 3D microenvironment. While spheroids have been utilized for molecular and cellular studies to investigate the cell behavior in 3D, only a few studies have employed multicellular spheroids that have revealed a key role for the mechanical properties of the microenvironment in a wide variety of cellular processes such as the malignant progression of cancer (Lucio *et al* 2017). Although the effect of the mechanical cues on cellular behavior is well-established, based on numerous approaches utilizing 2D cell culture systems for the investigation of cell mechanical properties, the spatial and temporal alterations in the endogenous cellular forces when the cells are embedded in growing multicellular aggregates still remain elusive. Using cell-sized oil droplets of known and tunable physicochemical properties as markers for the transduction of forces in mesenchymal cell aggregates, the magnitude of cell-generated stresses can be determined and has been observed to vary only slightly with spatial location within the spherical aggregate, whereas the stresses increases considerably over time during the compaction and growth of the entire aggregate (Lucio *et al* 2017).

Moreover, it has been shown that the temporal increase in cellular stresses is evoked by increasing cell pulling forces, which are transmitted to the stroma through integrin-facilitated cell adhesion that is consistent with the requirement of higher intercellular pulling forces for the compaction of cell aggregates.

The development of cell culture techniques has revealed many insights on molecular and cell biological effects, which are investigated under highly controlled biochemical conditions, compared to the complex and rather uncontrolled *in vivo* systems. In particular, 2D cell monolayers have been extensively utilized in cell culture studies, however, these conditions poorly mimic *in vivo* tissue conditions (Eyckmans and Chen 2017, Baker and Chen 2012). Therefore, in multiple cases, there exist key differences between 2D cell cultures and 3D tissues such as altered cellular shape and morphology, size, gene expression and proliferation (Edmondson *et al* 2014). Advanced 3D cell culture techniques can eliminate various problems of 2D cell cultures, as they more closely replicate the physiological conditions within the tissue, while still allowing high-throughput screenings for various applications such as drug testing (Breslin and O'Driscoll 2013, Friedrich *et al* 2009). Multicellular spheroids are composed of 3D aggregates of adherent cells, exhibit an overall spherical morphology and still possess the defining features of 3D tissues through their cell–cell and cell–matrix interactions (Fennema *et al* 2013, Lin and Chang 2008). Additionally, chemical gradients can be established in spheroids larger than 150–200 μm in diameter due to their 3D geometry, which makes them suitable to model systems for the formation of tumors, as they are often even subject to internal hypoxic regions and proliferative gradients (Oudin and Weaver 2017, Hirschhaeuser *et al* 2010). In addition to these chemical gradients, alterations in physical quantities such as cellular forces may even provide a key feature in the formation and the homeostasis of 3D multicellular spheroids (Oudin and Weaver 2017).

The exertion of external forces and alterations in the local mechanical properties of the microenvironment challenge cellular behavior in 2D cell culture systems (Wyatt *et al* 2015, Grashoff *et al* 2010, Trepat *et al* 2009, du Roure *et al* 2005, Tan *et al* 2003, Dembo and Wang 1999), during cellular development (Mammoto *et al* 2013, Miller and Davidson 2013, Nelson and Bissell 2006) and the progression of cancer disease in 3D tissues (Bissell and Hines 2011, Wirtz *et al* 2011, Kumar and Weaver 2009, Huang and Ingber 2005, Paszek *et al* 2005). Mostly, our knowledge of the ability of cells to generate forces and respond to mechanical cues has been gained from sophisticated advances in biophysical approaches to determine the cellular forces in 2D multicellular systems, such as cell monolayers cultured in *in vitro* 2D systems (Grashoff *et al* 2010, Trepat *et al* 2009, du Roure *et al* 2005, Tan *et al* 2003, Dembo and Wang 1999, Raupach *et al* 2007) and in 3D systems, where the cells are embedded in artificial 3D gel matrices (Polacheck and Chen 2016, Legant *et al* 2010). In contrast, our knowledge of the force generation and transmission in cells in 3D multicellular systems is not yet clearly understood. Various studies have a focus on the role of the mechanical extracellular microenvironment on the development of multicellular 3D aggregates and the endogenous distributions of the cell-transmitted stresses that assemble and continuously maintain the emergence of multicellular aggregates and their growth in 3D microenvironments, whereas their function remains elusive.

Indeed, it has been proposed that there exist internal, endogenous spatial variations in cellular stresses within 3D cellular spheroids and tumors (Roose *et al* 2003, Delarue *et al* 2013, 2014, Dolega *et al* 2017), which have been directly detected by using droplets embedded in the spheroids (Lucio *et al* 2017). Internal stresses have been estimated by physically cutting excised primary tumors, which is a rather crude non-physiological method, but nonetheless using the resulting deformation of the tissue and associated mathematical modeling the amount of these forces can be deduced (Stylianopoulos *et al* 2012). Based on these experimental and mathematical procedures, compressive stresses within murine tumors can estimated to be between 0.37–8.01 kPa (Stylianopoulos *et al* 2012). In addition, less invasive methods are employed to determine the maximal value of stresses generated by the cancer cell spheroids. In particular, the resistance to spheroid growth through the usage of elastic microcapsules (Alessandri *et al* 2013) or gels of varying stiffness (Khavari *et al* 2016, Helmlinger *et al* 1997) and the osmotic pressure exerted on the surface of spheroids (Delarue *et al* 2014) are utilized to determine the bulk isotropic mechanical stresses that spheroids can generate on the surrounding space, such as the microcapsules. Moreover, elastic microbeads have been employed to analyze the effects of mechanical compression on the propagation of isotropic stresses within the tumor spheroids (Delarue *et al* 2014). Although these mechanical stresses within the aggregates have been assessed only through confinement and external compression, they can also be used to determine the spatiotemporal fluctuations of endogenous stresses during the initial spheroid growth and subsequent compaction. An advanced technique uses oil droplets instead of microcapsules to analyze the cell-generated stresses *in situ* within the 3D multicellular spheroids (Campàs *et al* 2013). The technique employs fluorescently labeled cell-sized oil droplets as force transducers, which are directly injected between the neighboring cells in a multicellular 3D spheroid (Shelton *et al* 2018). Indeed, in proof-of-principle experiments, the droplets can be utilized to determine the cell-generated stresses within cellular spheroids of mammary epithelial cells and tooth mesenchymal cells (Campàs *et al* 2013). However, the limitations are on the precise control of the physical and chemical properties of these droplets and the confidence in non-commercial (self-produced components) reduced the practical use of droplets as force sensors in various situations. In order to overcome these limitations, a new system of droplet stress sensors has been established, which is fully based on commercially available components and with precisely controlled physical and chemical properties (Lucio *et al* 2017). The usage of these precisely defined droplets enables the quantification of the spatiotemporal alterations in endogenous, anisotropic, cell-generated stresses in growing multicellular spheroids of mouse tooth mesenchymal cells (Campàs *et al* 2013). In order to independently regulate the droplet interfacial tension and the interactions between the cells and the droplet, a two-surfactant system has been used that enables stress measurements in chemical environments with various ionic strengths or in the presence of small active surface-molecules, which are present in the cell culture media. When these droplets are injected in growing spheroids of tooth mesenchymal cells at different spatial locations and stages (at various time points), the spatiotemporal alterations in endogenous cell-generated stresses can be

determined. The magnitude of cell-generated anisotropic stresses does not vary pronouncedly with the location within the spheroid, whereas the magnitudes increases over time-scale, as the entire spheroid grows and becomes compact. The comparison of the stresses measured using droplets, which are either coated with or without ligands targeting distinct integrin receptors, has revealed that the pulling forces exerted through integrin-dependent adhesion increase with time the during the spheroid compaction process. These pioneering measurements provide a first glance into the spatiotemporal alterations in cell-generated stresses inside multicellular spheroids and have the capacity to be established as a widely used model system for cell behavior analysis in 3D microenvironments (Lucio *et al* 2017). The use of the two-surfactant coatings of the droplets and the microfluidics system for droplet generation establish a controlled system for droplet production. The new two-surfactant system of droplets is composed of the commerically-available fluorinated surfactant Krytose PEG (600) regulating the interfactial tension and a commercially available functionalized phospholipid surfactant DSPE-PEG(2000)-biotin facilitating the cell-droplet interactions through streptavidin-conjugated ligands aiming cell surface adhesion receptors. The droplet interfacial tension can be altered by varying the concentration of fluorosurfactant or the type of fluorocarbon oil employed. In particular, increasing the fluorosurfactant concentration reduced the droplet interfacial tension, such as a ten-fold decrease by using Novec 7700. Apart from a slight reduction in interfacial tension in the presence of cell culture medium of approximately 8% compared to Novec 7700 fluorocarbon oil, the new two-surfactant system largely protects the interface from alterations in ionic strength and the presence of small, surface-active molecules of the medium. Previous droplet stress sensors (Campàs *et al* 2013) relied solely on ionic surfactants and hence the interfacial tension of the droplet was highly dependent on the ionic strength of the medium. The constant and precisely controlled interfacial tension of the new two-surfactant system and the ability to functionalize the droplet surface with biotinylated molecules provides a helpful tool to measure of cell-generated stresses in a wide range of systems, such as living embryos (Nelson and Bissell 2006, Campàs *et al* 2016, Servane *et al* 2017 Heisenberg and Bellaiche 2013), tissue explants, biopsies or resections (Campàs *et al* 2013, Campàs *et al* 2016, Zhou *et al* 2015), embryoid bodies (Pettinato *et al* 2014, Itskovitz-Eldor *et al* 2000), organoids (Fatehullah *et al* 2016, Lancaster and Knoblich 2014) and also multicellular spheroids of cancer cells (Oudin and Weaver 2017, Paszek *et al* 2005, Delarue *et al* 2013, 2014, Butcher *et al* 2009).

The spatial dependence of the magnitudes of anisotropic stresses is minor within the spheroid and reveals that cell-generated stresses are similar throughout the spheroid. The anisotropic stresses measured with drops coated with ligands targeting integrins revealed that the cells generate increased pulling, integrin-dependent forces over time, when the spheroid grows and compacts then drops without coating. This result suggests that the cells cannot transmit forces through integrin receptors in loose aggregates (at 24 h) compared to the situation in more compact aggregates (after 48 h). However, various other mechanisms may be responsible for the increase of integrin-dependent forces, such as a mechanical feedback mechanism by which cells are able generate larger stresses in response to enhanced cellular contacts, an increased amount

of extracellular matrix content or to alterations in the mechanical properties of the local cellular microenvironment. Regardless of the origin of these increased integrin-dependent pulling forces, they mainly contribute to the compaction and cohesiveness of the spheroid. Indeed, integrins have been demonstrated to facilitate pronounced cohesion between mesenchymal cells in the surrounding 3D extracellular matrix (Robinson *et al* 2003). Droplets coated with RGD peptides targeting integrin receptors and non-adhesive droplets coated with PEG (control) have been compared and the temporal enhancement in anisotropic stresses revealed that purely integrin-facilitated cell-generated forces account for the differences. However, it needs to be considered whether other forces of different origin may also be involved in droplet deformations, which is not easy to rule out.

The magnitude of the average anisotropic stresses is on the order of 0.5 kPa (Lucio *et al* 2017), at first glance different to measurements of anisotropic stresses in mesenchymal cell spheroids using simple oil droplets (Campàs *et al* 2013), in which values of the maximal (rather than average) anisotropic stresses of about 1.5 kPa are determined. However, analysis of the distribution of anisotropic stresses revealed that the maximal values are approximately 1 kPa, which shows that both studies are in agreement and the differences are within experimental error despite a five-fold difference in the droplet interfacial tensions used in both studies (Campàs *et al* 2013, Lucio *et al* 2017). Besides these measurements of the endogenous, anisotropic stresses of spheroids, several works have estimated the isotropic stress component generated by the entire growth of the spheroid (Alessandri *et al* 2013, Helmlinger *et al* 1997). Tumor spheroids confined in elastic microcapsules exerted isotropic stresses on the order of 2 kPa (Alessandri *et al* 2013). When spheroids are cultured within agarose gels of different stiffness, the isotropic stress levels are in the range of 3.7–16.0 kPa (Helmlinger *et al* 1997). However, these values are larger than the droplet results, but they measure a different mechanical quantity, the bulk isotropic stress, which is applied by the entire spheroid onto the surrounding confining substrate. In general, the local stress anisotropy at the cellular scale (the cell-generated anisotropic stresses) and the global isotropic stresses generated by the entire spheroid are different quantities and may hence exhibit largely different values. Elastic microbeads have been utilized to analyze spatial variations in the transmission of supracellular, isotropic stresses evoked by externally applied osmotic pressures within spheroids of cancer cells (Delarue *et al* 2014). Under an applied surface stress of 5 kPa, the isotropic stresses inside the spheroids of cancer cells ranged from <1–4 kPa, with the highest stress in the core of the spheroid.

Indeed, the investigation of mechanical stresses in *in vitro* cell culture systems such as multicellular spheroids increases the understanding of how the mechanical cues together with biochemical cues regulate cellular behavior in 3D multicellular environments. In particular, the development of novel biophysical tools, such as droplet sensors or elastic microbeads, help to gain access to complementary information on different physical quantities within 3D multicellular spheroids compared to the overall surrounding microenvironmental properties. All these novel approaches will even help to increase current knowledge on how molecular cues affect cell behavior, such as cell migration and force exertion within these systems. Finally, these physics-based

tools have the potential to deliver significant new insights into the role of mechanical properties in tumor initiation and malignant progression.

13.5 Biomechanical alterations of extracellular matrix stroma in cancer

The malignant progression of cells is associated with specific alterations of the extracellular mechanical properties such as stiffness and adhesion strength (Katira *et al* 2013). When transformed cancer cells grow into primary solid tumors, they interact with their surrounding matrix microenvironment through the physical connections to matrix proteins and the exertion of forces, and thereby this interaction strongly modulates various properties of the extracellular matrix, such as stiffness, pore size, structure and crosslinks. Thus, biomechanical alterations do not only affect single cells during malignant cancer progression, they also affect the extracellular matrix and the entire microenvironment surrounding the tumor. In various soft tissues such as breast tissue, cancer can usually be diagnosed at an early stage by palpation, due to the rigid nature of malignant cancerous tissues compared to the soft healthy neighboring tissue. In particular, breast tissues can become at least ten times stiffer than normal tissue during tumorigenesis (Levental *et al* 2009). Many factors such as chemical or mechanical parameters can cause the stiffening of the tissue. In particular, lysyl oxidase (LOX), which is an enzyme that crosslinks collagen and elastin, is found to be elevatedly expressed in many cancerous tissues (Le *et al* 2009, Barker *et al* 2011). The LOX overexpression elevates the stiffness of the extracellular matrix and facilitates cancer cell invasion and malignant progression (Levental *et al* 2009). Additionally, cancer cells can enable the recruitment of various cells such as fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells and lymphocytes into the nearby surrounding stroma. The massive recruitment of distinct cells may also facilitate the stiffening of the primary tumor and finally leads to tumor hardness at a macroscopic scale. Simultaneously, cancer-associated fibroblasts (CAFs) remodel the tumor stroma, first by secreting additional extracellular matrix proteins and enzymes that covalently crosslink the collagens fibers of the stroma and, second, by pulling the entire collagen network (Egeblad *et al* 2010, Kalluri and Zeisberg 2006), which results in a stiffer stromal network (Clark and Vignjevic 2015).

13.6 Stromal influence on the behavior of cancer cells

The extracellular matrix consists of a broad variety of molecules such as collagen, laminin, elastin, glycosoaminoglycans (GAG), proteoglycans and adhesive glycoproteins. Differences in the combination and the spatial localization of these substances causes a broad variety of scaffold types that identifies and distinguishes the different tissues and organs. The extracellular matrix fulfills a fundamental role in cellular behavior and fate, as it not only sustains and connects cells, but also regulates multiple cellular functions such as differentiation, migration and mechanical properties, under physiological and pathological conditions such as cancer (Stern *et al* 2009, Ulrich *et al* 2009). Among all these, the cellular mechanical properties are pronouncedly altered by the properties of the local matrix through

sensing and adapting to different mechanical or topographical signals. The strong coupling between mechanical properties of the substrate and those of cells has been extensively analyzed. In particular, in the field of mechanobiology, a large number of studies has proposed that matrix stiffness affects the cell's mechanical properties through phenomena termed mechanotransduction processes (Wells 2008).

Indeed, cells are able to sense the softness or the rigidity of the surrounding matrix and adapt their mechanical properties (Chen *et al* 2012). Under healthy conditions, the cells usually increase their stiffness when the stiffness of the matrix is accordingly elevated and cells try to adapt their mechanical properties close to the mechanical properties of their substrate, if the stiffness of the matrix is too high and hence non-physiological (Solon *et al* 2007). This behavior seems to be strongly guided by alterations in the internal structure and organization of the cytoskeleton, as on stiff matrices cells assemble more pronounced stress fibers (Pourati *et al* 1998, Georges and Janmey 2005), increase the expression of integrins (Yeung *et al* 2005), display a more spread phenotype (Pelham and Wang 1997) and elevate the size and the amount of focal adhesions (Balaban *et al* 2001). However, the response to matrix stiffness still depends on the specific cell type (Georges and Janmey 2005) and mainly on the natural properties of the tissue in which these cells are usually located *in vivo*. Extreme examples of the differences in spreading behavior are neurons and fibroblasts that become maximally spread at substrate stiffness of 0.5 or 10 kPa, respectively (Yeung *et al* 2005, Flanagan *et al* 2002). In general, most cell types spread and adhere more strongly to stiffer matrices and these factors seem to be regulated by their mechanical properties (Zhu *et al* 2000, Reinhart-King *et al* 2005).

In fact, the matrix stiffness fulfills a key role in cancer and hence is involved in the regulation of tumor progression including cell growth, cancer cell invasion and the formation of metastases (Seewaldt 2014, Clark and Vignjevic 2015). Alterations in cellular and extracellular mechanical properties of the stroma during the malignant transformation of cells in turn cause alterations in the forces acting on the cancer cells and thereby alter their morphogenetic evolution, proliferation and invasiveness (Katira *et al* 2013, Lopez *et al* 2008). The matrix stiffness has been shown to regulate proliferation and the chemotherapeutic resistance of hepatocellular carcinoma cells (Schrader *et al* 2011). The viability of breast cancer cells has been reported to be precisely correlated inversely to the substrate elasticity, as when the number of cells decreases, the substrate elasticity is enhanced (Cavo *et al* 2016). Thus, the highest proliferation rate of cells can be found on the softest gel. Moreover, elevated matrix stiffness affects the induction of a malignant phenotype in mammary epithelial cells (Paszek *et al* 2005) and regulates the motility and the organization of the cytoskeleton of glioma cells (Ulrich *et al* 2009).

Regarding the effect of matrix stiffness on cancer cell mechanical properties, thyroidal cancer cells displayed an altered response on different stiffness gels than their normal counterparts (Rianna and Radmacher 2016b). In particular, both elastic and viscous properties of normal thyroidal cells are increased with enhanced substrate stiffness, whereas the cancer thyroidal cells are insensitive to alterations in matrix stiffness tuned using purely elastic polyacrylamide gels. In addition, normal cells cultured on plastic culture dishes with an elastic modulus of

approximately 1–2 GPa are stiffer than cancer cells, whereas normal cells cultured on soft gels with an elastic modulus of 3–5 kPa are softer than the cancer cells, which further supports the crucial role of the matrix in the comparison of cell mechanical properties between various cell types. In line with this result, another study demonstrated the limited response of cancer cells to be adapted to variations in stiffness, as the proliferation rate of several cancer cell types has been analyzed (Lin *et al* 2015, Tilghman *et al* 2010). The behavior of normal, cancer and metastatic renal cells has been investigated on substrates mimicking the mechanical properties of the local surrounding tissue (Rianna and Radmacher 2017). Indeed, it has been shown that metastatic cells are stiffer than their normal counterparts when cultured on 3 kPa soft gels. Therefore, these experiments need to be refined by using gel indentation for the analysis of the behavior of the metastatic cancer cells. Moreover, the interactions between cells and serum proteins have not been ignored, and they may have an impact on the alterations between normal and cancer cell mechanical properties.

In addition to mechanical modifications, alterations of the tumor microenvironment are additionally caused by topographical restructuring, which pronouncedly affects cancer cell behavior. In particular, at the tumor–stroma interface, collagen fibers translocate, align and orientate perpendicularly to the tumor boundary (termed tumor-associated collagen signatures TACS-3), whereas the collagen fibers in normal healthy stroma are still randomly oriented and anisotropic (Provenzano *et al* 2006, Conklin *et al* 2011, Conklin and Keely 2012). Moreover, the aligned collagen bundles correlate positively with a poor prognosis and decreased treatment efficacy (Provenzano *et al* 2006, Conklin *et al* 2011). Therefore, the investigation of cancer cell function on structured collagen bundles or other aligned biomimetic fibers has become the focus of several reports. Examples are both collagen fibers (Egeblad *et al* 2010) and polycaprolactone nanofiber scaffolds manufactured via electrospinning (Nelson *et al* 2014) that indeed served as highways or migration tracks for cancer cells.

In summary, the investigations of the mechanotransduction phenomena and mechanobiology of cancer cells cannot simply be based on the investigation of cells under non-physiological conditions of very stiff and homogeneously flat cell culture supports such as conventional cell culture dishes. Moreover, the crucial role of the extracellular matrix needs to be taken into account in terms of mechanical, topographical and biochemical properties. However, there is still more needed in the development of new culture tools to reveal phenomena that are currently not well understood.

13.7 How can the extracellular matrix of the stroma be mimicked?

A required first step for the establishment of connections among biomechanical pathways in the context of human diseases is to investigate cellular phenomena under conditions closely mimicking the *in vivo* conditions within living tissues. Hence, the recruitment of synthetic or biological approaches are needed in order to mirror the physiological state of tissues in cell culture systems.

13.7.1 2D and 3D matrices

In the field of cancer research, most *in vitro* analysis has been performed in 2D cell culture models such as glass or plastic cell culture dishes. Although the advantages of 2D models are their simplicity, lower cost and broad distribution in the field of biology and bioengineering, they still fail in mirroring the native tumor microenvironment adequately in terms of cell–cell and cell–matrix interactions. The standard culture dishes are very stiff and hence fail to mimic the mechanical properties of the natural extracellular matrix and, additionally, they are flat and thus cannot mimic the topographical structures of natural tissues. Thus, culture systems mimicking biological conditions are required to bridge the gap between the standard conventional 2D culture systems and the highly complex native structures. Indeed, the field of biomaterials has produced advances in 2D cell culture systems that can replicate some of the natural ECM properties, such as patterned glass substrates, elastomeric films, hydrogels, ceramics and foams (Lutolf and Hubbel 2005).

Hydrogels, which consist of crosslinked polymer networks with high water content, are optimal candidates in mimicking the mechanical properties of the extracellular matrix and are hence ideal candidates, as they support cell adhesion, facilitate protein absorption and, importantly, their stiffness and swelling can easily be tuned and varied (Tibbitt and Anseth 2009, Caliari and Burdick 2016). Specifically, hydrogels are classified into natural, such as collagen, fibrin or alginate, or synthetic, such as polyacrylamide or polyethylene glycol materials, or hybrid systems such as hyaluronic acid or polypeptides. Most of these hydrogels can be used both as 2D and 3D systems, as the cells can be cultured on top of or embedded within these matrices, respectively. However, among these hydrogels are polyacrylamide gels that can only be used as a 2D matrix, as the components are toxic before polymerization. In both dimensionalities, hydrogels are used in various cancer applications and have revealed critical phenomena determining cellular behavior and drug treatment. In particular, healthy mammary epithelial cells displayed tumorigenic potential in a 2D monolayer culture, whereas they assembled into multicellular spherical structures when embedded in a 3D basement membrane-derived hydrogel (Petersen *et al* 1992). Using more sophisticated hydrogels as a model for drug screening, the cancer cells possess larger resistance to chemotherapies when cultured on stiff collagen-rich substrates compared to cultures on softer materials (Nguyen *et al* 2014). 3D matrices based on hyaluronic acid have been utilized to investigate human glioblastoma multiforme, which is the most aggressive and invasive type of brain cancer (Pedron *et al* 2013, Rape *et al* 2014, Ananthanarayanan *et al* 2011). The choice of an appropriate system is critical and depends on the type of investigation. Indeed, cancer cells grown as 2D monolayers loose specific signaling pathways which affect their behavior (Luca *et al* 2013, Baker and Chen 2012). Cancer cells act differently when cultured on a 2D or 3D matrix in terms of gene expression, growth factor inhibition and response to drug treatments (Cavo *et al* 2016, Luca *et al* 2013, Benton *et al* 2009, Shin *et al* 2013, DelNero *et al* 2015). All of this demonstrates the necessity to refine the experiments

performed in 2D systems in 3D extracellular matrices, such as the analysis of the cancer cell invasive potential or the testing of anticancer drugs for cancer therapy.

13.7.2 Collagen-based hydrogel matrices

At least 30% of total mammalian protein mass is provide by variants of collagen types, which are the major component of the extracellular matrix (Myllyharju and Kivirikko 2004). Among these matrix proteins, type I collagen is the main structural component in the interstitial extracellular matrix, whereas type IV collagen is a key component of the basement membrane (Boot-Handford and Tuckwell 2003) that aggressive and invasive cancer cells penetrate at the beginning of the metastatic cascade involving cell migration (Bhowmick *et al* 2015). As collagens play a crucial role in providing cellular functions, they are commonly used for the fabrication of natural 2D and 3D matrices in order to analyze cell proliferation, migration and invasion (Schor 1980, Takata *et al* 2007) as well as the contraction of entire tissue resections (Ngo *et al* 2006). Another example of cancer research studies is a 3D *in vitro* model containing type I collagen gel in order to investigate the effect of the extracellular matrix in the determination of phenotype of MCF-7 breast cancer cells (Krause *et al* 2010). In more depth, the kinetics of MCF-7 breast cancer cell and HT29 colon cancer cell invasion into 3D collagen I gel was revealed using time-lapse microscopy and real-time analysis (Sakai *et al* 2011). A fully automated 3D tracking set-up based on collagen gels has developed to analyze the infiltration and migration of the human HT-1080 fibrosarcoma and MDA-MB-231 adenocarcinoma cells (Demou and McIntire 2002) and advanced 3D tracking has been shown that allows the analysis of several thousand cells in a fixed time point invasion assay (Fischer *et al* 2017, Kunschmann *et al* 2017). Moreover, even distinct migratory and infiltration modes of the two cell types can be identified, such as the high motility phenotype on the smooth collagen matrix surface of HT-1080 cells (Demou and McIntire 2002). In order to reveal the capacity of individual cancer cells to migrate and evaluate how effectively pharmaceutical drugs can impair the first step of invasion, it has been shown that the migration of cancer cell into reconstituted type I collagen gel through a combination of frozen sectioning and azan staining is suitable (Fukuda *et al* 2014). Indeed, matrix elasticity plays a key role in cancer cell invasion and migration, as stiff 3D collagen I matrices have been shown to enhance the tumorigenic prolactin signaling that is involved in the progression of breast cancer and facilitated other protumorigenic outcomes such elevated matrix metalloproteinase-dependent invasion and the realignment of the collagen scaffold (Barcus *et al* 2013). Taken together, these studies demonstrated that matrices based on natural extracellular matrix components such as collagen and also fibrin (Liu *et al* 2012) are a promising tool for the investigation of biological processes of normal and cancer cells such as tissue invasion, which is not possible to be analyzed on stiff and impenetrable matrices.

13.7.3 Cell-free tissue extracellular matrices

Another emerging strategy is the fabrication of matrices that can mimic the natural extracellular matrix, as decellularized matrices of natural biological tissues are

utilized for cell culturing. The removal of the cells from their tissue and subsequently slicing the remnant matrix using a cryostat, enables the generation of a natural network that is available for the investigation of cell–extracellular matrix interactions under physiological conditions. When the primary cells are removed, these decellularized matrices contain only extracellular matrix components and indeed mimic the natural surroundings of cells' *in vivo* confinements. Dissimilar to 3D hydrogels, which mimic only a few properties of the extracellular matrix, decellularized tissue matrices display the full range of variation and complexity of the natural extracellular matrix network. The decellularization of tissue can include several steps, such as physical, chemical or enzymatic procedures. The physical procedures include mainly freezing/thawing processes, sonication and mechanical agitation. The enzymatic procedures utilize trypsin and the chemical approaches use detergents such as Triton X100 and sodium dodecyl sulfate (SDS), however, often both procedures are combined to obtain optimal results.

Several tissue types have been utilized to produce and characterize diverse decellularization methods. In particular, the mechanical properties of lung decellularized tissues have been analyzed using AFM (Jorba *et al* 2017), which revealed the usage of 0.1% Triton X100 and 1% SDS as an effective chemical decellularization process, which maintains the extracellular matrix structure, its composition and the mechanical properties. To obtain human decellularized dermal matrix, primary cells need to be removed using a freezing and thawing protocol and finally the surface topography of the matrix can be characterized using peak force tapping mode of AFM. Then, these matrices can be directly employed for usage as a cell culture scaffold, in which the cells are placed on top and migrate vertically into them to build an artificial tissue.

Indeed, biological response has been reported on decellularized tissues. Human fibroblasts have been analyzed during their culture on six different groups of decellularized matrices (all with enzymatic decellularization procedures) in order to reveal a specific candidate of decellularized dermal matrix that is highly useful as an autologous skin graft (Labus *et al* 2017). Decellularized matrices have been utilized as xenogenous dermal matrices for breast reconstruction and hernia repair (Mirastschijski *et al* 2013). Indeed, the vertical proliferation, apoptosis and differentiation of fibroblast cells has been analyzed on human decellularized dermal matrix and matriderm, which represents a commercially available matrix (Mirastschijski *et al* 2013). In addition to decellularized matrices derived from human tissues, the usage of decellularized plant leaves, such as so-called green technology, has emerged in tissue engineering for the preparation of natural scaffolds for biological applications. Indeed, human mesenchymal stem cells and human pluripotent stem cell, which are derived cardiomyocytes, managed to adhere to and maintain their functionality on these plant-based scaffolds (Gershlak *et al* 2017).

Dissimilar to synthetic or hybrid 2D and 3D systems, native extracellular matrices precisely present a large number of signals and components that are fundamental for revealing the intriguing mechanisms at the cell–matrix level. Indeed, a comparative study on 2D cell monolayers, 3D spheroids and 3D native

decellularized tissue models has found that 3D decellularized models serve as a more suitable platform to mimic the mechanical properties of human native dermal tissue and conduct biological high-throughput experiments (Pillet *et al* 2017). To maintain fundamental features of the natural extracellular matrix such as composition, stiffness, ligand presentation and topography, the decellularized matrices seem to be promising for gaining deep insights in cellular functions. In particular, for enlightened processes of complex diseases such as cancer the usage of decellularized matrices is highly recommended and may help in understanding critical processes such as cell mechanical properties, invasion and migration during metastasis formation. Until now, only few applications have been observed in the field of biological cancer research, such as the engineered acellular tumor 3D scaffolds, which have been utilized to investigate the proliferation and growth factor expression of MCF-7 breast cancer cells (Lu *et al* 2014). Indeed, comparing the repopulation behavior of different cancer cells on an acellular tumor matrix treated with Tris–Trypsin–Triton (a combination of enzymatic and chemical procedures) revealed an improved modeling of the 3D tumor scaffold in performing cancer cell analyses. An increase in chemoresistance during tumor progression on decellularized matrices has been identified (Hoshiba and Tanaka 2016). Additionally, these decellularized matrices have been directly generated from tumor tissues at different stages of malignancy in order to investigate the 5-fluorouracil resistance of these diverse matrices. Thus, in cancer research, these decellularized 3D matrices may provide a realistic scenario that will help in obtaining deep insights in cellular behavior and functions, compared to previous studies on cell cultures on plastic substrates or 2D cell monolayers, rebuilding the natural microenvironment and evaluating the impact of the *in vivo* context.

Currently, the focus in research on the microenvironment of tumors is on how the mechanical properties of the cancer cells are changed and what impact it has on cellular behavior and biochemical properties or signal transduction processes. In particular, tumors are recognized as heterogeneous tissues, which are composed not only of uncontrollable cells but also of various microenvironments that actively regulate malignant cancer progression. When investigating the cancer cell's mechanical properties and comparing normal and cancerous cells, the key role of extracellular matrix properties should be adequately addressed. However, the hypothesis that cancer cells are really softer than their normal counterparts needs to be reconsidered, since this property may be related to the substrate stiffness. In several cases, it has been shown that cancer cells are stiffer than normal cells, when these two cell types are plated on substrates with stiffness comparable to the physiological tissue environment. Mechanical alterations of cancer cells and the extracellular matrix emphasize the key role of extracellular matrix properties for the morphology and function of cancer cells, and therefore some of the main strategies mimic the natural extracellular matrix in the investigation of cancer mechanobiology, such as synthetic or natural 2D as well as 3D matrices and decellularized tissues. All these approaches will greatly help to unravel the complex regulatory networks of cancer, which cannot be studied on stiff and hence non-physiological substrates. The fact that the mechanical properties of distinct cancer cells changed,

such as the hypothesis that cancer cells are softer than normal cells when seeded on stiff cell culture plastic, can be still utilized for diagnostics and fast discrimination among cell states. However, biomimetic platforms, which are able to mimic the *in vivo* context, are of great interest and will help in gaining deep insights into various crucial cancer cell mechanisms, such invasion and metastasis formation in tissues and the response to drug treatments.

13.8 The stroma decreases malignant cancer progression

What types of physical and molecular interactions exist between cancer cells and their extracellular matrix microenvironment? In order to support the homeostatic maintenance of tissue integrity and function, a continuous process of interaction and feedback between cells and the extracellular matrix is required, which consists of various structural and functional proteins (Humphrey *et al* 2014, Holle *et al* 2016, Seager *et al* 2017). Moreover, the physical determinants of the extracellular matrix can affect the behaviors of cells, such as growth and migration (Spill *et al* 2016, Wolf *et al* 2013, Mak *et al* 2016).

13.8.1 Stiffness

Cancer cells are able to adapt their migratory behavior in response to stiffness alterations and stiffness gradients of the surrounding stroma microenvironment (Zaman *et al* 2006). There exist at least two cooperative processes providing the stiffening of the extracellular matrix, such as matrix deposition and the crosslinking of the matrix network (Bonnans *et al* 2014, Zaman *et al* 2006). An example are breast cancer cells, where the crosslinking of collagen promotes tumorigenesis, together with the associated stiffening of the extracellular matrix and increased focal adhesion assembly. Conversely, when the crosslinking of an extant collagen matrix is stimulated, it results in a stiffer extracellular matrix, which is characterized by increased focal adhesions and simultaneously a more invasive phenotype for the constituent and embedded cancer cells (Levental *et al* 2009). Increased collagen density in the extracellular matrix of breast cancer significantly enhances tumor formation and causes an even more invasive phenotype in tumors (Provenzano *et al* 2008). From the mechanical point-of-view, this increase in collagen density generates a stiffer matrix, which in turn induces the invasive migratory behaviors of the cancer cells (Provenzano *et al* 2009b, Acerbi *et al* 2015). The analysis of these molecular processes showed that in regions of higher matrix density and stiffness, the cells displayed increased focal adhesion assembly and elevated activity of the focal adhesion kinase (FAK)-Rho signaling pathway. This signaling cascade causes a hyperactivation of the Ras-mitogen-activated protein kinase (MAPK) signal transduction pathway leading to increased proliferation of cancer cells (Provenzano *et al* 2009b).

13.8.2 Fiber alignment and structure

The stromal extracellular matrix contains high amounts of fibrillary glycoproteins such as fibronectin, collagens I and III and is usually organized into a network of

randomly associated fibers creating an isotropic network (Malik *et al* 2015). In cancer, the architecture of the tumor stroma is usually altered through mechanical forces and abundant CAF activity, which subsequently causes a higher degree of fiber alignment in a direction generally perpendicular to the tumor front. These fibers provide guidance cues for cancer cell migration away from the primary tumor and hence the orientation of the fibers within the extracellular matrix stroma surrounding cancer cells is a promotor of their migratory behavior. Moreover, cancer cells exhibit preferentially directed migration, which is provided by the extracellular matrix architecture (Guan *et al* 2015). Moreover, the migratory and invasive behavior of cancer cells can actually be impaired by altering the orientation of the extracellular matrix fibers (Grossman *et al* 2016).

13.8.3 Force, external stress and mechano-sensing mechanisms

During cancer cell migration, the cells exhibit sensitivity towards traction stresses caused by their internal contraction. When these cellular stresses increase based on the increases in tissue stiffness, the cells switch their migration mode in response. They shift from a blebbing-mode of migration, which relies purely on cellular deformations enabling the cells to squeeze through pores of the extracellular matrix, to a proteolytic degradation-dependent migration mode in which invadopodia-like membrane protrusions are indented into the stroma (Menon and Beningo 2011, Aung *et al* 2014). Another source of mechanical forces regulating cancer cell behavior is applied pressures. In metastatic tumors in bone tissue, the sensing of the pressure is mainly facilitated by osteocytes, which represent the primary mechano-transducing cells in bone tissue and enhance the production of MMPs and CCL5, a chemokine for the attraction of immune cells, due to increased stresses (Sottnik *et al* 2015). These results show that this mechano-sensitivity is manifested in the cancer cells themselves or seems to be a result of their interaction with non-cancerous cells within the tumor microenvironment. Alterations in the migratory and contractile behavior of cells can in turn physically change the structure and mechanical phenotype of the surrounding extracellular matrix. Among the most apparent phenotypic markers of the contraction-facilitated extracellular matrix remodeling is the alignment of extracellular matrix collagen fibers in a direction generally perpendicular to the invasive tumor front, which leads to the formations of tube like structures within the tumor stroma providing a highway for aggressive and invasive cancer cells to migrate out of the primary tumor and spread into the surrounding stroma tissue to metastasize subsequently in targeted organs (Lee *et al* 2017). Moreover, this mechanism can be utilized by both main types of cell migration. Collective and individual single-cell migration mechanisms can both evoke a mechanical strain stiffening in the extracellular matrix stroma that causes a more dense and aligned extracellular matrix along the migratory path of the cancer cells (figure 13.3) (van Helvert and Friedl 2016). In particular, the local invasion originating at these tumors is guided in its direction mainly through the alignment of migrating cells along these bundled collagen fibers, which leads to the hypothesis that the degree of the tumor-surrounding extracellular matrix collagen fiber

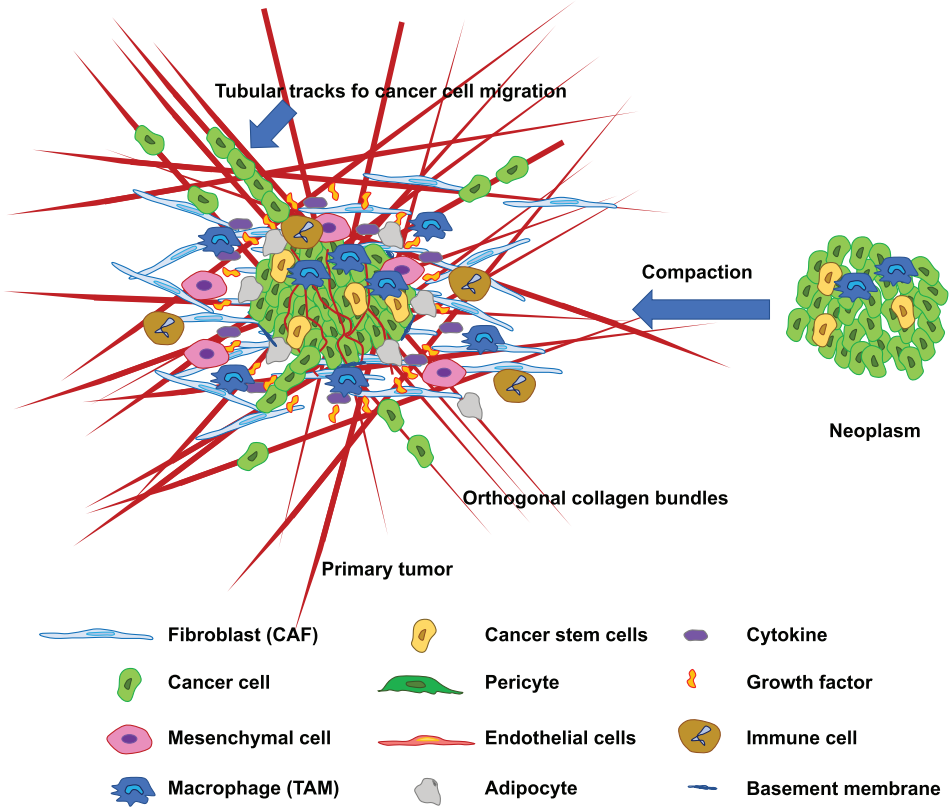

Figure 13.3. Tubular tracks of cancer cell migration out of a primary tumor can be formed.

alignment serves as a marker for the invasive potential of cancer type (Provenzano *et al* 2006). This hypothesis is supported by the finding that in LKB1 mutant cancer cells, the enhanced alignment correlates with increased invasiveness (Lee *et al* 2017). In line with this result, in lung cancer the phenomenon of tumor-centered matrix alignment has been shown to be caused through a malfunction at the molecular signaling level where the disruption of the LKB1-MARK1 pathway leads to the occurrence of an enhanced remodeling event of the extracellular matrix (Li *et al* 2017).

Distinct from the contraction-facilitated forces is the phenomenon of solid stress. In more detail, solid stress is caused by tumor growth and is transmitted by the solid and elastic components of the cancer cells and the extracellular matrix. The solid stress accumulates in the surrounding tumor stroma (Nia *et al* 2016). Moreover, these contraction-driven forces are significant only in tumors, as opposed to normal healthy tissue and are generally not correlated with overall tissue mechanical properties such as stiffness (Nia *et al* 2016). Additionally, the solid stresses may be different between various primary solid tumors and metastases, but it is positively correlated with the size of the primary tumor. Subsequently, the normal tissue surrounding the tumor has even been revealed to contribute to the solid stress of the primary tumor (Nia *et al* 2016).

13.8.4 The role of interstitial flow

When the model of tumor growth-induced forces is revisited, the mechanical force during the growth of the primary tumor transmits stress on the surrounding tissue and hence causes the establishment of stress gradients within the primary tumor. Additionally the plasma filtrate leaks from disordered blood vessel formation based on the tumor-induced neo-angiogenesis and hence also increases the interstitial fluid pressure within the primary tumor (Swartz and Lund 2012, Munson and Shieh 2014). This gradient in the fluid pressure evokes the flow of interstitial fluid through the entire tumor, including the tumor stroma, at higher flow rates than under normal physiological conditions (Munson and Shieh 2014). Hence, this interstitial flow affects the migratory capacity of cancer cells and is assumed to additionally affect the tumor growth and the process of metastasis (Polacheck *et al* 2011). Moreover, the interstitial flow facilitates the CAF migration and the associated remodeling of the extracellular matrix, that in turn promotes the migration and invasion of cancer cells, which leads to the hypothesis that *in vivo*, similar conditions will elevate the metastatic potential of the primary tumor (Shieh *et al* 2011).

13.8.5 Molecular interactions between cancer cells and the stroma

Cancer cells sense, respond to and interact with their microenvironment and hence they also actively and dynamically restructure their local extracellular matrix environment. This behavior of cells is facilitated by a combination of actomyosin-based mechanical contraction, protease-driven matrix degradation, *de novo* synthesis of extracellular matrix proteins and actin polymerization-based exertion of cell protrusions and the subsequent deformation of the surrounding extracellular matrix (Malik *et al* 2015, Bonnans *et al* 2014).

Remodeling of the extracellular matrix

The dynamical remodeling processes performed by enzymes are frequently facilitated by MMPs and depend on the initial physical properties of the surrounding extracellular matrix at the time point of tumor initiation and on the invasive capacity of the cancer cells, which is determined by the ability to move to another targeted location and the ability to degrade the extracellular matrix through enzymatic proteolysis (Harjanto *et al* 2011). In particular, when MMP-9 and Tenascin-C, which is a tumor extracellular matrix protein regulating cell behavior by binding to cell surface receptors, are found to be co-expressed in cancer, the clinical prognoses under these circumstances are significantly lowered compared to cancers in which only one of the two are expressed. Hence the interaction with the surrounding extracellular matrix as performed by membrane proteins such as tenascin-C and the ability to enzymatically degrade the surrounding extracellular matrix by proteases such as MMP-9 are both strongly correlated with the invasive potential and overall metastatic potential of cancer types or single cancer cells (Xu *et al* 2015).

Finally, the structural remodeling of the extracellular matrix is based on the metabolic aberrations, which are caused by mutations within the cancer cell's

genomic phenotype. Examples are the LKB1 loss-of-function mutations that abolish the regulation of lysyl oxidase (LOX) in lung cancers, which in turn causes excessive secretion and deposition of collagen in the extracellular matrix of the tumor stroma. Moreover, this result facilitates the enhanced activation of β1 integrin signal transduction pathways, which in turn provides the proliferation and invasion of cancer cells (Gao *et al* 2010). Additionally, the LOX-driven crosslinking of the collagen fibers leads to fibrosis-enhanced cancer cell proliferation, growth and subsequently elevated cancer metastasis levels (Cox *et al* 2013).

Signal transductive interactions with the stroma

How integrins function in providing the response to external mechanical stimuli from their surrounding local microenvironment seems to be crucial for understanding how they facilitate the phenotypic alterations in cancer cells, and how the bidirectional interactions between cancer cells and the tumor stroma emerge (Seguin *et al* 2015). Due to their transmembrane structure, the integrins couple intracellular signaling of the cell with the external physical interactions through their adhesive connection to the extracellular matrix microenvironment. However, this coupling is not unidirectional from intracellular signals to the stroma, it can also be bidirectional from the stroma to the cell's interior and thereby intracellular signaling and cellular mechanical properties alter the extracellular matrix through integrin signaling (Seguin *et al* 2015).

In cancer cells and healthy normal cells, the integrins induce the activation of intracellular signaling pathways upon response to extracellular ligand-binding, which is termed 'outside–in' signaling (Seguin *et al* 2015). This enables cells to respond to their external microenvironment through adaption of the activation of internal signaling pathways facilitating cellular behavior (Miranti and Brugge 2002). In more detail, a specific feature in outside–in signaling is the recruitment of the tyrosine kinase FAK to physically engaged integrins, which in turn increases the activity of FAK and induces further downstream signal transduction cascades (Sulzmaier *et al* 2014). These distinct signaling cascades and outcomes can vary frequently in cancer biology, however, the alterations in migratory and invasive behaviors of cells are highly promising in affecting and hence predicting the entire metastatic potential of the cancer type. An example are lung cancer cells, in which the outside–in signaling through the integrin β1 subunit induces cell invasion upon response to focal adhesion assembly by the surrounding extracellular matrix (Ungewiss *et al* 2016). The Gα-interacting, vesicle-associated protein (GIV), which is required for outside–in signaling and hence is upregulated, is found in a heightened activation of trimeric G proteins due to integrin ligand-binding, which in turn increases the PI3K signaling and the migration of cancer cells. In cancer, GIV belongs to the positive feedback loop increasing integrin–FAK signaling (Leyme *et al* 2016).

Cancer cells such as blood-borne circulating tumor cells are able to provide an 'inside–out' activation, in which intracellular signals control the ligand affinity of integrins through the coupling of the cytoplasmic and transmembrane domains of the integrin subunits to focal adhesion proteins with connection to the actin

cytoskeleton (Kato *et al* 2012). Among these cytoplasmic effectors are p130Cas, Src and talin, which regulate the integrin binding activity to drive the invasion of cancer cells and the chemoresistance in carcinomas (Sansing *et al* 2011). In glioblastoma, specific integrins such as $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha v\beta 8$ are involved in the regulation of transforming growth factor (TGF)-beta signaling, which has been identified as a key regulator of brain cancer (Roth *et al* 2013). In line with this observation, human metastatic tumors are found to have elevated levels of the integrin $\beta 1$ subunit. Moreover, in cancer, integrins can provide the activation of intracellular signaling pathways such as the progression of cancer without the activation by an integrin-bound ligand. Examples are breast, lung, and pancreatic carcinomas, in which the integrin $\alpha v\beta 3$, when the integrin is not occupied with a ligand, facilitates the recruitment of KRAS and RalB to the cell membrane of cancer cells, leading to the activation of TBK1 and NF-kB signaling which induces tumor initiation, the de-adhesion from the extracellular matrix, the stemness phenotype of cancer cells and their resistance to epidermal growth factor receptor (EGFR) inhibiting pharmacological reagents (Seguin *et al* 2014). In non-small cell lung carcinoma, integrin $\beta 1$ is required for the controlled expression of EGFR, whereas integrin $\beta 1$ deficiency causes deregulated EGFR signaling in cancer such as increased proliferation, inhibition of apoptosis, enhanced cell motility and increased invasiveness (Morello *et al* 2011). In particular, elevated levels of the integrin $\beta 1$ are connected to enhanced metastasis in head and neck squamous cell carcinomas (Wang *et al* 2012).

In addition to the MMP-9 structural remodeling capacity of the membrane collagen fibers, they are also involved in a variety of signaling pathways affecting cell behavior and the response to altered external conditions, which indicates that they are required for healthy tissue growth, however, they also play a prominent role in cancer progression and metastasis (Kessenbrock *et al* 2015, Gialeli *et al* 2011). A broad variety of anticancer drugs affect the expression of MMP-9 or inhibit their activation in various cancer types (Yeh *et al* 2012). MMP-9 has been established as a reliable promoter of the progression of cancer, which promotes the development of a metastatic phenotype in distinct breast cancer types (Mehner *et al* 2014). Moreover, elevated MMP expression such as MMP-1, MMP-9, MMP-11 and MMP-13 is correlated with a higher grading in breast cancer. A differential MMP expression also is correlated with both the histological cancer grade and patient outcome (Merdad *et al* 2014). MMP-facilitated extracellular matrix degradation is associated with the invasiveness of cancer cells, whereas excessive MMP expression reduces migratory activity compared to cells with lower MMP expression, which implies that there exists an optimal MMP expression level to acquire an invasive phenotype (Cepeda *et al* 2016). Tumor-associated neutrophils (TANs) secrete a unique form of MMP-9 that can induce tumor neo-angiogenesis and the intravasation of cancer cells. MMP-9 is secreted as a proenzyme that needs to be processed in order to become proteolytic enzymatic activity and hence fully functional as a proteolytic enzyme. In most cases, the proenzyme for MMP-9 is expressed in a complex together with the tissue inhibitor of metalloproteinase (TIMP)-1 that can in turn abolish the MMP-9 activation. An exception are neutrophils, which do not express TIMP-1, and hence MMP-9 is easily activated and available to promote the

progression of cancer and metastasis (Bekes *et al* 2011). Within the tumor microenvironment, neutrophils are a primary source of MMP-9 that secrete more proMMP-9 than tumor-associated macrophages (TAMs), which have been known as the main source of proMMP-9 (Deryugina *et al* 2014).

13.9 How is the dual role of the stroma affected?

In mammals, the cells are restricted by the topography of the tissue microenvironment in which they are located. The cells can generally sense their tissue confinement, transduce signals from the outside to the inside of the cells and, in addition, they are able to transduce signals from intracellular stimulatory events to the outside local surrounding tissue microenvironment, which in the case of cancer cells is the tumor stroma (Mierke *et al* 2017). In more detail, the stroma around a primary solid tumor is altered in terms of topography and composition, which has been presented by two-dimensional (2D) or three-dimensional (3D) matrix remodeling assays (Wolf *et al* 2003, Lagoutte *et al* 2016). Moreover, the topography of the stroma is also affected by other cells surrounding the primary tumor and non-living constituents such as the extracellular matrix, which is either secreted by stromal cells or cancer cells. In particular, the extracellular matrix is composed of rather randomly orientated and entangled fibers that are under certain circumstances tightly connected via crosslinking proteins such as fibronectin, and hence represent more complex topographies than simple 1D or 2D guidance cues for the migration and invasion of cells (Friedl and Alexander 2011). Hence it has been established that the 3D structure provides the spatial growth of cells and, beyond that, it enables spheroid, hydrogel or scaffold-based cell cultures and thereby supports cells with microenvironmental cues which are more closely related to those available in *in vivo* tissues and found in physiological or pathological tissue (Ravi *et al* 2015, Lee *et al* 2007, Edmondson *et al* 2014). Cells *in vivo* are in close proximity to other cells and are able to interact with these neighboring cells and the surrounding extracellular matrix. These interactions can occur in two ways, either initiated from the cancer cells or from the stroma, including embedded cells and substances. These reciprocal interactions need to be integrated in current *in vitro* tumor models in order to address the high variability of cancer cells and stroma cells (Dondajewska *et al* 2018). Moreover, primary solid tumors are no longer treated as masses of uncontrolled and uniformly proliferating cancer cells, but instead they can be considered as well-organized pathological organs (Egeblad *et al* 2010) containing various cell types such as fibroblasts, adipocytes, endothelial cells and immune cells (Hanahan and Coussens 2012). Hence, improved *in vitro* models need the integration of a coculture of cells, which are derived from different origins. Indeed, coculture studies revealed several mechanisms of different important biological processes, such as the epithelial–mesenchymal transition (EMT), cancer metastasis, neo-angiogenesis and the transformation of fibroblasts into cancer-associated fibroblasts (CAFs) or the transition of cancer-associated macrophages into tumor-associated macrophages (TAMs) (Kim *et al* 2015, Angelucci *et al* 2012, Sethi *et al* 2015, Rama-Esendagli *et al* 2014, Sung *et al* 2013). The pathology of the tumor

microenvironment is still not clearly understood. However, the details of the interaction between cancer cells and the stroma is crucial for the diagnosis and staging of tumors, and the development of new, effective and personalized cancer therapies.

Within the tumor stroma, cancer cells can utilize different migration mechanisms and hence cancer cells possess a high plasticity. In particular, cancer cells can adapt their signal transductions pathways and dynamically restructure their intracellular cytoskeleton and focal adhesions that provide the interaction with the surrounding physical confinements through connecting the cell's cytoskeleton to the extracellular matrix (Acerbi *et al* 2015, Friedl and Wolf 2010, Hung *et al* 2013, Tong *et al* 2012, Stroka *et al* 2014, Oudin and Weaver 2017). The physical parameters facilitating the alterations within the cells due to their physical constraints need to be determined for the impairment of the accessible cell motility and subsequently the malignant progression of cancer (Pickup *et al* 2010, Acerbi *et al* 2015, Friedl and Alexander 2011, Condeelis and Segall 2003). Knowledge of these basic principles helps us to reveal and understand the developmental processes and the morphogenesis of organs (Montell 2003, Herbert and Stainier 2011). Moreover, the specific behavior of cells can be altered, such as the immune response of cells (Luster *et al* 2005, Friedl and Weigelin 2008) and the growth and the structure of tissues can be directed using engineering or 3D printing of distinct structured tissues *in vitro* (Wrobel and Sundararaghavan 2014, Mandrycky *et al* 2016). The assessment of all these issues regarding the cell response to natural confinements *in vivo*, such as in living tissues, is less easily available, most costly and the reproducibility is low due to the low numbers of repeated experiments, the large variability between specimens and the low throughput. Hence, some issues, such as the effect of physical parameters on cellular motility, need to be investigated by using *in vitro* in model systems, such as artificially engineered microenvironments or tissue biopsies as well as resections.

However, the key question of whether the tumor stroma acts as a tumor inducer as well as promotor or as a steric hindrance and hence a confinement of tumor initiation, growth and malignant progression remains not fully understood. The tumor microenvironment is crucial for the successful initial establishment and progression of cancers, such as prostate, lung, liver, intestine, kidney or breast cancer. Indeed, the tumor stroma fulfills a precise function, as it induces and promotes the growth of microvessels and the formation of a relatively dense microvascular network surrounding and invading the primary tumor (figure 13.4). In addition, the tumor stroma facilitates the recruitment of fibroblasts (termed stromal fibroblasts), lymphocytes, neutrophilic, basophilic or eosinophilic granulocytes and macrophages, and stores or releases small peptides or proteins acting as signaling molecules as well as proteases (figure 13.4) (Rowley and Barron 2012, Tuxhorn *et al* 2002a). In particular, cancer-associated fibroblasts (CAFs) enable the production and structural alteration of extracellular matrix networks supporting the EMT of embedded cells, and even other types of cellular behaviors, providing an aggressive and invasive phenotype in surrounding normal neighboring epithelial cells, which exhibit a formerly non-aggressive and normal epithelial phenotype (Tuxhorn *et al* 2002b, Labernadie *et al* 2017). How can the stromal cells surrounding the primary tumor switch from a

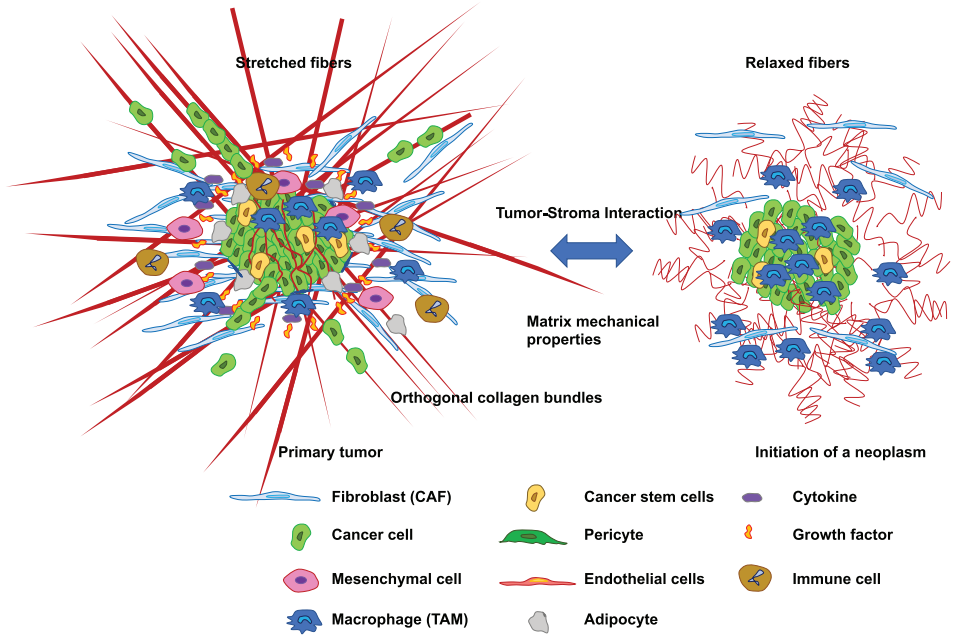

Figure 13.4. Tumor–stroma interaction.

non-malignant to an aggressive phenotype? The answer remains elusive (Pickup *et al* 2010). Moreover, distinct proteins are increasingly expressed in stromal cells and hence provide the prerequisites for the interaction between stromal and epithelial cells, whereas there is still a lot be revealed. In particular, it is known that the cytokine transforming growth factor β (TGF-β) and its signal transduction processes fulfill a key role in the interaction process (Ao *et al* 2006, Franco *et al* 2011). For example, in prostate cancer, the myofibroblastic and highly reactive phenotype of stromal cells surrounding the primary prostate cancer is not yet clearly understood, whereas the predominant cell population of the tumor stroma elevates the expression of vimentin and fibroblast activation protein expression and reduces the expression of alpha-smooth muscle actin, desmin and calponin 1 (Labernadie *et al* 2017, Dakhova *et al* 2009). In turn, the prostate cancer interacting stroma expresses and secretes increased levels of collagen type I, tenascin-C and TGF-β, which facilitates the tumor growth and hence tumorigenesis (Labernadie *et al* 2017, Ao *et al* 2006). Moreover, these proteins may be employed as marker proteins for prostate cancer, but they are still controversial (Labernadie *et al* 2017, Dakhova *et al* 2009, Weigelin and Friedl 2010). Thus, other more universal, specific and reliable parameters, such as the mechanical properties of the cells, need to be identified and characterized for use in predicting the malignant progression of cancer. Moreover, it needs to be revealed whether there exists a tumor stroma surrounding an aggressive cancer type, which adapts a dedifferentiated phenotype such as embryonic or tissue stem cells to promote the progression of cancer.

A candidate for an inverse tumor marker protein is Cavelin 1 (Cav-1), the expression of which is decreased in the stroma of prostate cancer (Dakhova *et al* 2009, Pascal *et al* 2009, Orr *et al* 2012, Di Vizio *et al* 2009). The decreased expression of Cav-1 correlates positively with the formation of metastases, whereas within the primary tumor the expression of Cav-1 is, together with the expression of another marker protein such as active Akt, increased (Di Vizio *et al* 2009, Li *et al* 2003, Ayala *et al* 2003). The knockdown of Cav-1 in the tumor stroma serves as an inductive signal for the extracellular matrix migration and invasion of epithelial-originated cancer cells and subsequently the malignant progression of cancer disease. These results indicate that a universal and reliable marker is still required for the identification and characterization of the onset of malignant cancer progression.

Based on the EMT, several factors such as the cell–cell adhesiveness and strength, the overall cellular stiffness and cell's shape regulate cellular motility in mixtures of cell populations undergoing the cell sorting processes that are required for physiological processes, such as the developmental of organs or tissues, and pathological processes, such as the formation of boundaries and the internal organization of solid tumors. When the differential adhesion hypothesis (DAH) (Foty and Steinberg 2004) is applied to living objects such as cells, they are treated as immiscible Newtonian fluids and it has been revealed that surface densities (excluding the activation) of adhesive molecules such as E-cadherin, P-Cadherin or N-cadherin actually do not correlate with the intercellular adhesion strength, whereas the simple cell shape, cellular stiffness and rate of cell sorting are correlated positively with the strength of cell adhesion (Pawlizak *et al* 2015). Additionally, the final sorting of the cell populations has been shown to be uncorrelated in its efficiency with the epithelial or mesenchymal origin of the cells, as it is equally efficiently performed by both cell types (Pawlizak *et al* 2015). In particular, active living tissues do not behave similarly to immiscible passive fluids, as dynamical effects such as directional motility, frictional forces and jamming account for the compartmentalization of tissue across the EMT. There are energies connected with tensile forces along the cell–cell adherence junctions and there are additionally energies dependent on the cell–cell adhesive forces of neighboring cells (Farhadifar *et al* 2007, Bi *et al* 2014, 2015, 2016). Hence, tissue surface tension and the actomyosin contractility drive the segregation of distinct cell populations within tissues (Park *et al* 2016). However, when the cell–cell adhesive forces become larger than the tensile forces, the energy barrier disappears (Rodríguez-Franco *et al* 2017, Park *et al* 2016, Sadati *et al* 2013). When the energy barrier is abolished, the cell collective shows, as a whole cell cluster, the behavior of a fluid which can flow continuously, and individual cells can easily exchange their places with their immediate neighbors. Thus, each individual cell is then uncaged and the collective can be considered as unjammed. In particular, epithelial cell aggregates or sheets can migrate collectively and in a certain manner display their disordered state and their cooperativity, which are both major characteristic features of the jamming behavior of tissues or cell aggregates. The cell jamming behavior is based on differential adhesion, the epithelial–mesenchymal transition and the reverse, the mesenchymal–epithelial transition. In summary, cell sorting and tissue assembly processes are affected by collective cell migration, in

which each cell migrates in highly cooperative packs, stripes, strands and sheets. Taken together, the physiological or occasionally pathological processes of cell sorting, tissue assembly or disassembly, and collective cellular migration are all emergent phenomena, as they are inherently collective and are not explained by the movement of single cells under isolated single-cell migration conditions (Sharma *et al* 2018).

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Part VI

The impact of the mechanical and biochemical interaction of cancer cells with other cells in transendothelial migration

Most cancer-related deaths are not evoked by the primary tumor, instead they are caused by the malignant progression of cancer, such as the process of metastasis. One of the main steps of cancer metastasis is the transendothelial migration of cancer cells, which is a complex event in which cell adhesion and the transmigration of cancer cells need to be precisely regulated. In particular, the transmigration involves biochemical and biomechanical interactions of metastatic cancer cells with the endothelial cell lining of blood or lymph vessels. Under normal conditions, the endothelium acts as a barrier against the invasion of cancer cells in order to decrease cancer cell migration and consequently cancer metastasis. However, certain cancer cells can overcome the endothelial cell monolayer by activating alterations within endothelial cells, including reduction of endothelial cell stiffness, regulation of adhesion molecules and the remodeling of the endothelial cytoskeleton. In turn, cancer cells need to dynamically alter their cytoskeleton and their cell shape and they may apply forces toward the endothelium in order to facilitate their transmigration. Thus, the biomechanical properties of cancer and endothelial cells seem to play an important role in transendothelial migration. Moreover, it has been suggested that mechanical alterations are necessary in both cancer and endothelial cells in order to regulate cancer cell invasiveness. However, the detailed regulatory mechanisms are not well understood and, in particular, the role of forces exerted by aggressive and invasive cancer cells on the endothelium and the specific force application mechanisms remains elusive. Finally, part 6 considers the question of how such a mechanically based mechanism can produce cancer cell invasion, and in the case of certain cancer cells, how it may even increase their invasiveness after transendothelial migration. In addition to this, we address how cancer cells are supported to find their metastatic niche by cellular particles such as exosomes, or other cell types such as immune cells or stroma cells.

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Cellular and microenvironmental effects
Claudia Tanja Mierke

Chapter 14

The role of endothelial cell–cell adhesions

Summary

During the process of cancer metastasis, the transendothelial migration of cancer cells seems to be crucial in providing the formation of secondary tumors in targeted organs, the so-called metastatic niches. Whether these cancer cells transmigrate transcellularly (through the living endothelial cell of the confluent endothelial monolayer lining blood vessels) or paracellularly (through cell–cell adhesions of neighboring endothelial cells) is not yet clear. It seems to be the case that cancer cells are able to use both migration routes at different cellular loci of the endothelial monolayered vessel lining. However, it is not yet clear what effect the mechanical cues of the endothelial cells or cancer cells have on the choice of the migration path and on the type of transmigration path. Moreover, the biochemical and mechanical cues of the surrounding microenvironment are still elusive, but seem to have a non-neglectable effect. How these transmigration routes and loci are selected is discussed and hypothesized below. Moreover, the exosomes may even play a role in supporting the efficient and successful settlement of cancer cells into the pre-metastatic niche in targeted organs.

14.1 The expression of cell–cell adhesion molecules

Invasive cancer cells regulate the expression of endothelial cell–cell adhesion molecules

The expression of endothelial cell–cell adhesion molecules is important for inter-endothelial adhesion strength and hence for the integrity of the endothelial cell monolayer (figure 14.1). However, it has not been shown how the expression of these molecules is regulated by aggressive and invasive cancer cells, but not by non-invasive cancer cells. There are still many questions that need to be raised and answered in order to understand the transendothelial migration step of the metastatic cascade, for example: Do only aggressive and invasive cancer cells alter the expression of cell–cell adhesion molecules on endothelial cells? The question of how specific cancer cells transmigrate through the endothelium remains

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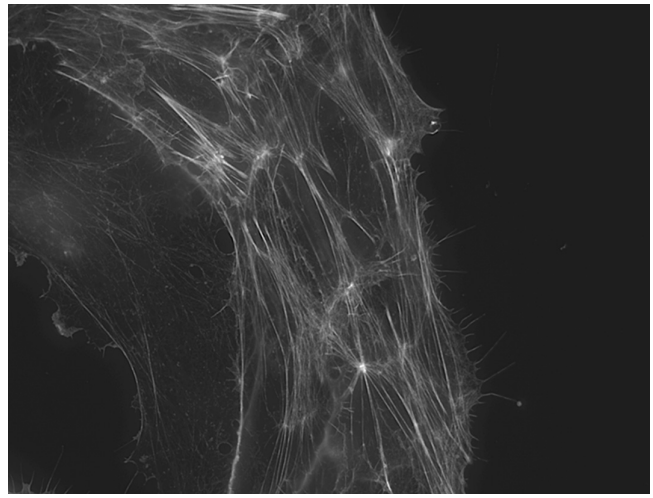

Figure 14.1. Endothelial cell monolayer. The cells are stained for F-actin using Alexa Fluor 546 Phalloidin.

controversial, however, the question of whether highly invasive cancer cells are able to regulate the cell–cell adhesion molecule expression on human microvascular endothelial cells during co-culture has been investigated. Primary human pulmonary microvascular endothelial cells derived from the lung (called HPMECs) were co-cultured for 16 h with highly invasive MDA-MB-231 and weakly invasive MCF-7 cells. Using the flow cytometry technique, the co-culture of microvascular endothelial cells with highly invasive MDA-MB-231 breast cancer cells revealed that the platelet endothelial cell adhesion molecule-1 (PECAM-1) and the vascular endothelial-cadherin (VE-cadherin) were both down-regulated during co-culture with highly invasive MDA-MB-231 cells compared to mono-cultured endothelial cells. In contrast, the co-culture of endothelial cells with weakly invasive MCF-7 cells showed no effect on the expression of the two endothelial cell–cell adhesion proteins, PECAM-1 and VE-cadherin (Mierke *et al* 2011). These findings suggest that the down-regulation of endothelial cell–cell adhesion molecules seems to be cancer-cell-specific and may depend on their individual invasive potential as well as possibly on their mechanical properties. Taken together, these results indeed show that the co-culture of endothelial cells with highly invasive cancer cells evokes a down-regulation in the cell surface expression of the cell–cell adhesion molecules VE-cadherin and PECAM-1, thus altering the biomechanical properties of endothelial cells and effecting the break-down of the endothelial barrier function (figure 14.2).

In order to investigate which particular mechanism facilitates the reduced endothelial cell–cell adhesion molecule expression during co-culture with MDA-MB-231 cells, the membrane shedding of these adhesion molecules was inhibited during the trans-endothelial migration of the MDA-MB-231 cells. In more detail, it was investigated whether the decreased expression of PECAM-1 and VE-cadherin receptors on human pulmonary microvascular endothelial cells (HPMECs) during co-culture with MDA-MB-231 cells is due to membrane shedding of these receptors. Thus, the co-culture of cancer cells and endothelial cells was performed in the presence and absence of the broad

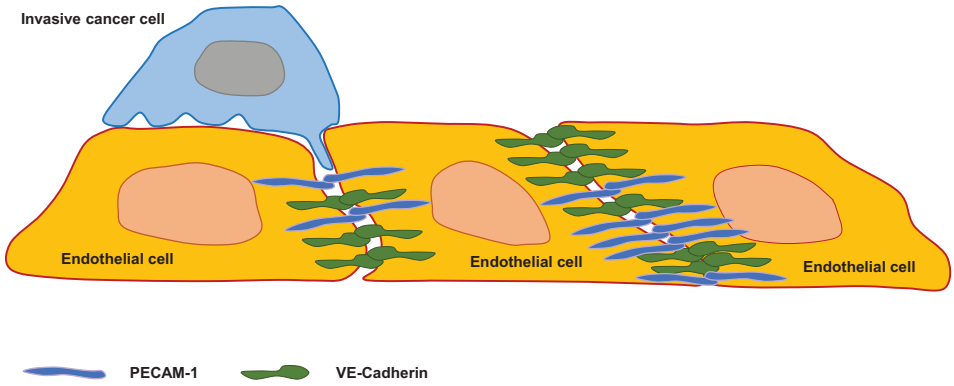

Figure 14.2. An aggressive and invasive human MDA-MB-231 breast cancer cell can transmigrate through cell–cell interendothelial adherence junctions through the down-regulation of cell–cell adhesion molecules such as PECAM-1 and VE-cadherin through direct contact with the aggressive cancer cell.

matrix-metallo-proteinase inhibitor GM6001. Indeed, the reduction of the cell–cell adhesion receptor, such as PECAM-1 and VE-cadherin, expression on endothelial cells during the co-culture with MDA-MB-231 cells has been shown to be caused by increased membrane shedding of these cell–cell adhesion receptors.

14.2 The strength of cell–cell adhesions

Vascular leakage is a hallmark of many inflammatory and often life-threatening diseases and hence contributes to disease severity in disorders such as sepsis, cancer, diabetes and atherosclerosis (Weis and Cheresh 2005). Despite the tremendous medical importance of vascular leakage, only a few specific therapies are available in order to counteract it and current therapies often fail (Groeneveld 2002). However, the *in vivo* molecular targets are not yet fully understood, although a wealth of data obtained from *in vitro* studies is available for the signal transduction pathways that regulate vascular permeability (Mehta and Malik 2006, Jacobson and Garcia 2007). Among various new agents that potentially reduce endothelial hyperpermeability, such as cholesterol-lowering statin drugs, some have been proposed to reduce vascular leakage, as they are able to inhibit RhoA proteins (Jacobson *et al* 2005, van de Visse *et al* 2006). In a proof-of-principle it has been shown that increased RhoA activity fosters vascular hyperpermeability *in vivo* (Gorovoy *et al* 2007). In more detail, it has been found that an increase of RhoA activity by deletion of one of its inhibitory proteins such as RhoGDI leads to a reduction of endothelial junctional integrity and finally reduces the vascular endothelial cell barrier function.

Rho GTPases such as RhoA, Rac-1 and Cdc42 have been revealed as key regulators of cell shape, movement and proliferation. However, *in vitro* studies have shown that the balance of activities of these small G proteins regulates the blocking potential of the endothelial barrier (Wojciak-Stothard and Ridley 2002) (figure 14.3). Cdc42 enhances the process of recovery of a disturbed barrier (Kouklis *et al* 2004), Rac1 is required to establish a tight barrier function (Wojciak-Stothard and Ridley 2002) and RhoA is involved in the induction of the endothelial

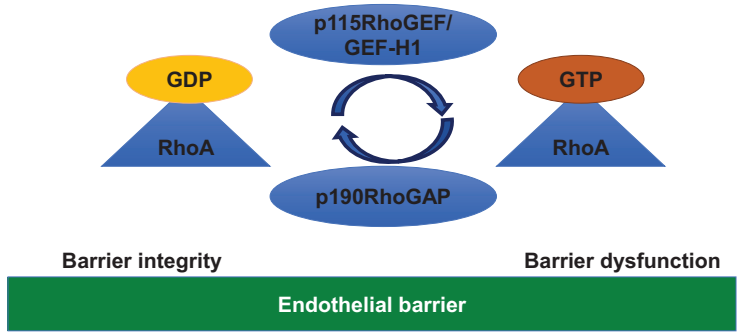

Figure 14.3. The endothelial barrier function is tightly regulated through a precise balance of the individual activities of small G proteins. In particular, vasoactive agents such as VEGF and thrombin, and interaction with leukocytes, can impair the endothelial barrier function through specific receptors. Indeed, several signal transduction mechanisms are simultaneously activated, such as the influx of calcium ions, the activation of small Rho GTPases and various other kinases and the phosphorylation of adherence junctional proteins. Among the small GTPases, RhoA seems to be mainly involved in inducing endothelial hyperpermeability, whereas Rac1, Cdc42 and Rap1 foster an intact endothelial cell barrier function. The activity of the small GTPases has been shown to be precisely determined by three classes of regulatory proteins, GDIs, GEFs and GAPs. In more detail, RhoGDI has been identified as a novel therapeutic target and evidence has been provided that in the healthy vasculature RhoGDI is a key regulatory point, restricting the RhoA activity to low levels.

hyperpermeability caused by various stimuli, such as thrombin, VEGF, angiopoietin-2 and LPA (Essler *et al* 1998, van Nieuw Amerongen *et al* 1998, Parikh *et al* 2006). In addition, these Rho GTPases regulate other vascular cells and leukocytes and hence facilitate other vascular functions.

The inhibition of the RhoA-target ROCK1/2 (Rho kinase) by inhibitors revealed the involvement of RhoA/ROCK activation in embryonic development/cytokinesis (Lai *et al* 2005) and in various vascular pathologies, such as (pulmonary) hypertension, atherosclerosis, stroke and even heart failure (Shimokawa and Rashid 2007). Indeed, the first evidence has been obtained that ROCK inhibition by Y-27632 can reduce pulmonary edema in animals after LPS stimulation or re-expansion of the lung (Tasaka *et al* 2005, Sawafuji *et al* 2005). Because of the central importance of ROCK in the regulation of many basal cellular functions, such as migration and proliferation, it has been suggested that the activity of Rho proteins is strongly controlled by regulatory proteins. In more detail, guanine dissociation inhibitors (GDIs) hold Rho proteins in their inactivated GDP-bound mode (DerMardirossian and Bokoch 2005), while in contrast guanine exchange factors (GEFs) activate Rho proteins by facilitating the exchange of GDP for GTP, and GTPase activating proteins (GAPs) inactivate Rho proteins by inducing the conversion of Rho-bound GTP to GDP. These regulatory proteins act in close concert: the dissociation of RhoA from RhoGDI is a prerequisite for its activation by RhoGEF. Regulatory proteins of all three classes (RhoGDIs, RhoGEFs and RhoGAPs) are involved in the regulation of thrombin-enhanced *in vitro* endothelial permeability (Holinstat *et al* 2006, Mehta *et al* 2001, Birukova *et al* 2006). Epac, an analogous cAMP-activated exchange factor for Rap1

(a small GTPase not belonging to the Rho family of small GTPases), plays a crucial role in increasing vascular endothelial-cadherin-facilitated cell–cell contacts (Cullere *et al* 2005, Fukuhara *et al* 2005).

In order to investigate the pulmonary vasculature, a model of isolated mouse lungs was selected. An increase in capillary permeability is the basic underlying abnormality of acute lung injury or acute respiratory distress syndrome (ALI/ARDS), which builds a continuum of mild to severe lung damage. ARDS often develops in septic patients or after trauma and thus is a major cause of death in the intensive care setting. It is thus important to investigate the effect of treatment with endotoxin, as a model for sepsis, inducing RhoA activity in the lung.

An important finding was that increased permeability in RhoGDI$^{-/-}$ mice was completely reversible by pharmacological inhibition of the Rho kinase. First, this indicates that the hyperpermeability in RhoGDI$^{-/-}$ mice was indeed caused by enhanced RhoA/Rho kinase signaling, excluding possible side-effects. Second, this brings the concept of RhoGDI/RhoA-facilitated vascular leakage into the focus of medical research, as Rho kinase inhibitors with a reasonable safety profile (such as fasudil) are available.

However, no signs of edema were detected in the intact animals, which was attributed to the presence of safety factors such as lymphatic drainage. It remains to be investigated whether the vasculature in an intact animal is hyperpermeable, using appropriate dye extravasation experiments.

The data are challenging and should be interpreted with care. It is tempting to conclude that the absence of RhoGDI and the accompanying effect of RhoA on vascular leakage was caused by activation of RhoA in endothelial cells. Indeed, it has been shown that the endothelial junctions in capillaries and postcapillary venules become disturbed, but the biochemical measurements were performed in whole lung homogenates and therefore are inconclusive. Although the siRNA approach in cultured endothelial cells confirmed that deletion of RhoGDI by itself is sufficient for barrier dysfunction, the present study does not exclude the possibility that nonendothelial effects in RhoGDI$^{-/-}$ mice may also contribute to the enhanced vascular permeability in the intact lung. In particular, future studies are needed to investigate whether rescuing of RhoGDI specifically in endothelial cells or leukocytes excludes the possibility that the effect on vascular junctions is not indirectly evoked by an alteration in leukocyte influx after LPS challenge, or an alteration in resident leukocytes and mast cells due to life-long depletion of RhoGDI. However, even if such indirect effects contribute to vascular leakage in RhoGDI$^{-/-}$ mice, this study provides fuel for the suggestion that inhibition of RhoA is a potential target for reducing vascular leakage. This has been reported for statins, which in addition to the inhibition of cholesterol synthesis, abolish the isoprenylation of proteins such as RhoA, required for their membrane anchoring. Moreover, it has been suggested that vascular leakage induced by sepsis may benefit more from treatment by statins than other, similar leakages caused by other stimuli in cardiovascular patients would (Jacobson *et al* 2005, van de Visse *et al* 2006). Due to the contribution of vascular leakage to many other nonpulmonary disorders, future studies are required to reveal whether similar mechanisms apply to other vascular beds and other disease states.

It remains a future challenge to develop therapies that increase RhoGDI activity. The answer may be found in the inhibition of the kinases that phosphorylate RhoGDI and stimulate the release of RhoA from RhoGDI. Two candidate kinases are Src and PKC (Holinstat *et al* 2006, DerMardirossian *et al* 2006). Interestingly, in line with this, it has been suggested that inhibition of p190RhoGAP by Angiopoietin-1 reduces endotoxin-enhanced vascular permeability in the mouse lung, indicating that targeting of Rho-regulatory proteins seems to be a feasible approach for reducing vascular leakage (Mammoto *et al* 2007). However, information about the activity status of the different Rho-regulatory proteins in (human) disease would provide valuable information for directed therapy. In summary, the concept of RhoA being very important in the regulation of vascular endothelial leakage is approaching clinical applicability.

14.3 The cancer cell transmigration route

How can cancer cells transmigrate through an endothelium?

Most cancer-related deaths are caused by cancer metastasis, a process that starts with dissociation of cancer cells from the primary tumor, followed by tissue invasion, entrance into blood or lymph vessels (intravasation) and transport to remote sites (figure 14.4). The transmigration through the endothelial cell layer of

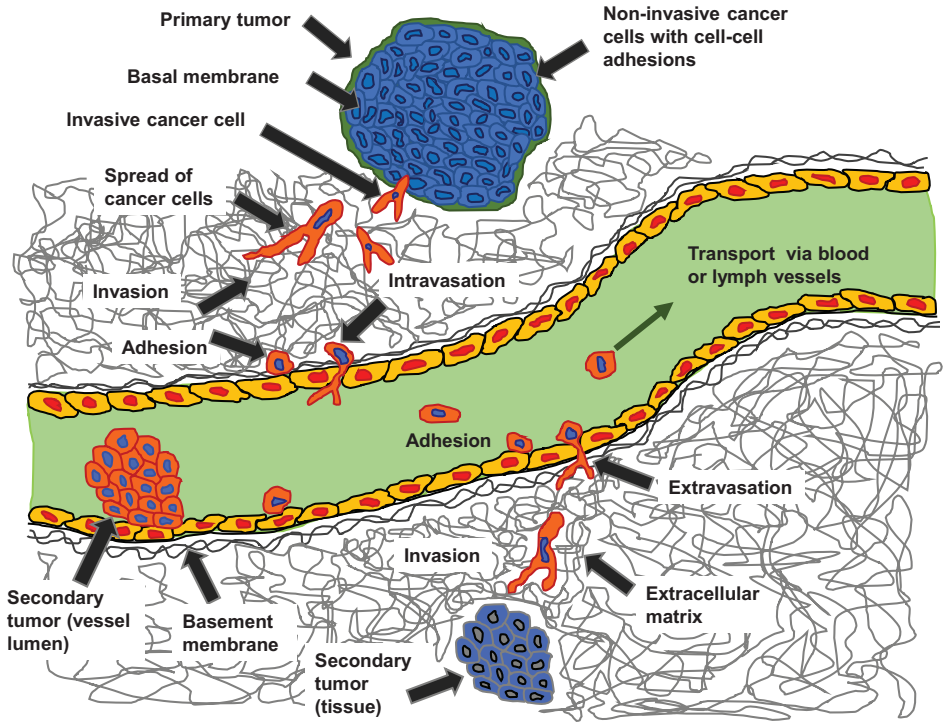

Figure 14.4. The metastatic cascade. Cancer cells spread from the primary tumor and migrate into the surrounding matrix microenvironment, where they reach blood or lymphoid vessels, intravasate and get transported through the vessels.

blood or lymph vessels is a major step in the metastatic cascade of malignant tumor progression. However, the transmigration of cancer cells through the endothelial cell lining of blood vessels is not yet understood in detail. It is assumed that cancer cells can then escape from the microvasculature (called extravasation), invade the target tissue and form secondary tumors in distant organs (Liotta *et al* 1991, Langley and Fidler 2007, Steeg 2006). A potentially rate-limiting step in the metastatic cascade, therefore, would be the extravasation process that involves adhesion of cancer cells to endothelial cells and their transmigration through the endothelial cell monolayer and finally the basement membrane (Steeg 2006, Nicolson 1989, Stetler-Stevenson *et al* 1993, Luzzi *et al* 1998). Indeed, specific cancer cell types have been demonstrated, both *in vitro* and *in vivo*, to be able to overcome the endothelial barrier (Luzzi *et al* 1998, Weis *et al* 2004, Voura *et al* 2001, Tremblay *et al* 2006, Sandig *et al* 1997, Fidler and Hart 1982). However, cancer cell extravasation need not be the only mechanism for metastasis formation, as has been pointed out by Al-Mehdi and colleagues (Al-Mehdi *et al* 2000), who reported that cancer cells can adhere and grow onto the endothelial layer and form intraluminal metastases without ever leaving blood or lymph vessel confinement. Either way, the role of the endothelial monolayer of blood or lymph vessels in this process seems to be crucial in that it can actively regulate metastasis formation by either allowing or blocking the adhesion, and possibly transmigration, of cancer cells (Voura *et al* 2001, Tremblay *et al* 2006, Sandig *et al* 1997). The details of the endothelial cell functions in this process, however, are poorly understood and the extent to which the endothelium restricts or even promotes the process of metastasis is also not yet clearly understood.

Transmigrating cancer cells are thought to be able to overcome the endothelial barrier by inducing alterations within endothelial cells, such as the up-regulation of adhesion molecule receptor expression (Laferriere *et al* 2001), the reorganization of the cytoskeleton (Rousseau *et al* 1997), Src-mediated disruption of endothelial VE-cadherin-beta-catenin cell–cell adhesions (Weis *et al* 2004), the formation of 'holes' within the endothelial layer (Li and Zhu 1999) and even the induction of apoptosis (Heyder *et al* 2002). However, cancer cell invasion seems to be similar to leukocyte trafficking, for which the endothelium acts as a passive barrier, greatly reducing invasion rates (Wittchen *et al* 2005). For instance, the function of the endothelial cell barrier against both leukocyte trafficking and cancer cell transmigration is reduced in the presence of inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (Voura *et al* 2001, Laferriere *et al* 2001, Chandrasekharan *et al* 2006, McGettrick *et al* 2006). In more detail, these cytokines are known to trigger an up-regulation of the adhesion molecule E-selectin (Laferriere *et al* 2001). The subsequent adhesion of cancer cells to E-selectin leads in turn to an up-regulation of stress-activated protein kinase-2 (SAPK2/p38) in endothelial cells (Laferriere *et al* 2001), finally triggering actin polymerization and reorganization into stress fibers in endothelial cells (Rousseau *et al* 1997). These results indicate that the mechanical properties of endothelial cells may be altered by invasive cancer cells adhering and transmigrating through an endothelial cell monolayer, which represents an endothelial cell barrier lining blood or lymphoid vessels.

Chemokines and their receptors are also important for leukocyte trafficking (Gallatin *et al* 1983, Hillyer *et al* 2003) and cancer cell invasion (Reiland *et al* 1999). In more detail, chemokines are a superfamily of small cytokine-like proteins that induce cytoskeletal rearrangements in endothelial cells and leukocytes, the firm adhesion of leukocytes to endothelial cells and the directional migration of leukocytes (Gallatin *et al* 1983). The involvement of chemokines in tumor–endothelial interactions and their effect on cancer cell mechanics during matrix invasion are considerably less well understood and hence require further investigation.

Thus, it has been investigated whether the endothelium is able to regulate the transmigration and invasion of cancer cells into an extracellular matrix. The invasion of several human cancer cell lines into a 3D collagen gel matrix covered with an endothelial cell monolayer was performed. Interestingly, in the presence of an endothelium, the invasion of special cancer cell lines increased pronouncedly. Moreover, gene expression analysis of endothelial cells co-cultured with invasive cancer cells revealed an up-regulation of Gro-β and IL-8 chemokines compared with endothelial cells co-cultured with non-invasive cancer cells (Mierke *et al* 2008). Finally, it was demonstrated that Gro-β and IL-8 receptor (called CXCR2) expression on cancer cells serves as a key mediator responsible for the break-down of the endothelial barrier function through enhancing cancer cell force generation and cytoskeletal remodeling dynamics (Mierke *et al* 2008).

14.3.1 The paracellular transendothelial migration route

It has been hypothesized that the endothelial cell's actin cytoskeleton may provide a migration scaffold for transmigrating cancer cells (figure 14.5). The endothelial cell lining of vessels represents a strong barrier against the invasion of specific cancer cells and is thus a key rate-limiting step against the transmigration, invasion and

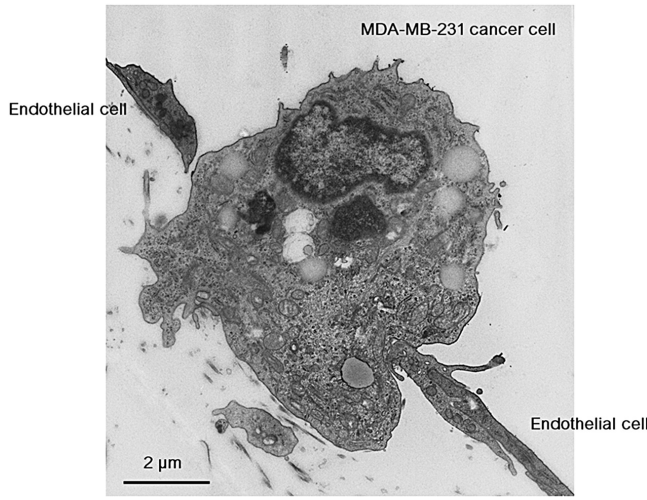

Figure 14.5. Paracellular migration. The transmission electron microscopic image shows a human MDA-MB-231 breast cancer cell that transmigrates through cell–cell adherence junctions between two neighboring endothelial cells.

metastasis of aggressive and invasive cancer cells (Zijlstra *et al* 2008). In particular, the endothelial vessel wall has been commonly considered to be a strong tissue barrier against the dissemination of cancer cells through pronouncedly reducing their invasiveness and consequently eliminating their metastatic potential (Wittchen *et al* 2005). However, recent results have led to the establishment of a novel and unexpected role for the endothelial cell lining of vessels. By fulfilling this role, endothelial cells enhance the invasiveness of certain cancer cells (Mierke *et al* 2008). First, breast cancer cells showed increased dispersion and clearance through hematogeneous dissemination adjacent to blood vessels (Kedrin *et al* 2008). Second, the invasiveness of special cancer cell lines is endothelial-cell-dependent and is thus enhanced in certain highly invasive cancer cells, whereas for the weakly invasive cancer cells the endothelium acts as a classical barrier confinement impairing cancer cell invasion (Mierke *et al* 2008). Although the process of cancer cell invasion and metastasis has been the subject of numerous research papers, the molecular and mechanical mechanisms of cancer cell transendothelial migration are not yet precisely understood and thus require further investigation.

The physical and biochemical aspects of the cancer cell intravasation process involve the interaction of at least three cell types: an invasive cancer cell, a macrophage and an opposing endothelial cell representing the barrier function. In more detail, all three cell types will engage the mechano- and biochemical-transduction properties of the cytoskeleton of all three neighboring cells. In order to reveal the cancer-cell-induced signals in endothelial cells, a 3D transmigration and invasion assay can be used in which the real-time intra-endothelial signaling events evoked by invasive cancer cells or macrophages are analyzed and compared to mono-cultured endothelial cells (Khuon *et al* 2010, Dovas *et al* 2013, Roh-Johnson *et al* 2014). In particular, this assay involves the assembly of a vasculature network within a 3D collagen matrix using endothelial cells that express a fluorescent resonant energy transfer-based biosensor reporting the activity of myosin light chain kinase (MLCK) in endothelial cells in real time (Chew *et al* 2002). As expected, endothelial cells react to mechano-sensing events in the 3D collagen matrix. For example, the 3D microenvironment induces lumen formation of endothelial cells and endothelial cells show basal–apical polarity in the proper orientation indicated by α4 laminin deposition. As hypothesized before, it was confirmed that invasive cancer cells affect the MLCK-facilitated actomyosin function within the underlying endothelium. In addition, cancer cells can trans-migrate through the endothelial barrier confinement in at least two different cellular ways: through transcellular routes (by transmigrating directly through the cytoplasm of an adjacent underlying endothelial cell) and through paracellular routes (by transmigrating between the endothelial cell–cell junctions of two neighboring individual endothelial cells) (Khuon *et al* 2010).

14.3.2 The transcellular transendothelial migration route

The transcellular transmigration route has for a long time been regarded solely as an artifact, but this mode has turned out to be real, as in several reports this transmigration

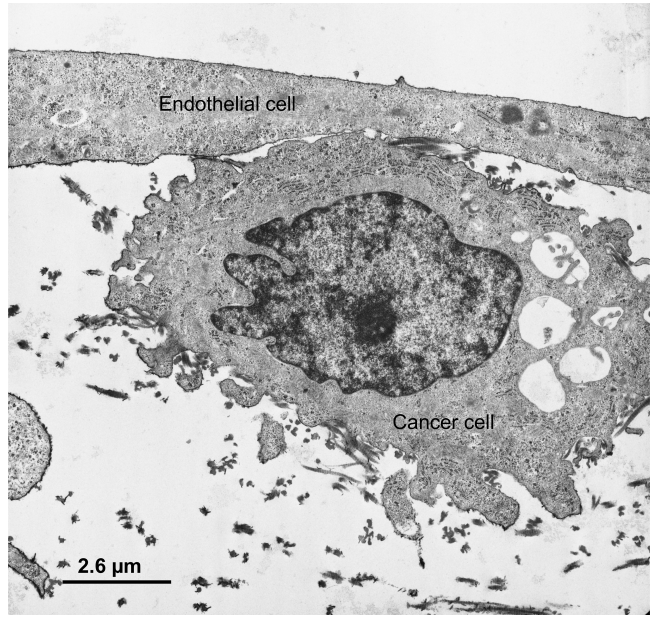

Figure 14.6. Transcellular migration. The transmission electron microscopic image shows a human cancer cell that has transmigrated, possibly transcellularly, through the neighboring endothelial cell into a 3D collagen fiber matrix.

step has even been presented in videos. Thus, this method of transendothelial migration is now established for cancer cells (figure 14.6). However, the precise regulatory mechanisms are not yet well understood and require further investigation. Moreover, this transcellular mode of transendothelial migration of cancer cells seems to be involved in the generation of forces and utilizes the actomyosin cytoskeleton of the endothelial cell to migrate through its cytoplasm in a directed manner using the confinement of the endothelial cell as a migration grid.

However, when cancer cells use a transcellular invasion path, they trigger the activation of MLCK in this individual endothelial cell, which correlates with increased locally and spatially restricted phosphorylation of the myosin-II regulatory light chain (RLC) and followed by localized endothelial myosin contraction. Indeed, this has been functionally analyzed using endothelial cells expressing a RLC mutant that cannot be phosphorylated; the intravasation events of cancer cells migrating intracellularly (transcellularly) through the endothelial cell body are drastically reduced. In summary, (i) invasive cancer cells are capable of undergoing transcellular migration; (ii) cancer cells induce transient and local MLCK activation, as well as myosin contraction in adjacent endothelial cells at the site of transmigration and tissue invasion; and (iii) the transcellular invasion path through endothelial cells depends on the phosphorylation of myosin-II RLC. However, this result has to be confirmed through investigating more cancer cell types and cancer cells isolated from different stages of cancer disease. Nonetheless, all these findings demonstrate that the endothelium fulfills an exceptional and active role in cancer cells' intravasation—and possibly also in their extravasation.

14.4 The role of cancer cell exerted invadopodia during transendothelial migration

Cancer cell extravasation is a key step during cancer metastasis, yet the precise mechanisms that regulate this dynamic process are still unclear. A high-resolution time-lapse intravital imaging approach has been utilized to visualize the dynamics of cancer cell extravasation *in vivo*. During intravascular migration, cancer cells build protrusive structures identified as invadopodia through their enrichment of MT1-MMP, cortactin, Tks4 and, importantly, Tks5, which can be localized exclusively to invadopodia (figure 14.7). In more detail, cancer cells exert invadopodia through the endothelium into the extravascular stroma prior to their total extravasation at endothelial junctions. Genetic or pharmacological inhibition of invadopodia initiation (cortactin), maturation (Tks5) or function (Tks4) results in the elimination of cancer cell extravasation and metastatic colony formation in an experimental mouse lung metastasis model. Thus, this provides direct evidence for a functional role of invadopodia during the process of cancer cell extravasation and distant cancer metastasis and moreover reveals an opportunity for therapeutic intervention in this important process.

Metastasis is a complex scenario consisting of a multistep process that represents the most deadly aspect of cancer. Cancer cells that successfully disseminate from the primary tumor and survive in the vascular system eventually extravasate across the endothelium to colonize secondary sites. However, the process of cancer cell extravasation is the least understood step in the metastatic cascade, as it is difficult to investigate as no appropriate human microvascular endothelial cell lines exist and hence primary human endothelial cells have to used. Immune cell extravasation or diapedesis relies on ligand–receptor interactions for adhesion to the endothelium, assembling specialized structures called podosomes to promote their transmigration

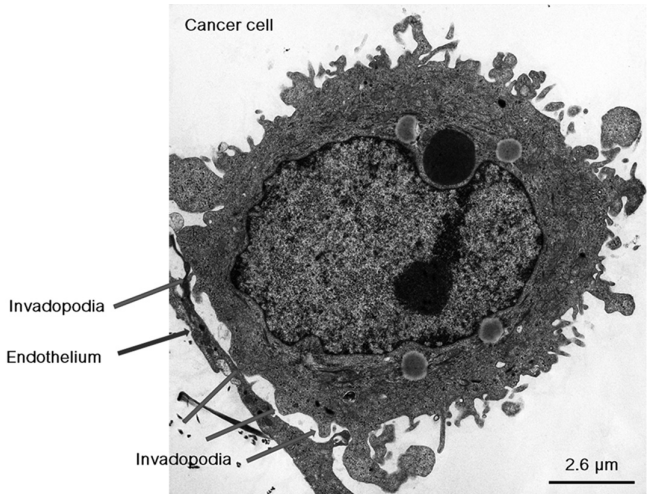

Figure 14.7. Invadopodia formation at the onset of transendothelial migration of a cancer cell.

across the endothelial layer (Carman *et al* 2007). Morphologically, these podosomes have been described as invadosome-like protrusions that are regarded as normal counterparts of subcellular protrusions commonly referred to as invadopodia in cancer cells (Carman and Springer 2004, Carman *et al* 2007, Murphy and Courtneidge 2011). Indeed, podosomes have been observed *in vivo* during atherogenesis of the intimal layer of mouse aorta (Quintavalle *et al* 2010) and in neural crest migration during embryonic development (Murphy *et al* 2011), however a physiological role for invadopodia in cancer disease has not been revealed.

Invadopodia are cancer-specific protrusive and adhesive structures, and were initially observed *in vitro* as flat protrusions on the basolateral side of cancer cells. Extensive efforts to characterize invadopodia and podosomes have shown that these two structures are composed of proteins such as cortactin, N-WASP, Tks4 and Tks5 (Clark *et al* 2007, Murphy and Courtneidge 2011, Oser *et al* 2009, Weaver *et al* 2002). However, Tks5 is exclusively present within podosomes, suggesting that it fulfills a specific role in invadopodia formation and maturation (Abram *et al* 2003, Seals *et al* 2005). Invadopodia contain proteases (such as MT1-MMP, MMP9 and MMP2) for local directed release and/or activity during extracellular matrix breakdown (Clark *et al* 2007) and, in particular, Tks5 is required in both invadopodia and podosomes to degrade the extracellular matrix *in vitro* (Caldieri *et al* 2009, Furmaniak-Kazmierczak *et al* 2007, Magalhaes *et al* 2011, Pignatelli *et al* 2012, Seals *et al* 2005).

The visualization of cancer cell invadopodia in living organisms has been elusive due to the challenges associated with distinguishing them from other invasive structures, such as lamellipodia (Gligorijevic *et al* 2012). However, established components of invadopodia, such as cortactin, MT1-MMP and Tks4, are also expressed in other protrusive structures, such as lamellipodia. Recent evidence suggests that invadopodia can indeed be specifically inhibited through loss of function of Tks5 (Burger *et al* 2014, Diaz *et al* 2009, Sharma *et al* 2013a). Moreover, cytoplasmic extensions assembled by cancer cells during intravascular arrest in capillary beds have been observed in zebrafish models (Stoletov *et al* 2010, Yamauchi *et al* 2006), although a functional link to extravasation has to be established through further experiments. The visualization and characterization of invadopodia structures in models of breast cancer (Kedrin *et al* 2008, Roh-Johnson *et al* 2014) have suggested that invadopodia are key mediators of intravasation (Eckert *et al* 2011, Gligorijevic *et al* 2012). Indeed, invadopodia have been observed in *ex vivo* experiments (Schoumacher *et al* 2010, Weaver *et al* 2013), leading to additional support for a role *in vivo*. However, despite the evidence that invadopodia are prevalent in metastatic cancer cells and fulfill a major functional role in the invasion and metastasis of cancer, direct evidence for their contribution *in vivo* is still elusive and needs further investigation.

As podosomes are involved in immune cell extravasation and invadopodia are prevalent in metastatic cancer cells, the role of invadopodia should be investigated in cancer cell extravasation *in vivo*. In particular, to visualize these dynamic cell interactions in high-resolution in real time, an intravital microscopy platform specifically developed to investigate cancer cell migration *in vivo* has been utilized

in the *ex ovo* chicken embryo model (Arpaia *et al* 2012, Leong *et al* 2010, 2012a, 2012b). Moreover, real-time 3D time-lapse intravital imaging was performed to visualize the behavior and dynamics of cancer cell extravasation *in vivo*. Indeed, direct evidence of the role of invadopodia during cancer cell extravasation has been provided. Moreover, it was demonstrated that disruption of invadopodia assembly via RNA interference with structural proteins (such as cortactin, Tks4 and Tk5), by either genetic or pharmacological means, results in reduced extravasation rates and finally the elimination of cancer metastasis.

Intravital imaging of human cancer cells and endothelium

The chorioallantoic membrane (CAM) of the chicken embryo, with its highly organized capillary bed network supported by arteries, veins and stromal cells, is an ideal model for visualizing the behavior of disseminating human cancer cells *in vivo* (Deryugina and Quigley 2008, Koop *et al* 1996). In order to investigate this, an intravital microscopy platform capable of capturing high-resolution 3D time-lapse imagery of human tumor growth, cell migration and extravasation using the *ex ovo* chicken embryo model was utilized (Arpaia *et al* 2012, Goulet *et al* 2011, Leong *et al* 2012a, 2012b, Palmer *et al* 2014, Zijlstra *et al* 2008). In more detail, visualization of the luminal surface of the CAM endothelium was achieved upon intravenous (IV) injection of fluorescent *Lens culinaris* agglutinin, which interacts specifically with the glyocalyx of avian endothelial cells (Jilani *et al* 2003). Moreover, the blood volume was visualized using IV fluorescent dextran. Extravascular regions of the CAM, marked by stromal cells were identified during intravital imaging through their lack of lectin–rhodamine staining. As has been reported previously (Arpaia *et al* 2012), a significant accumulation of lectin–rhodamine staining is located at endothelial junctions during intravital imaging experiments, allowing their precise localization within the tissue. In order to confirm this, the avian endothelium was transduced with cytoplasmic zsGreen *in vivo* using IV lentivirus prior to injection of lectin–rhodamine, which indeed resulted in cytoplasmic GFP expression in roughly 5% of the CAM endothelial cells. Thus, strong lectin staining corresponded precisely with the endothelial cell–cell junctions.

Cancer cells intravasate

In order to define the characteristics of cancer cell arrest and extravasation in this model, time-lapse intravital imaging was performed after the IV injection of fluorescent human epidermoid carcinoma (HEp3-GFP) cells into the vitelline vein of *ex ovo* chicken embryos, which were preinjected with lectin–rhodamine. Circulating cancer cells initially arrest at the distal end of CAM arterioles where they meet the capillary plexus. Cancer cells within the vascular lumen adopt an amoeboid morphology, maintaining close contact with the endothelium during intravascular migration, before extravasating into the adjacent targeted stromal layer of the CAM. During this process, highly dynamic cytoplasmic protrusions are exerted at the leading edge of intravascular HEp3-GFP cells. Although the majority of these dynamic protrusions are intravascular, a proportion of them extend through the entire endothelium into the adjacent stroma.

Cancer cells exert invasive cytoplasmic extensions, breaching the endothelium

Intravital imaging of the early stages of cancer cell extravasation revealed that cancer cells form cytoplasmic protrusions that extend through the endothelial cell lining into the extravascular stroma. In particular, it has been observed that cells typically migrate intravascularly along the endothelium for 6–8 h and then cease migration over the endothelium. At this point, protrusions are observed that cross the endothelium into the extravascular stroma. Approximately 1 1/2 h after initial contact, the cancer cell has begun extravasation and over the next 3 h, the extravascular portion grows larger, while the intravascular portion gradually shrinks as the cell translocates from the vessel lumen into the stroma. It has been observed that even single cancer cells form two distinct protrusions into the extravascular stroma. However, in all the cases observed, cancer cells follow a single protrusion in order to complete their extravasation. These invasive structures were identified in a substantial fraction of a range of cancer cells, as quantified by intravital imaging.

In particular, the precise route of extravasation was also investigated using high-resolution intravital imaging. When individual planes of 3D image volumes from extravasating HEp3-GFP cells were examined, it was clear that the majority of the invasive protrusions extended through endothelial junctions have separated them slightly, and moreover that these separations dissolve once extravasation is completed. Indeed, a range of cancer cell lines including HT1080 (fibrosarcoma), B16F10 (melanoma), MDA-MB-231LN (breast) and T24 (bladder) were evaluated and also found to extravasate mainly at endothelial junctions using the paracellular transendothelial migration mode. In some cases, it was observed that cancer cells extensively remodeled the local endothelium, appearing to displace endothelial cells to breach the endothelium in order to access the extravascular stroma. However, in these cases, the endothelial cells remained viable and no evidence of interendothelial transit was revealed (Carman and Springer 2008).

Cancer cells exert invadopodia through the endothelium

It has been hypothesized that components of invadopodia are present within the cytoplasmic protrusions of extravasating cancer cells observed *in vivo*. Indeed, histology was performed on frozen cross-sectional slices of CAM containing extravasating HT1080 fibrosarcoma cells. Visualized by fluorescence microscopy, extravasating cells exerted protrusions projecting through the endothelial layer and extending into the extravascular stroma. In more detail, these cellular protrusions contain both F-actin and cortactin, indicating that they may indeed be invadopodia.

In order to further characterize the identity and dynamics of these protrusions during extravasation, structural components known to be localized or concentrated in invadopodia, such as cortactin, Tks4 and Tks5 (Buschman *et al* 2009, Oser *et al* 2010, Sung *et al* 2011, Abram *et al* 2003, Seals *et al* 2005), were assessed in extravasating cancer cells using intravital imaging. Constructs encoding the fusion proteins cortactin-zsGreen (CTTN-zsG) and Tks4-zsGreen (Tks4-zsG) were stably transfected into three aggressive human cancer cell lines—epidermoid carcinoma HEp3, fibrosarcoma HT1080 (HT1080-tdT) and breast cancer MDA-MB-231LN (231LN-tdT). The localization of each of these cancer cells was confirmed using fluorescence imaging.

During extravasation of 231LN-tdT cells *in vivo*, cortactin-zsGreen was abundant in cytoplasmic protrusions extending through the endothelium. Although cortactin-zsGreen is also present in foci throughout the cell, the cytoplasmic extension exerted into the stromal layer contains organized structures of cortactin-zsGreen that would be expected in invadopodia. Indeed, localization of cortactin-zsGreen in HEp3 cells was present through diffuse puncta throughout the cell, as well as within thin invasive structures at the site of cancer cell extravasation. Indeed, a significant enrichment in cortactin-zsGreen was observed within protrusions that extended through the endothelium.

Moreover, the localization of Tks4-zsG in HEp3, HT1080-tdT and 231LN-tdT cell lines was also evaluated during extravasation, using intravital imaging. In intravascular 231LN-tdT breast cancer cells, the Tks4-zsGreen construct was present as a diffuse signal throughout the cytoplasm. During extravasation, however, Tks4-zsGreen localized to the apical tips of cytoplasmic protrusions, which are exerted into the CAM stroma. The localization of Tks5-GFP, by contrast, was concentrated at the base of the protrusion and even throughout the protrusion invading into the extravascular stroma. However, a similar concentration at the base of invadopodia was not observed in cells expressing Tks4-zsG. The proteolytic activity of MT1-MMP is a key marker of invadopodia and indeed it has also been observed in protrusions formed by extravasating 231LN-tdT cells and HT1080 cancer cells expressing MT1-MMP-GFP. The enrichment of cortactin, Tks4, Tks5 and MT1-MMP in these invasive protrusions further suggests that they really display invadopodia and moreover the intravital imaging experiments suggest that cancer cells extend invadopodia between endothelial cells and into the extravascular stroma prior to and during extravasation.

Tks4, Tks5, and cortactin are required for invadopodia formation

To reveal the impact of invadopodia on extravasation *in vivo*, loss-of-function experiments that targeted Tks4 and Tks5 individually were performed; these were reported to inhibit invadopodia function *in vitro* (Buschman *et al* 2009, Diaz *et al* 2009). However, it has been hypothesized that RNAi knockdown of Tks4 or Tks5 can inhibit invadopodia assembly in cancer cells, thus inhibiting cancer cell extravasation and finally cancer metastasis. In particular, cancer cell lines with stable small hairpin RNA (shRNA) knockdowns of cortactin, Tks4 and Tks5 were evaluated for their ability to form invadopodia, to extravasate and to establish metastatic colonies *in vivo*. These results were compared with cells lacking the key cell migration regulator RhoA. When assessed for the cancer cell's ability to degrade extracellular matrix, loss of cortactin, but not loss of RhoA, a reduced the fraction of cells able to degrade gelatin.

In more detail, HEp3-GFP cells expressing RhoA or cortactin shRNAs were assessed in extravasation assays with the chick embryo CAM using an intravital imaging approach. Thus, cancer cells arrested in the CAM were visualized and classified according to their location as intravascular, in the process of extravasating, and extravascular. The majority of shLuc HEp3-GFP cells (40%–50%) extravasate within 24 h, with very few cells present in the intravascular space. In contrast, a

significantly greater proportion of shCTTN cells remained in the intravascular space at each time point, whereas very few shRhoA cells were present within the intravascular space. Moreover, very few shRhoA and shCTTN cells successfully extravasated 24 h after intravenous injection. Because the numbers of extravasating cells can be monitored over time in each embryo, the number of cells that have been lost or have died can also be determined. Cells expressing shRhoA exhibited the greatest loss between the zero hour to six hour time points, indicating that most of these cells probably died in the intravascular space. Cell damage was much lower in the first six hours for shLuc and shCTTN cells, indicating that the significant difference in the numbers of intravascular and extravascular cells after 24 h was not due to cell death, but to an inhibition of the shCTTN cells to extravasate.

The impact of cortactin on subsequent metastatic colony formation was then investigated using intravital imaging and an experimental metastasis approach. Two hundred thousand cells per embryo were injected intravenously and after seven days the number of metastases, micrometastases and single cancer cells present throughout the entire CAM organ was determined. In more detail, embryos injected with shRhoA and shCTTN cells displayed the fewest metastases, micrometastases and single cancer cells compared to both parental and shLuc control cells. In summary, these data indicate that RhoA depletion inhibits metastatic colony formation primarily through cell damage, whereas the inhibition of invadopodia through the depletion of cortactin impairs metastasis as a result of abolished cancer cell extravasation.

Although Tks4 is predominantly localized to invadopodia, it has also been observed within lamellipodia, whereas Tks5 has only been observed in invadopodia (Abram *et al* 2003, Buschman *et al* 2009, Seals *et al* 2005). To establish the requirement of invadopodia for extravasation and metastatic colony formation, 231LN-tdT cell lines with stable shRNA knockdowns for Tks4 and Tks5 were evaluated for their ability to extravasate in the CAM of avian embryos over a 24 h time period. In particular, 231LN-tdT cells lacking RhoA, CTTN, Tks4 or Tks5 had significantly reduced extravasation rates compared to control cells. Moreover, these cells were also observed to have decreased incidence of protrusions formed by cells arrested in the intravascular space three hours after intravenous injection of cells. Taken together, this provides strong evidence that invadopodia are indeed required for efficient extravasation in the CAM. These *in vivo* loss-of-function experiments revealed a functional requirement for CTTN, Tks4 and Tks5 in the formation of invadopodia and finally cancer cell extravasation.

Src kinase inhibition eliminates invadopodia formation

Src kinase regulates invadopodia formation via phosphorylation of cortactin to its active state (Evans *et al* 2012, Mader *et al* 2011, Oser *et al* 2009), while also phosphorylating a number of other targets (Ferrando *et al* 2012). The treatment of HEp3-GFP cells with the Src kinase inhibitor Saracatinib at 1.0 mM significantly altered *in vitro* cell morphology from a stellate to cobblestone morphology. Marked reductions of cortactin-rich invadopodia and the associated adhesion-type structures that are characteristic of invadopodia were detected. The impact of Src kinase

inhibition on the extravasation kinetics of HEp3-GFP cells in the avian embryo CAM was analyzed *in vivo* over a 24 h time period, comparing Saracatinib at 1.0 mM versus vehicle. As expected, no *in vivo* or *in vitro* cytotoxic effects were observed when Saracatinib was administered to a final concentration of 1.0 mM. Saracatinib-treated cancer cells at 3–6 h after intravenous injection exhibited a significant reduction in invadopodia assembly compared to vehicle-treated cells. Over 24 h, Saracatinib treatment resulted in significantly decreased extravasation rates compared to vehicle control after 24 h and thus was accompanied by a retention of Saracatinib-treated cells in the intravascular space after 24 h. The interpretation of the data is complicated due to the fact that more Saracatinib-treated cells were lost or died in the first three hours compared to vehicle-control-treated cells, resulting in significantly fewer cells successfully extravasating after 24 h. However, these data suggest that Saracatinib directly affects cancer cell extravasation, which indicates that it might be a useful therapeutic reagent.

Inhibition of Tks4 or Tks5 reduces metastatic colony formation in a mouse model
Following the observations in the chicken embryo CAM model, a mammalian adult cancer model was used to confirm them. Clonogenic MDA-MB-231LN-tdT cell lines were generated with stable shRNA knockdowns for Tks4 and Tks5 and evaluated for their ability to extravasate and form metastatic colonies in murine lungs after tail vein injection in nude beige mice. Extravasation efficiency was determined by comparing the number of cells that extravasated 24 h after intravenous injection to the number of cells that initially arrested in the lung zero hour after intravenous injection. According to the histological sections of injected murine lungs, the majority of cells were intravascular immediately after injection, and thus all cells that were present in the lungs after 24 h had successfully extravasated, as determined by confocal microscopy. The knockdown of Tks4 or Tks5 led to significant decreases in extravasation compared to the shLUC control. Indeed, the majority of the Tks4 and Tks5 shRNA clones exhibited negligible rates of extravasation, with no extravasated cancer cells after 24 h post-injection. Moreover, the metastatic burden was also evaluated four weeks post-injection, where knockdown of Tks4 or Tks5 resulted in a significant reduction in the number of macrometastases, micrometastases and extravascular single cells. In summary, these data demonstrate that in two xenograft models of human cancer metastasis invadopodia are required for extravasation and metastatic colonization of distant sites.

The extravasation of cancer cells at distant sites occurs predominantly within capillary beds (Chambers *et al* 2002) and is thought to be a key step in the metastatic cascade, preceding metastatic colony formation. In the past, a putative role for invadopodia in cancer cell migration and invasion has been extensively reported using *in vitro* approaches (Artym *et al* 2006, Buschman *et al* 2009, Diaz *et al* 2009, Linder 2007, Mader *et al* 2011, Oser *et al* 2009), whereas a lack of direct *in vivo* evidence for invadopodia has raised questions regarding their physiological relevance to cancer disease. Using cortactin, MT1-MMP and Tks4/5 fusion expression constructs, and shRNA-facilitated knockdown, it was determined that invadopodia

are formed early in the extravasation process, manifesting as protrusions that breach the endothelial layer, and that they are required for successful extravasation. Taken together, cancer cell extravasation is a highly coordinated and dynamic process that occurs within 24 h, consistent with other observations during the initial intravital imaging experiments (Koop *et al* 1996). However, the inhibition of the structural or functional components of invadopodia results in a pronounced reduction in metastatic colony formation in two different experimental models of metastasis. Finally, by providing direct evidence of the functional importance of invadopodia in cancer cell extravasation *in vivo*, these studies demonstrate that invadopodia are crucial in the metastatic cascade and represent a potential therapeutic target for antimetastasis strategies.

The assembly of invadopodia is a precisely regulated and sequential process that is characterized by the initial formation of the nondegradative invadopodium precursors that are enriched in actin regulators such as cortactin, Arp2/3 and cofilin (Clark *et al* 2007, Oser *et al* 2009). Next, these precursors mature through a sequence of events involving stabilization through Tks5 interactions (Blouw *et al* 2008), actin polymerization and the recruitment of matrix proteases such as MT1-MMP, whose localization and stability is in turn regulated by factors such as Tks4 (Buschman *et al* 2009). Moreover, this results in mature, matrix-degrading invadopodia that regulate the remodeling of the extracellular matrix to increase cell migration and translocation. Based on these observations regarding the ability of invadopodia to facilitate cancer cell transmigration through the endothelial layer in the early steps of cancer cell extravasation, it has been hypothesized that their inhibition may prevent extravasation and consequently inhibit the formation of metastatic colonies. Thus, it has been investigated how the inhibition of distinct steps of invadopodia initiation (cortactin), maturation (Tks5) and function (Tks4) impacts on the extravasation of metastatic cancer cells. In addition, these results have been compared to the results regarding the loss of RhoA, which has been suggested to impair cell migration in an invadopodia-independent way. Indeed, the extravasation rates were significantly reduced in the CAM of the avian embryo and in mouse lungs when any of these contributors to invadopodia were depleted in cancer cells. In particular, the depletion of Tks4 or Tks5 in cancer cells also led to a reduction of micrometastases and single migratory cancer cells compared to control cancer cells, indicating that the inhibition of invadopodia has additional anti-metastatic effects that finally lead to a further reduction in metastatic efficiency. These observations are consistent with other studies that have established a role for invadopodia in other steps within the metastatic cascade, such as tumor growth and intravasation (Blouw *et al* 2008, Gligorijevic *et al* 2012, Sharma *et al* 2013b), and which implicate these structures in a general mechanism for cancer cell motility and translocation.

Taken together, it has been established that the inhibition of extravasation by targeting invadopodia seems to be a viable antimetastasis approach. Nonetheless, a clinical window of opportunity for anti-metastatic therapies may not exist for all cancer patients, especially considering the primary tumor's ability to disseminate cancer cells into the circulation at early stages of progression. However, there may be possibilities for therapeutic opportunities at other distinct stages of cancer

development and treatment, but this is not addressed here. A substantial body of clinical evidence suggests that cancers such as prostate cancer acquire metastatic potential during the course of progression. Thus, some benefit from antimetastasis agents might be provided for patients who are identified as being at increased risk of metastasis (Palmer *et al* 2014). Finally, based on the growing evidence that cancer cells are shed into the circulation after core-needle biopsy (Hansen *et al* 2004) or surgery (Juratli *et al* 2014), it would be worth exploring whether an antiextravasation approach might help to eliminate a potential metastasis risk from these procedures.

The understanding of cancer cell extravasation has been advanced to a significant extent by well-characterized mechanisms of immune cell transendothelial migration or diapedesis. In particular, this both clarifies and substantiates the postulated concepts of cancer cell extravasation through the dynamic visualization of individual cancer cells at high resolution. Although the processes of cancer cell extravasation and leukocyte diapedesis share several features in common (Carman and Springer 2008), there seems to be no evidence from intravital imaging experiments that cancer cells undergo transcellular migration, or migration through pores created in endothelial cells, which has been observed with leukocytes. This raises the question of whether this transcellular mode is indeed a commonly used transmigration mode for cancer cells. The examination of thousands of cells from a panel of cancer cell lines undergoing extravasation demonstrated clearly that cancer cells in this model system only use a paracellular mode of transendothelial migration into the extravascular stroma. The simplest explanation for this observation is that cancer cells are typically much larger in volume than leukocytes or endothelial cells, which may cause a large hole within the endothelial cell upon transcellular migration that might eventually destroy its cytoskeletal architecture. As the cytoskeletal architecture is required for the transcellular transmigration of cancer cells, this mode does not seem to be the most common to be used. It has been observed that a minority of extravasating cells gained access to the extravascular stroma through a more pronounced displacement of endothelial cells. However, this did not appear to affect the viability of the endothelial cells and is consistent with previous observations in an *in vivo* model (Stoletov *et al* 2010), suggesting that cancer cells can play an active role in remodeling the local endothelium, which has been observed in an *in vitro* transendothelial migration model (Mierke *et al* 2008, Mierke 2011). This is particularly interesting, as it has been suggested that invadopodia biogenesis is linked with the secretion of exosomes (Hoshino *et al* 2013). In the intravital imaging experiments, invadopodia have been revealed to be highly dynamic in morphology as they extend into the extravascular stroma and they are even sporadically associated with the transient release of microparticles. The idea that invadopodia utilize a tightly regulated microvesicle release mechanism in order to influence the local microenvironment is a compelling one and thus requires further detailed investigation. Taken together, a powerful model has been established to visualize and dissect the key functional and structural components of invadopodia and provide significant evidence for an invadopodia role during cancer cell extravasation

14.5 Tumor extracellular vesicles and interaction with the vascular system such as endothelial cells and immune cells

Tumor-secreted extracellular vesicles (EVs) represent critical components of intercellular communication between cancer cells and stromal cells in local and distant microenvironments (Becker *et al* 2016). Thus, EVs fulfill an essential role in primary tumor growth and in the malignant progression of cancer such as metastasis. EVs play a role in multiple systemic pathophysiological processes, such as vascular leakiness, blood coagulation, and reprogramming of stromal embedded cells to provide the pre-metastatic niche formation and subsequent cancer metastasis. Moreover, in clinical approaches, EVs may serve as biomarkers and/or novel therapeutic targets for malignant cancer progression, possible for predicting and preventing future metastatic initiation, metastatic reoccurrence and development.

Various types of EVs exists and no clear discrimination has been made between the different types, and hence they are all termed EVs, such as microvesicles, exosomes, ectosomes, oncosomes and cytoplasts (Colombo *et al* 2014, Di Vizio *et al* 2012, Headley *et al* 2016, Thery *et al* 2009, van der Pol *et al* 2012). In detail, exosomes are EVs 30–150 nm in diameter and are derived from the multivesicular endosome pathway, however, the term is used widely in multiple studies for small EVs, which have been recovered by different protocols that cannot actually distinguished between endosome-derived and cell membrane-derived EVs. Hence, the term 'exosomes' is mostly used in studies without discrimination between an exclusively endosomal or exclusively cell membrane origin of the EVs. The EVs are not simply membrane enclosed objects containing water, instead they contain bioactive molecules such as nucleic acids (including DNAs, mRNAs, microRNAs, and other non-coding RNAs), proteins (including receptors, extracellular matrix proteins, transcription factors and enzymes) and lipids that are able to alter and hence redirect the function of an exosome accepting (recipient) cell (Raposo and Stoorvogel 2013). For instance, cancer cell-derived EVs induce angiogenesis, provide coagulation and alter the immune system, and can even restructure the surrounding parenchymal tissue, all of which enhances the malignant progression of cancer (Ciardiello *et al* 2016, Peinado *et al* 2011, Ratajczak *et al* 2006, van der Pol *et al* 2012). In clinical aspects, circulating exosomes and microvesicles that are obtained from cancer patients have been correlated with cancer metastasis or relapse, and hence can serve as possible diagnostic and prognostic biomarkers and also be the target of therapeutic drugs (Lener *et al* 2015).

Can tumor extracellular vesicles make a path through the extracellular compartment? EVs that have been shed from the primary tumor, get possibly via diffusion through the tumor stroma, enter blood vessels, circulate within the vessels and hence can be isolated from nearly all body fluids such as blood, saliva and urine (Boukouris and Mathivanan 2015, Ciardiello *et al* 2016). There is accumulating evidence that circulating EVs facilitate the so-called 'reprogramming' of multiple cell types at distant targeted sites and thereby affect various processes such as blood coagulation, the immune response, and subsequently the establishment of a pre-metastatic niche.

14.5.1 Tumor extracellular vesicles induce vascular leakiness and promote circulating tumor cell arrival to distant sites for metastasis

Vascular leakiness seems to represent a hallmark of the formation of a pre-metastatic niche (Huang *et al* 2009, Psaila and Lyden 2009). In particular, melanoma-secreted vesicles can evoke vascular leakiness, inflammation and the recruitment of bone marrow progenitor cells by upregulating factors such as S100a8, S100a9 and TNF-α (Peinado *et al* 2012). Moreover, isolated exosomes of human breast cancer cause vascular leakiness in the lung through the enhanced expression a subset of S100 proteins and the activation of Src kinase signal transduction processes (Hoshino *et al* 2015). Metastatic breast cancer cells secreting miR-105-containing exosomes can disrupt the tight junction protein ZO1 in interacting endothelial cells thus enhancing their vascular permeability (leakage) and subsequently susceptibility for the metastatic invasion of cancer cells into the targeted tissues or organs (Zhou *et al* 2014). Taken together, these findings lead to the hypothesis that tumor-secreted exosomes first increase the permeability of the vessels through the delivery of specific transport goods to endothelial cells, then diffuse through this weakened endothelial barrier and, finally, exosomes can fuse directly with parenchymal cells within the novel pre-metastatic niches supporting the metastatic colonization. However, more research effort is needed to reveal the exact mechanism through which EVs break down the integrity of the endothelial barrier and how the specificity of this targeting is altered within vasculature of different organs, as specific cancer cell type derived exosomes target only distinct regions for new metastatic niches.

14.5.2 Tumor extracellular vesicles help to establish new target sites for tumor growth termed the pre-metastatic niche

The pre-metastatic niche is well characterized by the development of an environmentally distant region from the primary tumor that is suitable for the survival and outgrowth of incoming circulating tumor cells. In particular, the concept of the pre-metastatic niche has been established and initiated by an early observation made by Stephen Paget in 1889, who observed that different cancer types metastasize to different organs, which leads to the suggestion that the microenvironment plays a prominent role in dictating and predicting the metastatic invasion and the secondary tumor formation (Paget 1989, Peinado *et al* 2011).

The role of tumor-secreted factors and EVs in initiation of a pre-metastatic niche and its evolution has received a great deal of attention. It has been demonstrated that the combined effects of soluble factors and exosomes derived from CD44 variant isoform (CD44v)-positive pancreatic cancer cells facilitate the establishment of a pre-metastatic niche within lymph nodes and the lung (Jung *et al* 2009). Moreover, CD105-positive microvesicles that are released from human renal cancer stem cells induce tumor angiogenesis and the initiation of pre-metastatic niches in the lungs through a defined subset of pro-angiogenic mRNAs and microRNAs (Grange *et al* 2011). Tumor-exosomal miR-494 and miR-542p were transported to lymph node stromal cells and lung fibroblasts, which causes the down-regulation of cadherin-17 and the up-regulation of matrix metalloproteinase up-regulation, such

as MMP2, MMP3, and MMP14 (Rana *et al* 2013). In addition, the alteration of the glucose metabolism through the transfer of the mir-122 from breast cancer-derived microvesicles to stromal cells has been demonstrated to be crucial for the establishment of the pre-metastatic niche (Fong *et al* 2015). By impairing the uptake of glucose in stromal cells through a miR-122-based inhibition of the pyruvate kinase, breast cancer cells build a pre-metastatic niche with higher glucose availability for their own utilization to form secondary tumors (Fong *et al* 2015).

The activation of TLR3 in lung epithelial cells through tumor-exosomal non-coding snRNA increases the expression of S100A8, A100A9, MMP9, Bv8 and fibronectin, which then fosters the formation of a pre-metastatic niche in the lung (Liu *et al* 2016). The up-regulation of TLR3 induces the secretion of chemokines that mobilize neutrophils ($CD45^{+}CD11b^{+}Ly6G^{+}Ly6C^{int}cKit^{+}$ $VEGFR1^{+}$), as well as macrophages ($F4/80^{+}$, which is a unique marker of murine macrophages) and monocytes ($VEGFR1^{+}Ly6G^{-}Ly6C^{+}$) that further enhance the pre-metastatic niche establishment (Liu *et al* 2016). Moreover, tumor-secreted exosomes have their own protein 'zip-codes', which are the specific integrin profiles that address them to specific targeted organs, thus hence determine metastatic organotropism (Hoshino *et al* 2015). Thus, tumor exosomes deliver signals that cause the upregulation of pro-inflammatory S100 molecules in resident cells of the targeted organs and thereby cause molecular and cellular alterations that induce the development of a pre-metastatic niche (Hoshino *et al* 2015). The imaging of tumor-secreted EVs in metastatic organs has revealed that the interactions of EVs with targeted cells are highly dynamic within pre-metastatic niches and support their role in facilitating phenotypic alterations within stromal cells at selected target sites of metastasis (Suetsugu *et al* 2013, Zomer *et al* 2015).

The recruitment of various cell types, such as fibroblasts, endothelial cells, macrophages and various populations of bone marrow-derived cells (BMDCs) to the pre-metastatic niche is driven by tumor-secreted exosomes (Costa-Silva *et al* 2015, Peinado *et al* 2012). These exosomes are secreted from pancreatic cancer cells and perform the stepwise progression of pre-metastatic niche establishment in the liver, which is targeted for metastasis (Costa-Silva *et al* 2015). In particular, Kupffer cells, which represent the resident macrophages in the liver, are the primary target for exosomes derived from pancreatic cancer cells that activate Kupffer cells upon their uptake (Costa-Silva *et al* 2015). Pancreatic tumor-derived exosomes containing the macrophage inhibitory factor (MIF) promote the secretion of TGF-β in Kupffer cells, which in turn stimulates neighboring hepatic stellate cells to secrete fibronectin that subsequently recruits BMDCs to the pre-metastatic niche and creates a fully active metastatic niche for hosting secondary tumors (Costa-Silva *et al* 2015). Moreover, melanoma-secreted exosomes promote the pre-metastatic niche formation in the lung by reprogramming of BMDCs, which then cause the recruitment and activation of lung cells (Peinado *et al* 2012). Mechanistically, the transfer of the single pass tyrosine kinase receptor (MET) from melanoma exosomes to $c\text{-}Kit^{+}$ $Tie2^{+}$ bone marrow progenitor cells results in a pro-vasculogenic behavior of these cells (Peinado *et al* 2012). Moreover, exosome-secreted MET has been proposed to induce the progression of hepatocellular carcinoma, which leads to the enhanced

mobilization of normal hepatocytes that themselves seem to facilitate the protrusive activity of HCC cells through liver parenchyma during the process of metastasis (He *et al* 2015). Evidence has provided that gastrointestinal stromal tumors release exosomes containing the oncogenic protein tyrosine kinase KIT, which induces the conversion of progenitor smooth muscle cells into tumor-supporting cells by inducing tumor invasion (Atay *et al* 2014).

Melanoma-derived exosomes facilitate the cancer cell recruitment, extracellular matrix deposition, and the proliferation of vascular cells in the lymph nodes (Hood *et al* 2011). Several genes related to the cell recruitment, such as Stabilin 1, Ephrin receptor β4 and αv integrin, related to extracellular matrix, such as Mapk14, uPA, Laminin 5, Col 18α1 and G-α13, and related to vascular growth factors, such as TNF-α, TNF-αip2, VEGF-B, HIF-1α and Thbs1, were enhancedly expressed, which is caused by tumor-secreted exosomes in lymph nodes (Hood *et al* 2011). In line with this, exosomes isolated from highly metastatic colorectal cancer facilitate metastasis by recruiting CXCR4-expressing stromal cells, providing the establishment of suitable metastatic microenvironment and hence reinforcing cancer metastasis (Wang *et al* 2015).

Taken together, these data suggest a prominent role for tumor-derived EVs in both early and late pre-metastatic niche assembly. It can be assumed that their major contribution to the development of the metastatic niche upon the arrival of cancer cells and also to the enhanced progression of cancer, is to switch from a micro-metastatic to macrometastatic state.

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Claudia Tanja Mierke

Chapter 15

The mechanical properties of endothelial cells altered by aggressive cancer cells

Summary

The mechanical properties of cells have long been ignored and only the structural and the compositional aspects of cellular components have been analyzed. When analyzing the mechanical properties of cells, the microenvironment in which they are cultured plays a crucial role. When replicating the distinct tumor microenvironment there is indeed a central challenge in the development of novel experimental *in vitro* models for cancer. To develop and establish a reliable tool for analyzing the drug development and for investigating a personalized cancer therapy, it is crucial to mimic and also to implement certain key features of the original tumor development and progression. Based on this effort, 3D cellular aggregate models are being extensively developed and improved in terms of reproducibility and high throughput and hence employed for co-cultures or multiple cell cultures.

The process of mechanotransduction has become a research focus for biophysical research, and especially for cancer research, and it is still of high importance. Moreover, it has been reported that the mechanical properties of cancer cells support their invasive and aggressive potential in order to promote the malignant progression of cancers. However, the mechanical properties of the endothelial barrier of blood or lymph vessels has not yet become the focus of cancer metastasis research, and only a few papers have investigated the role they play in providing the passive endothelial barrier function or promoting the transmigration of special cancer cells.

In addition to the investigation of single cells or co-cultures, spheroids become a subfocus in investigating the mechanical properties of cells in a collection or aggregate. In particular spheroids are self-assembled cell aggregates, which possess several important components promoting the physiological spatial growth and cell–cell interactions. Moreover, using more sophisticated hybrid spheroids, the interconnection between cancer cells and endothelial cells can be analyzed. For this

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assembly and preparation of hybrid spheroids, either established cancer cell lines or patient-derived cancer cells can be employed in addition to single-cell morphologies. Additionally, the overall morphology of the spheroid can be analyzed and compared to tissue-like features of natural tumors, which can also be addressed. In addition to the simple co-culture within the hybrid spheroids, also capillary-like structures can be built through the induction of the endothelial cell assembly to capillaries. A special feature of these spheroids is that the spheroids' shape and surface texture can be used as an indication of spatial invasiveness of cancer cells or other spheroid cell types to migrate in and invade through the surrounding the extracellular matrix, which is an additional parameter that can be altered. The establishment of a model such as hybrid tumor/stroma spheroids seems to be crucial for personalized medicine and may provide a reliable, suitable and low-cost high-throughput method for improving the prediction of drug effects and therapy success.

15.1 The role of endothelial cell stiffness

How is endothelial cell stiffness impaired by substrate stiffness in 2D and 3D?
Over the last two decades, there has been growing experimental interest in the impact of passive mechanical properties—such as the viscosity (Edwards *et al* 1996), microstructure (Sieminski *et al* 2002), and especially the stiffness (Discher *et al* 2005, Peyton *et al* 2007)—of the local microenvironment on cellular functions relevant to development, homeostasis and disease. For instance, in fibroblasts the substrate stiffness has been shown to affect the rate (Pelham and Wang 1997) and directionality (Lo *et al* 2000) of cell migration, the assembly of focal adhesions (Pelham and Wang 1997) and the formation of actin stress fibers (Halliday and Tomasek 1995, Yeung *et al* 2005). In addition, neurons display increased branching densities when cultured on compliant substrates, whereas glia cells, which are normally co-cultured with neurons, do not even survive on deformable substrates (Flanagan *et al* 2002). Thus, these results show how import the stiffness of the substrate for the culture of cells is. Moreover, the substrate stiffness can no longer be ignored and many 'old' experiments performed on flat 2D substrates need to be refined using 3D substrates. How can cells form stress fibers and focal adhesions in 3D extracellular matrices? Is the quantity of stress fibers and focal adhesion different to that for 2D substrates? What about structures such as filopodia and lamellipodia, do they also assemble in 3D matrices?

Substrate stiffness pronouncedly influences the differentiation of mesenchymal stem cells, with soft, intermediate and stiff materials being neurogenic, myogenic and osteogenic, respectively (Engler *et al* 2006). Moreover, it has been shown that sensitivity to substrate stiffness requires nonmuscle myosin II activity, which applies tension to the actin cytoskeleton and hence stiffens cortical actin structures. It has been shown consistently that mesenchymal stem cells (such as C2C12 myoblasts and human osteoblast (hFOB) cells cultured on collagen-laminated polyacrylamide gels with a substrate stiffness of 1000, 10 000 and 40 000 Pa) have a matrix stiffness that is dependent on the increase in cellular stiffness (Engler *et al* 2006). It has been reported that in the case of fibroblasts grown on fibronectin-coated polyacrylamide

gels, the cells' elastic moduli were equal to (or slightly lower than) those of their underlying substrates for a range of substrate stiffness up to 20 kPa (Solon *et al* 2007).

These studies used cells cultured on top of protein-laminated polyacrylamide gels, where the cells interact with a 2D surface. In particular, this system provides excellent control of the substrate stiffness, while it does not provide an option for the cells to be cultured within a 3D matrix, which for many cell types is more representative for their native microenvironment. For these cell types, cell culture within 3D extracellular matrix gels would provide a more realistic microenvironment, with the stiffness of the gels controlled by the gel concentration, crosslinker concentration or the mechanical boundary conditions of the gels (Nehls and Herrmann 1996, Roeder *et al* 2002, Sieminski *et al* 2004). For instance, the stiffness of collagen gels regulates the morphology of embedded fibroblasts, with compliant gels supporting elaborate dendritic extensions. Indeed, the proliferation and migration of mammary epithelial cells is regulated by the stiffness of the surrounding gels (Paszek *et al* 2005). In particular, endothelial cells within 3D collagen gels form microvascular networks, with the average length of the network and the average lumen area altered by the stiffness of the gel (Sieminski *et al* 2004). Moreover, endothelial cells display similar stiffness-induced alterations in network morphology when cultured in self-assembling peptide gels (Sieminski *et al* 2007).

While there is indeed evidence that substrate stiffness can regulate cellular functions, little is known about how a given response to alterations in 2D substrate stiffness might correlate with cellular responses to alterations in 3D substrate stiffness (Peyton *et al* 2007). Thus, the effects of 2D and 3D substrate stiffness on the regulation of endothelial cell stiffness can be investigated. In addition, the substrate stiffness effects on actin can be investigated, as actin is a major determinant of the cell's mechanical properties, with both the abundance of actin and its tension evoked by myosin playing important roles (Pourati *et al* 1998). Endothelial cells are an ideal cell type for these studies, as they reside *in vivo* in both types of microenvironments: the endothelial monolayers lining blood or lymph vessels resemble the 2D *in vitro* microenvironment and capillaries surrounded by the basement membrane resemble the 3D *in vitro* microenvironment Mierke 2011.

Endothelial cells can alter their cell stiffness (figure 15.1) in response to the stiffness of the underlying substrate that they are adhered to. Indeed, these observations confirm the results of earlier studies of fibroblasts, mesenchymal stem cells and immortalized osteoblast and myoblast cell lines (Engler *et al* 2006, Solon *et al* 2007). In addition, it has been reported that the stiffness of the 3D matrix in which cells are cultured also alters the stiffness of the embedded cells. As technical restrictions prevent the measurement of cell stiffness within 3D gels, for these cells stiffness measurements can be performed directly after they have been isolated from gels of different stiffness. For the following reasons, it is reasonable to expect that the cell stiffness measured on cells directly isolated from gels reflects their stiffness within the gels. First, quantification of the actin staining performed on cells still within the 3D gels reveals differences in the intensity of the actin staining consistent with the trends observed in the stiffness measurements. The knowledge that

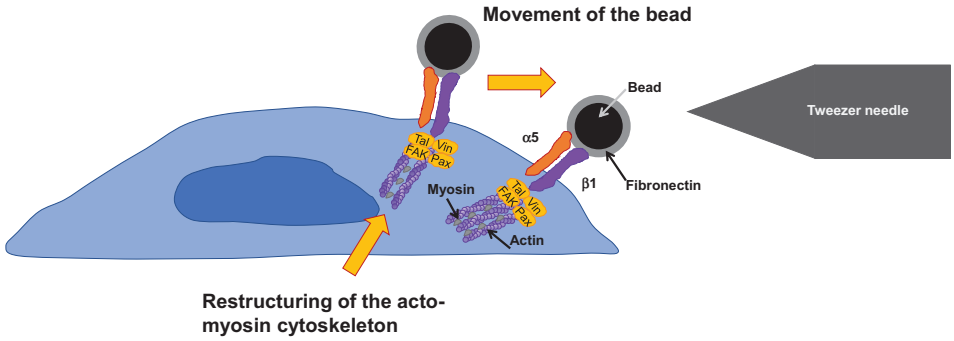

Figure 15.1. Endothelial cell stiffness can be measured using magnetic tweezers.

alterations in cell stiffness are accompanied by alterations in the actin cytoskeleton was gained from a recent study in which fibroblasts were cultured on fibronectin-coated polyacrylamide gels of different stiffness (Solon *et al* 2007). Second, cells isolated from gels of different stiffness had different initial stiffness and these differences remained stable for several hours, which is all that is required to perform microaspiration measurements. Finally, over a period of 8–16 h, the stiffness and actin content/structure of cells isolated from gels are stabile, as they adapt to their new microenvironment. The timescale of this stiffness adaptation process is much slower than is required for the measurements of cell stiffness using microaspiration.

The choice of the stiffness range for the 2D polyacrylamide gels was guided by the work of Yeung and colleagues, which showed that cells cultured on matrices with a stiffness of 1600 Pa or less lacked stress fibers or other actin bundles, whereas cells on matrices with a Young's modulus E of 3200 Pa or greater displayed significant stress fibers (Yeung *et al* 2005). In more detail, it has been shown that endothelial cells remain attached to the gels, but do not entirely spread on top of gels with an elastic modulus less than approximately 1000 Pa. Thus, a range of gel stiffness is selected that would affect endothelial cells while still ensuring that the cells can exhibit normal behavior (Yeung *et al* 2005) on top of the substrate. The results for endothelial cells on polyacrylamide gels confirm published results showing cells that are able to respond to alterations in stiffness on the order of 1000 or 10 000 Pa (Byfield *et al* 2009, Mierke *et al* 2008a). Thus, it may be suggested that alterations across a similar stiffness range would be required to stimulate alterations in endothelial cells within gels. However, there are several reasons for selecting a lower stiffness range for the 3D gels. First, it has been demonstrated that endothelial cells within 3D gels can alter their morphology, which may lead to alterations in this range of substrate stiffness (Sieminski *et al* 2004, Kanzawa *et al* 1993). Second, it has been shown that endothelial cells in significantly stiffer 3D gels (~1000 Pa) cannot form microvascular networks and hence remain essentially spherical (Sieminski *et al* 2007). Thus, as in the 2D studies, a range of gel stiffness is selected that affects endothelial cell behavior, while still ensuring that the cells exhibit normal behavior.

While endothelial cells displayed similar alterations in their cellular stiffness in response to alterations in either 2D substrate (cells on top of a gel) stiffness or 3D substrate (cells embedded in a gel) stiffness, the magnitude of the 2D-substrate-stiffness-induced effects was about an order of magnitude greater than those from 3D substrate stiffness. The idea that matrix dimensionality may facilitate the matrix stiffness effects is consistent with other concepts. In particular, it has been suggested that the matrix dimensionality and matrix stiffness both work together to regulate assembly of cell–matrix adhesions (Cukierman *et al* 2001). Cellular responses to alterations in either 2D substrate stiffness or 3D substrate stiffness have been detected and hence support the conclusion that the dimensionality of the substrate may indeed regulate stiffness effects. For instance, there are several studies that have investigated the effects of 2D or 3D substrate stiffness on different endothelial cell functions, however, those analyzing the cells within 3D gels observed responses at lower stiffness compared to the 2D situation. Similarly, those studies investigating fibroblasts in 3D collagen matrices saw effects attributed to stiffness at collagen concentrations in the range of those used by Grinnell and colleagues (Grinnell 2003), whereas those studying matrix stiffness effects in fibroblasts on top of extracellular matrix-laminated polyacrylamide gels used higher stiffness levels, ranging from 1000 to 30 000 Pa (Lo *et al* 2000, Jiang *et al* 2006, Kostic and Sheetz 2006).

In response to the notion that dimensionality is a key variable, it should be said that other differences between the cells in the collagen gel system and the cells on the polyacrylamide gel system may account for the observed difference in effectual stiffness. For instance, the ligand density, which will vary between these two systems, and the stiffness are known to interact, regulating cellular responses in other systems (Engler *et al* 2004). However, the apparent differences in the effectual stiffness that were observed may not represent true differences in the endothelial cells' response to their local microenvironment, but may rather result from the difficulty of characterizing the stiffness of a nonlinearly elastic material. Collagen gels, but not polyacrylamide gels, exhibit nonlinear elasticity with increased stiffness at greater strains (Storm *et al* 2005). In addition, the structure of the collagen matrices cannot be regarded to be homogeneous and isotropic. This special feature may also be concentration-, temperature-, pH- and cross-linker-dependent. Additionally, in strain-stiffening materials the stiffness of collagen gels is much higher at larger strains than at lower strains. Thus, it is possible that the stiffness used experimentally for collagen gels, based on the linear elastic region observed at relatively low strains, may be considerably lower than the actual stiffness in the pericellular microenvironment of tumors, when larger strains are present (Byfield *et al* 2009). In contrast, the viscoelastic property of collagen, which is opposed to the purely elastic nature of polyacrylamide, provides collagen with the ability to creep over longer times compared to purely elastic gels, which then decreases the stress and consequently lowers the collagen's effective stiffness. Regardless of the reasons for the apparent differences in the effective stiffness in the 2D and 3D systems, these data further support the notion that alterations in both 2D substrate stiffness or 3D substrate stiffness can have pronounced effects on cells, and they show that cells alter their cellular stiffness

due to alterations in the stiffness of their 3D microenvironment (Byfield *et al* 2009). Taken together, these results demonstrate that the magnitude of the stiffness initiating a response depends pronouncedly on the specific features of the microenvironment, which potentially includes the dimensionality of the matrix. Thus, when extrapolating the effectual stiffness obtained in one environment to another one, one must be very careful (Byfield *et al* 2009).

15.2 The role of the endothelial contractile apparatus

Do transmigrating invasive cancer cells regulate the biomechanical properties of the endothelium?

The impact of endothelial cells on the regulation of cancer cell invasiveness into 3D extracellular matrices is not yet well understood and requires further investigation. In particular, the regulation of cancer cell transmigration seems to be a complex scenario that has not yet been fully characterized. In numerous studies, the endothelium acts as a functional and passive barrier against the invasion of cancer cells (Al-Mehdi *et al* 2000, Zijlstra *et al* 2008). Moreover, the endothelium reduces the invasion of cancer cells and hence metastasis (Van Sluis *et al* 2009). By contrast, several recent reports have proposed a novel paradigm in which endothelial cells are able to regulate the invasiveness of certain cancer cells by increasing their dissemination through vessels (Kedrin *et al* 2008) or by increasing the individual invasiveness of cancer cells (Mierke 2008 Mierke *et al* 2008a, Mierke 2013). Although several adhesion molecules have been identified as functioning in tumor–endothelial cell interactions, hence promoting cancer metastasis formation, the role of endothelial mechanical properties during cancer cell transmigration and invasion is not yet known.

However, it has been suggested that the altered mechanical properties of endothelial cells may support one of the two main functions of the endothelium in cancer metastasis: they act as a passive barrier for cancer cell invasion and they serve as an active enhancer for cancer cell invasion. A main biochemical signal transduction pathway of the tumor–endothelial interaction has been shown to involve cell adhesion receptors and integrins, such as platelet endothelial cell adhesion molecule-1 (PECAM-1) and $\alpha v\beta 3$ integrins, respectively (Voura *et al* 2000). As integrins connect the extracellular matrix and the actomyosin cytoskeleton (Neff *et al* 1982, Damsky *et al* 1985, Riveline *et al* 2001), the link between both components is facilitated through the mechano-coupling focal adhesion and cytoskeletal adaptor protein vinculin (Mierke *et al* 2008b), which subsequently determines the quantity of cellular counter-forces maintaining cellular shape, morphology and cellular stiffness (Rape *et al* 2011). A biomechanical approach investigating the endothelial barrier break-down in the presence of co-cultured invasive cancer cells, in which both the cancer and endothelial cells' mechanical properties are determined, remains elusive. Microrheologic measurements (such as magnetic tweezer microrheology) have been adequate for analyzing the endothelial cell's mechanical properties, such as cellular stiffness during co-culture with invasive or non-invasive cancer cells compared to mono-cultured endothelial cells. As expected, it has been reported that the endothelial stiffness influences co-cultured invasive cancer cells. In more

detail, highly invasive breast cancer cells alter the cellular mechanical properties of co-cultured microvascular endothelial cells, as they lower the stiffness of endothelial cells, whereas in contrast non-invasive cancer cells have no effect on endothelial cell stiffness (Mierke 2011, Mierke 2012). In addition, nanoscale-particle-tracking-method-based diffusion measurements of actomyosin cytoskeletal-bound microbeads (which serve as fiducial markers for structural alterations of the intercellular cytoskeletal scaffold) are suitable for revealing the actomyosin-driven cytoskeletal-remodeling dynamics. Thus, the cytoskeletal-remodeling dynamics of endothelial cells have been found to increase during co-culture with highly invasive cancer cells, whereas endothelial cytoskeletal-remodeling dynamics are not altered by non-invasive cancer cells (Mierke 2011). Taken together, these findings demonstrate that highly invasive breast cancer cells can actively alter the mechanical properties of nearby co-cultured endothelial cells. Thus, these results may provide a proper explanation for the break-down of the endothelial barrier function of vessel wall monolayers.

15.3 Interaction between cancer cells and endothelial cells in 3D spheroids

The individual and so-called personalized therapy of cancer has become a focus of cancer research, and is being promoted as the next generation of therapeutic treatments. However, it is common knowledge that tumors are displaying a substantial heterogeneity in their specific type, tissue or organ site and disease stage. However, even patients with the same type of disease can exhibit broadly divergent tumor phenotypes (Ahn *et al* 2016, Niu *et al* 2016). In order to select an appropriate and efficient cancer therapy, the vast complexity of tumor biology needs to be taken into account. Several novel methods are currently developed and adapted to perform a personalized therapy, such as in silico prediction tools (Kar *et al* 2016), genetic analysis (Niu *et al* 2016, Ishikawa *et al* 2010) and experimental models including DNA microarrays (Sotiriou and Pusztai 2009). For example, the extraction of genetic information of a certain tumor or tumor stage by using deep sequencing techniques identifies distinct mutations in oncogenes, which may potentially direct clinicians towards a certain treatment approach (Dienstmann *et al* 2015), but it does not predict whether the outcome will be beneficial. However, most of the current genetic information is still poorly understood and artificially translated into clinical treatment approaches, which is due to the lack of specific key gene targeted drugs and also possibly due to the broad complexity of the gene regulatory mechanisms and the dependence of certain cancer types on more than one gene mutation. Additionally, the dramatic tumor heterogeneity often challenges the tumor mapping through a small biopsy, as there is even a large diversity in the genetic information that can be obtained from different biopsies of the same tumor (Gerlinger *et al* 2012). Based on these major complexities of cancer, it currently represents a great effort to develop and establish predictive drug performance tools possessing clinical relevance. Thus, reliable experimental cell-culture-based models predicting the overall cell functionality under physiologically relevant conditions, are highly required.

There are still cellular monolayer assays that are commonly utilized as research tools for high-throughput drug screening and are widely applied in molecular biology for the identification of different molecular pathways as well as other utilizations (Crawford and Leblanc 2014, Weigelt *et al* 2014). In particular, drug screening approaches of compound libraries for various activities, such as anticancer activity, rely mainly on cytotoxicity assays, which employed solely established cancer cell lines grown as 2D cultures on stiff surfaces that enable rapid and easily reproducible growth kinetics. These approaches have contributed significantly to an understanding of tumor biology and increased our knowledge for improving the anticancer drug discovery and further development. However, 2D cell cultures still lack key features of the primary tumor in a real tissue or organ that are crucial for recapitulating the physiological systems (Xu *et al* 2014), such as the spatial cell–cell interactions, the complex extracellular matrix tumor microenvironment (Lamichhane *et al* 2016), the dynamic metabolic demand and the elevated hypoxia in primary tumors based on the tumor mass growth and its surrounding tumor stroma (Klimkiewicz *et al* 2017), and other effects of tumor microenvironment such as tumor stromal fibroblast or endothelial cells recruited to the primary tumor (Cavo *et al* 2016). These differences may explain why there are distinct rates of proliferation and cell susceptibility to death signals in 3D models compared with 2D cultures, in response to a certain drug exposure. It has been revealed that there is even a reduced cell proliferation rate when cancer cells were grown in 3D cell cultures compared with 2D cell cultures (Liu *et al* 2010, Hsiao *et al* 2009). Moreover, the low level of physiological and clinical relevance of 2D cultures in cytotoxicity assays causes false positive results and also leads in certain cases to a misinterpretation and hence a poor prediction of *in vivo* behavior. However, these limitations of drug screening in monolayer-based cell culture models seems to be at least partly responsible for the high rate of clinical trial failures of novel compounds or drugs, although they reached excellent results in antitumor treatment *in silico* and *in vitro* (Pampaloni *et al* 2007).

Therefore, more sophisticated 3D cellular models are needed to improve the screening procedure and have indeed been developed and studied thoroughly (Hirschhaeuser *et al* 2010). Among these models is one that is most interestingly, the 3D multicellular tumor spheroid model. In more detail, spheroids are self-assembled cell aggregates or cell clusters that possess the potential to mimic several important components of the physiological spatial growth and cell–cell interactions (Sant and Johnston 2017). These 3D spheroids represent *ex vivo* microtissues containing metabolic activity that is guided by nutrient and oxygen diffusion mechanisms (Sant and Johnston 2017, Groebe and Mueller-Klieser 1991) similar to avascular tumors. However, the spheroid diffusion is limited to only 150–200 μm (Lin and Chang 2008) and in larger spheroids it is even less restricted and reaches values up to 400–500 μm in diameter and even the outer layer of the spheroids can still proliferate, whereas the core becomes necrotic due to hypoxia and nutrient deficiency. Indeed, these conditions are highly similar to hypoxic microtumors *in vivo*, which have been revealed to affect the outcome of a drug screening negatively, as they negatively affect the sensitivity of the tumor to anticancer drugs

and hence lead to the acquired resistance (Harris 2002, Semenza 2003). This finding represents a major advantage of spheroid cultures in drug testing.

In order to better imitate the primary tumors' microenvironment *in vivo*, cancer cells need to be cultured in the presence of stromal cells that are also present in the tumor niche. The crosstalk between cancer cells and endothelial cells facilitates critical processes such as the formation of new blood vessels (Folkman 1995), which is termed (neo)angiogenesis and is one of the hallmarks of tumorigenesis. The complex interconnection between cancer cells and endothelial cells provides the establishment of modifications in the gene expression profile of endothelial cells (Khodarev *et al* 2003), their mechanical properties (Mierke 2011) and their activation state, which all contribute to the initiation of angiogenesis and finally provide drug resistance (Hoffmann *et al* 2015, Theodoraki *et al* 2015).

A more complex stroma-mimicking spheroid model in which cancer cells and endothelial cells interact additionally with mesenchymal cells (stroma cells) has been shown to better sustain and mimic the local tumor microenvironment, representing an enhanced platform for investigating the effect of drugs *in vitro* (Lamichhane *et al* 2016). Indeed, it has turned out that, as a critical component of the tumor tissue, the endothelium needs to be an integral part of these *ex vivo* models. Therefore, great effort has been made in the development and establishment of tumor spheroids with endothelial cells, which still serve mainly as a model for tumor angiogenesis (Klimkiewicz *et al* 2017, Nakatsu *et al* 2003, Heiss *et al* 2015, Laschke and Menger 2017). Moreover, even sophisticated constructs of interacting capillary systems using microfluidic techniques have been developed within these spheroids (Kim *et al* 2015, Dereli-Korkut *et al* 2014, Niu *et al* 2014, Hockemeyer *et al* 2014). To reveal the cancer cell-specific behaviors in 3D multicellular structures and the cancer cell interactions with endothelial cells, they have been analyzed in 3D in different sources of cancer cells and in different ratios of cancer cells to endothelial cells. A procedure for the spheroid culture with endothelial cells has been successfully developed by using an optimized protocol for spheroid assembly. Using the spheroid array method, the characterization of spheroids derived from either cancer cell lines or patient cancer cells can be performed successfully. Thus, endothelial cells such as human umbilical vein endothelial cells (HUVECs) have been utilized for spheroid cultures (Shoval *et al* 2017), that are commonly used for modeling angiogenesis in 3D cultures (Khodarev *et al* 2003, Nakatsu *et al* 2003, Heiss *et al* 2015, Vitorino *et al* 2015, Wenger *et al* 2005, Laib *et al* 2009, Chen *et al* 2009). In addition, potential correlations between the spheroids' shape, surface texture and the spatial invasiveness of cells in the extracellular matrix have been demonstrated. Hence establishing a hybrid tumor/stroma spheroid model is important for personalized cancer treatment, which seems to provide a reliable and low budget procedure for rebuilding the tumor microenvironment in order to predict the effect of certain drugs.

Despite successful pre-clinical testing, approximately 85% of early clinical trials for novel drugs fail. Of those that reach phase III trials only 50% even pass the requirement for clinical approval (Ledford 2011). Moreover, among these clinical trials, oncological drug trials have been revealed to have the highest failure rates (Arrowsmith 2011).

The fast-growing advances in the field of tissue engineering and microfabrication techniques have brought some improved models for cell culture trials in the drug screening including 3D cell cultures, tissue fragments and organoids *ex vivo*. In particular, the 3D cell models, which are able to mimic the tumor microenvironment, have the greatest impact on substantially improving the validity of the drug screening assays in order to obtain improved predictions for drug success in cancer treatment (Breslin and O'Driscoll 2013, Baker and Chen 2012, Hutmacher 2010, Weigelt and Bissell 2008).

It has been reported that cellular behavior is altered by the presence of stromal cells, either alone (Franses *et al* 2011) or in combination with mesenchymal cells (Lamichhane *et al* 2016), which both are present in the local tumor microenvironment. In particular, altered invasion behavior and cytotoxic properties have been detected when analyzing the drug effect on a pure fibroblasts culture in comparison to co-culture of varying ratios of fibroblasts and cancer cells (Schreiber-Brynzak *et al* 2015, Jeong *et al* 2016). Moreover, interactions with inflammatory cells are crucial in an *in vivo* tumor microenvironment, such as macrophages and hence they are investigated *in vitro* at the spheroids' surface. Indeed, it has been demonstrated that they possess the ability to infiltrate the inner layers of these spheroids and disintegrate them after five days (Nyga *et al* 2016, Hauptmann *et al* 1993), which also may affect the outcome of a drug screening experiment.

Thus, the interactions between endothelial cells, which represent the vasculature of the tumor and are a major microenvironmental component, and cancer cells are included in these studies. The comparison of four different methods for spheroid formation has revealed that the 3D culture dish array is the most robust and generates standard spheroids, which are homogeneous in size and circular shape. A weakness is that in some cases the size of the spheroids was physically constrained by the well size, which then affects cellular processes such as proliferation and apoptosis (Helmlinger *et al* 1997, Bell *et al* 2001, Cheng *et al* 2009). Compared with the 3D culture dish array method, agarose-coated flat wells lead to the formation of heterogeneous spheroids and in many preparations multiple spheroids per well were detected. The formation of spheroids with the Lipidure® MPC-coated 96 well plates leads to better results than those of the agarose method, as single spheroids per well are obtained and the spheroids exhibit the same round shape. These results may be attributed to the U-shaped bottom of these culture plates impairing the cell spreading over the entire bottom surface and forces the individual cells to interact with each other. In addition, the 2-methacryloyloxyethyl phosphorylcholine (MPC) co-polymer coating prevents cells from cell adhesion to the surface of the plate. Apart from their rounded shape, spheroids were still polydispersed. The next method is the classical hanging-drop method, which is still commonly used for the formation of spheroids and leads to the generation of a wide range of spheroids regarding their size and shape, in most cells types. Moreover, the hanging-drop method applied to ovarian cancer cell lines revealed not very round spheroids (Sodek *et al* 2009), although another study reported rather round spheroids using the OVCAR8 cell line. This method seems to need specific adaption for each cell type, as the spheroid formation time is different for each cell type. These three methods, the hanging-drop assay, the U-shaped bottom coated

96-well plate and agarose-coated plates lead to the formation of spheroids of varying sizes and shapes and hence it reveals that each cell type requires a specific optimization. Another method for scaffold free spheroid formation represents the Perfecta3D® Hanging Drop Plate from 3D BiomatrixTM, which seems to be a standardization of the hanging-drop method using designated culture plates (Zanoni *et al* 2016).

In summary, the 3D culture dish array provides a better size and shape control of the spheroids independently of the cell origin. In particular, the special wells, which possess a re-usable mold and hence physically confine the spheroids in terms of growth and forces them to build spheroids of the same size.

Using the 3D culture dish array, the aggregates of two patient-derived cell types, such as M21 and BR-58, and two cancer cell lines of different origin, such as A375 and BxPC3, using different initial cell numbers per spheroid can be compared (Shoval *et al* 2017). In all four cell types it has been shown that the density of the spheroids is enhanced with the elevation of spheroid cell number. In particular, the BxPC3, a pancreatic cancer cell line, produced round spheroids even at high densities. This is consistent with the pathophysiology of pancreatic tumors, which produce due to fibroblast-driven inflammation dense stromal tissue fibrosis and possess a hypo-vascularity phenotype. Not only are these tumors poorly vascularized, the blood vessels surrounding the tumor within the tumor microenvironmental tissue are also commonly compressed due to high tissue pressure (Longo *et al* 2016). These tissue features pronouncedly effect the drug permeability into the primary tumor through simple diffusion (Provenzano *et al* 2012). Hence it can be suggested that the spheroid's shape and condensed structure are signs of cluster growth.

Indeed, a functional endothelial cell lining is helpful for the tumor's growth, and hence the structural patterns of the endothelium in the tissue may be an indicator of tumor aggressiveness and disease prognosis. However, the extravascular migratory metastasis in melanoma and the angiotropism such as the 'pericytic mimicry' or the replacement of pericytes by angiotropic cancer cells, are supposed to be suitable biomarkers for a worse outcome of cancer (Lugassy *et al* 2013, 2014). Hence, it has been hypothesized that angiogenesis is not required for these tumors, when they are in close neighborhood to pre-existing blood vessels, whereas the interaction with endothelial cells is indeed important.

As this spheroid model is static, the formation of functional capillaries cannot be suggested, however, the formation of the primitive vessel architecture can be analyzed in terms of its tumor-cell-type dependence and whether the capillary structures can be revealed without a steady flow. Hence, HUVECs and cancer cells are co-cultured using various ratios to create spheroids. In particular, for different cancer types, 'forcing' a fixed cancer-cell:endothelial-cell ratio *in vitro*, which may even differ from the *in vivo* situation. However, dramatic differences between cancer cell types have been observed. For instance, immunofluorescence staining showed that endothelial cells in BxPC3 spheroids cluster closer to the spheroids' periphery than to the core, which has been seen in most cases. Moreover, their structure cannot assemble tubular shapes, which is different to BR-58, MDA-MB-231 and A375 spheroids, which all originate from highly angiogenic tumors and hence they form a vessel-like network. As hypothesized, the vessel-like network cannot function as a

vessel. Spontaneously assembled 'open lumen'-like structures were found in M21 spheroids. In line with this, a similar pattern of CD31 positive stained cells, displaying the endothelial cell organization in the center of the spheroids, has been seen with patient-derived papillary thyroid cancer cells and normal thyroid spheroids cell culture (Cirello *et al* 2017). However, it has been found that the usage of a triple co-culture 3D microenvironment recreates this system. Thus, human mesenchymal stem cells were co-cultured with human pulmonary vascular microvascular endothelial (HPMECs) and lung epithelium A549 lung cancer cells, to build an *in vivo*-like model (Lamichhane *et al* 2016). The presence of mesenchymal stem cells can function as a critical factor in the long-term sustainability of viable endothelial cells in hypoxic regions, which represents a more sophisticated *in vivo* mimicking model.

In order to analyze whether the spontaneous assembly of capillary-like structures is innate, a 2D monolayer growth model has been utilized to investigate the interactions between the cells. When endothelial cells and cancer cells are co-cultured in a 1:1 ratio, it has been revealed that under confluent conditions cancer cells assemble as islands of aggregates, which are surrounded by a flat endothelial cell monolayer. In this 2D setting, when comparing BxPC3 with A375, the island growth pattern obtained is higher in the BxPC3 pancreatic cell line, which is related to the tendency of the cells to form distinct aggregates in the 3D spheroids.

Cellular invasiveness represents a critical step in the metastatic cascade. It has been found that with higher tumor HUVECs content, the spheroids possess rougher margins as more cells 'sprout' or even detach from the central aggregate, which indicates a higher invasive capacity. It needs to be revealed whether the innate potential of cancer cells to invade the 3D extracellular matrix can be kept in a 3D *ex vivo* structure and whether significant differences occur in the invasiveness of spheroids based on the individual tumor type. Indeed, it has been reported that each cell type has a different migratory potential, which is not preserved across all cancer types. For instance, A375 melanoma cell line is highly invasive in 3D collagen type I matrices, whereas M21, a patient-derived cell-type originating from the metastatic site of the lymph node, has been seen to be 45-fold less invasive than A375. This leads to the suggestion that the behavior of cancer cells pronouncedly differs in cell lines of different primary cancer cells and may therefore not be employed as a reliable model for the prediction of clinical outcomes.

Indeed, it has been shown that spheroids of M21 and BR-58, both patient-derived tumor spheroids, generated high levels of collagen. When considering the effect of the deposition and remodeling of extracellular matrix collagen on both tumor volume (Christensen *et al* 2015) and progression (Provenzano *et al* 2008), further studies are required to sample an even larger number of spheroids of different origin in order to enlighten those possible correlations.

In summary, it can be concluded that a 3D model of hybrid spheroids can be successfully constructed. However, as each cancer type generates different interactions with its nearest neighbor cells and endothelial cells, there is a requirement for a platform for personalized medicine accounting for these alterations. In addition, it has been revealed that the hybrid spheroid of endothelial cells and cancer cells can

mirror vascular-like structures and can even sprout when interacting with the extracellular matrix. Finally, this model can be used as a basis for rebuilding complex multicellular tumors and stromal structures and hence it can be employed as a more reliable method for the prediction of biological processes involved in cancer and the efficacy of drug treatment.

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Chapter 16

The role of macrophages during cancer cell transendothelial migration

Summary

A major step in the malignant progression of cancer and hence in the process of cancer metastasis is the intravasation of cancer cells into blood vessels. This key step of the metastatic cascade that follows the metastatic dissemination of the cancer cells from the primary tumor into the surrounding tumor stroma is the intravasation of cancer cells through the basement membrane of vessels and the endothelial vessel lining. How the triple-cell complex consisting of a macrophage, Mena over-expressing cancer cell and an endothelial cell, termed the tumor microenvironment of metastasis, facilitates the transendothelial migration of the cancer cell is yet not precisely understood. Moreover, it has been revealed that the physical contact between a macrophage and cancer cell leads to the formation of invadopodia, the exertion of actin-rich matrix degrading protrusions, which are required for cancer cell invasion and transendothelial migration, and cancer cell dissemination. This chapter discusses how tumor-associated macrophages guide cancer cells through the endothelial cell lining of blood or lymphoid vessels. Moreover, it covers the precise role of macrophages in the progress of cancer and examines whether macrophages affect the cellular functions and properties of cancer cells in order to provide cancer cell invasiveness into extracellular matrices of connective tissue. In addition, the function of macrophages during the intravasation of cancer cells into blood or lymphoid vessels through the endothelial vessel lining is discussed in detail. Some open questions are also discussed. Are macrophages necessary to provide cancer cell transendothelial migration and invasiveness? Why do the tumor-associated macrophages fulfill such a special function? Do macrophages also affect the properties of the endothelium in order to facilitate cancer cell transmigration and invasion?

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16.1 What is the role of macrophages during cancer disease?

Macrophages are terminally differentiated cells of the mononuclear phagocytic lineage, which have developed under the influence of their primary growth and differentiation factor, the colony-stimulating factor-1 (CSF-1). Although the mononuclear phagocytic lineage differentiates into heterogeneous populations, depending upon the tissue of residence, motility is an important feature of its function. In order to mediate their migration and invasion through tissues, macrophages express a unique adhesion range and contain specific cytoskeletal proteins. In addition, macrophages do not assemble large, stable adhesions or actin stress fibers, but they rely on small, short-lived point focal contacts, focal complexes and podosomes for generation and transmission of traction (figure 16.1). Thus, macrophages are present in their targeted tissue in order to respond rapidly to migratory stimuli. In addition to CSF-1 mediated increased growth and differentiation, it also acts as a chemokine supporting macrophage migration through the activation of the CSF-1 receptor tyrosine kinase. In particular, CSF-1R autophosphorylation of several intracellular tyrosine residues leads to its association and the activation of many downstream signaling molecules. Phosphorylation of only one residue, Y721, facilitates the association of PI3K with the receptor to activate the major motility signal transduction pathways in macrophages. However, dissection of these pathways will lead to the identification of possible drug targets for the inhibition of diseases in which macrophages contribute to adverse outcomes.

Macrophages reside in almost every tissue of the body and thus their adaptation to the different tissue microenvironments is exceptional; they can adopt a diverse

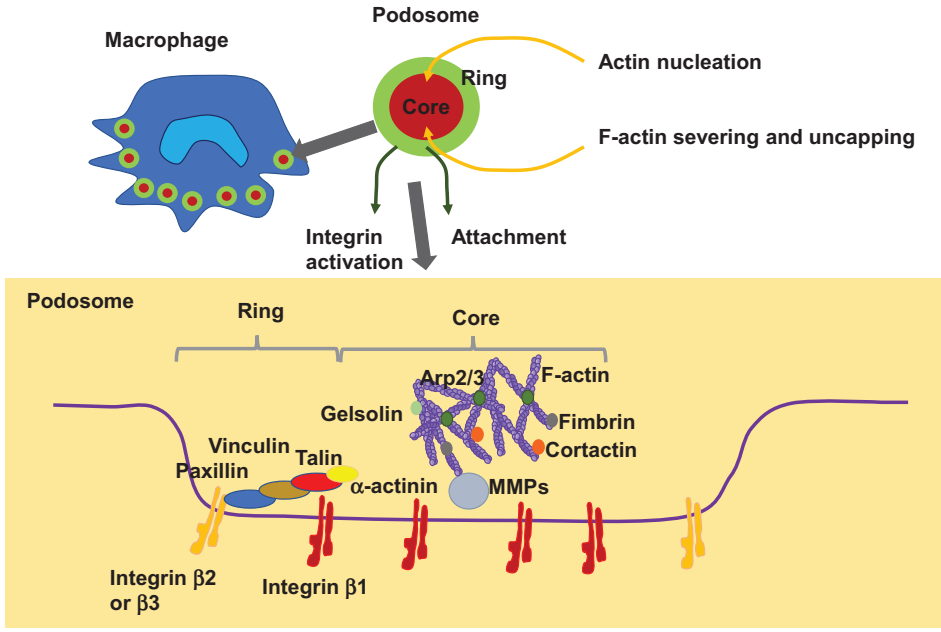

Figure 16.1. Structure of podosomes

range of morphologies and carry out a variety of functions. Despite their heterogeneity, macrophages all originate from the same pluripotent hematopoietic stem cell and, under the influence of hematopoietic growth factors, differentiate through several multipotent progenitor stages to a lineage called mononuclear phagocytic precursors, which is located in the bone marrow (Pixley and Stanley 2004, Gordon and Taylor 2005, Pollard 2009). The mononuclear phagocyte system is composed of mononuclear phagocyte precursors (such as monoblasts and promonocytes), circulating monocytes and fully differentiated, tissue-resident macrophages (Pixley and Stanley 2004, Pollard 2009, Gordon and Taylor 2005, van Furth *et al* 1972). CSF-1 has long been recognized as the primary growth factor regulating the survival, proliferation and differentiation of cells of the mononuclear phagocytic lineage (Pixley and Stanley 2004, Pollard 2009, Stanley and Heard 1977). It is also an essential differentiation factor for the bone resorbing osteoclast (Asagiri and Takayanagi 2007). A spontaneously occurring inactivating mutation in the mouse CSF-1 gene (osteopetrotic, Csf1op) has been shown to be associated with reduced tissue macrophage numbers and a marked reduction in osteoclasts, while it also causes osteopetrosis, together with other developmental defects (Pixley and Stanley 2004, Yoshida *et al* 1990, Wiktor-Jedrzejczak *et al* 1990, Cecchini *et al* 1994). The CSF-1 protein signals through the CSF-1 receptor tyrosine kinase (RTK), which is encoded by the c-fms proto-oncogene (Sherr *et al* 1985). In addition, RTK induces a series of phosphorylation cascades, facilitating cellular responses to CSF-1 (Pixley and Stanley 2004). While the phenotype of mice nullizygous for the CSF-1R ($CSF\text{-}1R^{-}/CSF\text{-}1R^{-}$) largely replicates that seen in the $Csf1^{op}/Csf1^{op}$ mouse lacking biological active CSF-1, it has an even more severe effect and this discrepancy has been explained by the discovery of a second partially redundant ligand for the CSF-1R, the interleukin-34 (IL-34) (Dai *et al* 2002, Lin *et al* 2008, Wei *et al* 2010).

Macrophages are professionally motile cells, which perform a variety of roles in immune surveillance and normal tissue development by secreting cytokines and growth factors, and also phagocytizing foreign material and apoptotic cells. Transendothelial and interstitial motility is an essential part of their function as they must be able to migrate to specific sites upon stimulation. From studies in primary macrophages and in CSF-1-dependent macrophage cell lines, it has been reported that CSF-1 is not only a mononuclear phagocyte lineage growth factor, in addition, it is also an important inducer of macrophage motility (Pixley and Stanley 2004, Wang *et al* 1988, Webb *et al* 1996, Pixley *et al* 2001). In more detail, the depletion of specific subsets of tissue macrophages in the $Csf1^{op}/Csf1^{op}$ mouse and their reconstitution upon restoration of CSF-1 expression revealed that CSF-1 facilitates the differentiation and migration of trophic and/or scavenger macrophages that are physiologically essential for normal development and tissue homeostasis rather than immune function (Pollard 2009, Cecchini *et al* 1994, Dai *et al* 2002, Ryan *et al* 2001). In more detail, CSF-1 or CSF-1R deficient mice demonstrate abnormal neural, skeletal and glandular development, not only due to reduced macrophage content and decreased osteoclast numbers, but even more caused by impaired matrix remodeling (Pollard 2009). Thus, CSF-1-induced motility seems to be an important element of macrophage function in development. Beyond their critical physiological

role, CSF-1-dependent macrophages can promote disease progression in conditions ranging from cancer to atherosclerosis and arthritis (Pixley and Stanley 2004, Pollard 2009, Chitu and Stanley 2006, Hamilton 2008). Reactivation of developmental macrophage functions seems to be possible and may underlie the progression of these pathologies (Pollard 2009).

In order to participate in the disease process, macrophages must initially migrate to the affected tissue. Moreover, in the case of an increase in tumor invasion, tumor-associated macrophages and mammary carcinoma cells have been reported to migrate together away from the primary tumor site (Wyckoff *et al* 2004). How the macrophage motility is facilitated remains elusive. How the motility machinery differs from other cell types and whether inhibition of macrophage motility may improve disease outcomes is not yet well understood. Moreover, CSF-1 can activate signal transduction pathways that activate molecules or protein isoforms selectively expressed in macrophages (Pixley and Stanley 2004); some of these may represent attractive therapeutic targets to specifically inhibit macrophage infiltration into disease sites. When considering the contribution of macrophages and CSF-1 to tumor dissemination and the progression of several inflammatory disorders (Pollard 2009, Chitu and Stanley 2006, Hamilton 2008), the focus is on understanding macrophage migration and its facilitation by CSF-1.

16.2 Impact of Mena on cancer cell invasion and macrophages

It has been reported that distinct Mena isoforms of the actin-regulatory protein are expressed in invasive and migratory cancer cells *in vivo* and that the invasion isoform ($Mena^{INV}$) potentiates carcinoma cell metastasis in murine models of breast cancer. However, the specific step of metastatic progression affected by this isoform and the effects on metastasis of the Mena11a isoform, expressed in primary cancer cells, have not yet been fully revealed. Evidence has been provided that elevated $Mena^{INV}$ increases coordinated streaming motility and increases the transendothelial migration and intravasation of cancer cells. Indeed, the promotion of these early stages of metastasis by $Mena^{INV}$ is dependent on a macrophage–cancer cell paracrine loop. In addition, it has been shown that increased Mena11a expression correlates with decreased expression of CSF-1 and leads to a dramatically decreased ability to participate in paracrine-mediated invasion and intravasation. These results illustrate the importance of paracrine-mediated cell streaming and intravasation for cancer cell dissemination and moreover show that the relative abundance of $Mena^{INV}$ and Mena11a helps to regulate these key steps of metastatic progression in breast cancer cells.

Cellular motility is essential for many aspects of metastasis. However, there are only a few molecular markers that can indicate and even predict the migratory potential of a cancer cell *in vivo*. The intravital multiphoton imaging technique can be used to characterize carcinoma and stromal cell behavior in detail within intact primary tumors in living animal models (Condeelis and Segall 2003, Wang *et al* 2007, Egeblad *et al* 2008, Kedrin *et al* 2008, Andresen *et al* 2009, Perentes *et al* 2009). Indeed, these imaging approaches deliver direct information at single-cell resolution and even permit the quantification of cellular motility and interactions

between cancer and stromal cells, as well as providing direct observation of the invasion, intravasation and extravasation steps that all play an important role in cancer metastasis. In mammary tumors, this technology was used to describe the microenvironments in which cancer cells can invade, migrate and intravasate, while also revealing essential roles for macrophages in these events (Condeelis and Segall 2003, Condeelis and Pollard 2006, Yamaguchi *et al* 2006, Kedrin *et al* 2007). In more detail, chemotaxis of cancer cells toward macrophages is an essential step for invasion in mouse primary mammary tumors (Wyckoff *et al* 2004, Goswami *et al* 2005), while chemotaxis of cancer cells toward perivascular macrophages is essential for their intravasation (Wyckoff *et al* 2007).

Expression profiling of invasive cancer cells isolated from the primary tumor was used to obtain molecular information regarding the pathways providing carcinoma cell invasion and intravasation (Wyckoff *et al* 2000a, Wang *et al* 2004, Wang *et al* 2007). The 'invasion signature' revealed by this profile provides sets of coordinated expression alterations associated with the enhanced invasive potential (Goswami *et al* 2004, Wang *et al* 2004, Wang *et al* 2006, Wang *et al* 2007, Goswami *et al* 2009). As suggested, Mena, a regulator of actin polymerization and cell motility, is upregulated in invasive cancer cells obtained from rat, mouse and human tumors (Di Modugno *et al* 2006, Goswami *et al* 2009, Robinson *et al* 2009). However, the conservation of Mena up-regulation in invasive cancer cells across species indicates that it plays an important role in the metastatic progression of cancer disease.

In patients, Mena expression correlates with metastatic risk: for instance, relatively high Mena expression has been observed in patient samples from high-risk primary and metastatic breast tumors (Di Modugno *et al* 2006), as well as cervical, colorectal and pancreatic cancers (Gurzu *et al* 2008, Pino *et al* 2008, Gurzu *et al* 2009). Mena is also a component of a marker for metastatic risk called tumor microenvironment for metastasis (TMEM) (Robinson *et al* 2009). TMEMs are identified by co-localization of Mena-positive cancer cells, macrophages and endothelial cells and, in particular, the TMEM score predicts the risk independently of the clinical subtype of cancer (Robinson *et al* 2009). The contribution of Mena to metastasis is independent of clinical subtype.

These findings highlight the importance of the mechanism by which Mena and its isoforms differentially facilitate metastatic progression. In particular, Mena is a member of the Ena/VASP family of proteins and can bind actin in order to affect the geometry and assembly of filament networks through: (i) anti-capping protein activity (Bear *et al* 2002, Barzik *et al* 2005, Hansen and Mullins 2010), which includes binding to profilin and both G- and F-actin; (ii) Mena tetramerization; and (iii) a decrease in the density of actin-related proteins 2 and 3 (Arp2/3)-facilitated branching (Gertler *et al* 1996, Barzik *et al* 2005, Ferron *et al* 2007, Pasic *et al* 2008, Bear and Gertler 2009, Hansen and Mullins 2010). Moreover, alternative splicing for the Mena gene has been shown: a 19 amino acid residue insertion just after the EVH1 domain leads to the Mena invasion isoform (named Mena^{INV}, formerly Mena^{+++}) (Gertler *et al* 1996, Philippar *et al* 2008), whereas an 21 residue insertion in the EVH2 domain leads to the Mena11a isoform (Di Modugno *et al* 2007). A comparison of the invasive and migratory cancer cells collected *in vivo*, with primary cancer cells

isolated from mouse, rat and human cell-line-derived mammary tumors, showed that Mena^{INV} expression is upregulated and Mena11a is down-regulated selectively in the invasive and migrating carcinoma cell population (Goswami *et al* 2009). Moreover, the differential regulation of Mena isoforms across species indicates that the two isoforms fulfill important roles in invasion and metastasis.

In previous studies, the expression of Mena^{INV} in a xenograft mouse mammary has been reported to promote tumor progression by increased formation of spontaneous lung metastases from orthotopic tumors and even to alter the sensitivity of cancer cells to epidermal growth factor (EGF) (Philippar *et al* 2008). This study was performed in order to identify the step(s) in the metastatic cascade regulated by Mena^{INV} expression and to analyze the effect of expression of the second regulated isoform, Mena11a, on metastatic progression. In more detail, each step of metastatic progression was investigated in order to determine which steps are regulated by expression of Mena^{INV}, which finally enhances the metastatic dissemination, and whether the same steps are also regulated by Mena11a expression in cancer cells.

MTLn3 cells have been selected in order to investigate the impact of these two Mena isoforms, as these cells are well characterized regarding cancer cell invasion, migration and metastasis (Levea *et al* 2000, Sahai 2005, Le Devedec *et al* 2009, Le Devedec *et al* 2010), tumor–stromal cell interactions (Sahai 2005), TGFβ signaling in metastatic progression (Giampieri *et al* 2009) and the functional role of Mena in breast cancer metastasis (Philippar *et al* 2008, Goswami *et al* 2009). Moreover, MTLn3 cells were obtained from the clonal selection of metastatic lung lesions from rats with mammary tumors (Neri *et al* 1982). In more detail, these rat mammary tumors were characterized as estrogen-independent and were observed to metastasize in the lymph nodes and lungs (Neri *et al* 1982). The evaluation of vimentin and keratins in MTLn3 mammary tumors, associated lymph nodes and lung metastases demonstrated that MTLn3 cancer cells are comparable to a basal-like subtype of breast cancer (Lichtner *et al* 1989).

Mena^{INV} promotes coordinated cell migration through streams of single migrating cells

It has been found that expression of Mena and Mena^{INV} increases *in vivo* cellular motility, and hence it has been hypothesized that this supports the increased lung metastasis detected in these cells (figure 16.2) (Philippar *et al* 2008). However, different types of motility seem to play diverse roles during the invasion of cancer cells (Wolf *et al* 2003, Gaggioli *et al* 2007, Ilina and Friedl 2009, Friedl and Wolf 2010, Wolf and Friedl 2011). Thus, it has been hypothesized that Mena^{INV} expression supports a type of motility that provides enhanced cancer cell invasion. In order to test this hypothesis, a multiphoton-based intravital imaging approach was used to examine the types of motility displayed by cancer cells expressing the different Mena isoforms. In all tumors, two patterns of movement were usually observed: coordinated cell movement, whereby the cancer cells align and move in an ordered single file line (called streaming), and random cellular movement, whereby cancer cells move independently of other cancer cells in a somewhat uncoordinated fashion, however, the movement can also be a highly persistent random walk in a 3D

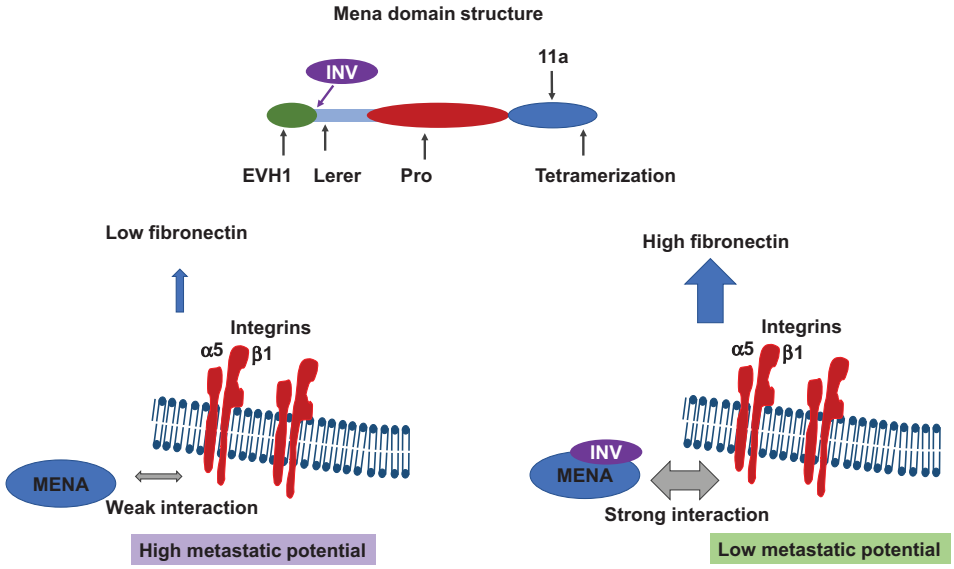

Figure 16.2. Mena domain structure and function.

microenvironment (Luzhanskey *et al* 2017). As expected, both Mena^{INV}-expressing and Mena11a-expressing cancer cells exhibited streaming and random movement *in vivo*. However, streaming movement was significantly more common in Mena^{INV}-expressing cancer cells *in vivo*. The quantification of cells moving within the primary tumor showed that Mena^{INV} expression significantly elevated both random and streaming cancer cell movement compared to GFP-expressing control cells and Mena11a-expressing primary mammary tumors. Both movement types were only slightly increased in Mena11a-expressing cancer cells. In addition, to characterize streaming motility more precisely, time-lapse images were recorded in order to investigate cell crawling, and photoconversion from green to red of a dye termed Dendra2 in a chosen population of cells by using a 405 nm laser for the photo-switching process was performed to measure the stability of the streams over a 24 h period, which provides a smaller number of dead cells during the measurement (Kedrin *et al* 2008). Carcinoma cell streams have been observed over 24 h after photoconversion, indicating that streaming is a long-lived behavior involving crawling cells. The results from the co-injection of MTLn3 cells expressing GFP–Mena11a–GFP or Cerulean–Mena^{INV} confirmed that the tissue architecture specific to each Mena isoform type was even preserved after injection, compared with the injection of either Mena11a- or Mena^{INV}-expressing cells separately.

Streaming cell movement is more efficient than random cell movement

The underlying motility parameters contributing to streaming and random movement were investigated by determining cell speed, net path length, directionality of the motion and the turning frequency *in vivo*. *In vivo*, streaming cells indeed migrated notably faster compared to randomly moving cells, regardless of Mena

isoform expression, exhibiting average speeds greater than 1.9 μm min^{-1}. All cell types participating in this random movement displayed a narrow distribution of velocities, whereas cells that performed the streaming movement showed a broad distribution of velocities. However, this suggests that random cell movement is autonomous, whereas streaming cells are restricted in their velocities due to their multiple cell–cell signaling interactions.

The directionality, net path length and turning frequency of a cell are all measures of its locomotion efficiency. In general, the net path length and directionality of streaming carcinoma cells *in vivo* were elevated, whereas the turning frequency was reduced compared to randomly moving cells. Thus, these results suggest that the streaming cell movement is more efficient, as it has been observed that streaming cells move faster and further and turn less frequently. The enhanced streaming found *in vivo* for cells expressing Mena^{INV} means that Mena^{INV}-expressing cells migrate more efficiently *in vivo*. In steady-state conditions *in vitro*, these cells only move randomly and the Mena isoform-expressing cells do not differ pronouncedly from each other in their speed and the directionality of their movement. Moreover, this suggests that additional factors are essential for streaming that are not present *in vitro* and which must hence be revealed in *in vivo* experiments.

In vivo invasion is enhanced by Mena^{INV} and suppressed by Mena11a
In order to analyze whether the enhanced streaming exhibited by Mena^{INV}-expressing cancer cells correlates with chemotaxis-dependent invasion, the *in vivo* invasion assay was performed to evaluate the capacity of cancer cells to migrate and invade towards EGF *in vivo* (Wyckoff *et al* 2000a). MTLn3 cells were shown to display a characteristic biphasic response to EGF, whereby maximal chemotactic invasion was achieved in response to 25 nM EGF (Segall *et al* 1996, Wyckoff *et al* 2000a). Moreover, expression of Mena^{INV} shifts this biphasic response and hence maximal invasion was achieved in response to 1 nM EGF (Philippar *et al* 2008). This result demonstrates that sensitivity to EGF chemotaxis is increased *in vivo* and is indeed consistent with the increased sensitivity of Mena^{INV}-expressing cells to EGF *in vitro* (Philippar *et al* 2008). *In vitro*, Mena^{INV}-expressing cells exhibit protrusive activity in response to EGF concentrations as low as 0.1 nM, whereas cells expressing GFP and Mena11a do not respond to stimulation with either 0.1 or even 0.5 nM EGF. Importantly, Mena^{INV} expression not only sensitizes cancer cells to EGF, it also significantly increases the number of invasive cells collected with the peak concentration of EGF, which in general indicates more efficient cell migration. However, Mena11a-expressing tumors did not invade significantly above background levels in response to a broad range of EGF concentrations. Taken together, Mena11a and Mena^{INV} seem to have opposite effects on chemotaxis-dependent invasion *in vivo*.

16.3 Impact of Mena on macrophages

Mena isoforms alter paracrine loop signaling with macrophages during invasion
Using the *in vivo* invasion assay, cancer cells have been observed to chemotax into needles containing either EGF or CSF-1 (Wyckoff *et al* 2004, Patsialou *et al* 2009).

This can only be detected if paracrine signaling is established with macrophages, because, in the absence of macrophages, the chemotactic signal cannot be transmitted over long distances and only a few cells are collected (Wyckoff *et al* 2004). Therefore, the question of whether the cancer cell–macrophage paracrine loop is involved in the increased *in vivo* invasion of Mena^{INV}-expressing cells and the suppression of invasion in Mena11a-expressing cells was addressed. Both macrophages and cancer cells enter collection needles during *in vivo* invasion (Wyckoff *et al* 2004) and typing of cells collected following *in vivo* invasion confirm the presence of both cancer cells and macrophages in tumors expressing GFP, Mena^{INV} and Mena11a.

To assess the impact of paracrine signaling between macrophages and Mena^{INV}-expressing carcinoma cells during *in vivo* cancer cell invasion, the *in vivo* invasion assay was performed in the presence of either 6.25 nM Erlotinib (Tarceva), an EGF receptor (EGFR) tyrosine kinase inhibitor, or a mouse CSF-1 receptor-blocking antibody (α-CSF-1R) (figure 16.3) (Wyckoff *et al* 2004). *In vivo* invasion of both Mena^{INV}- and GFP-expressing cells was reduced to background levels in assays with Erlotinib as compared with invasion toward needles containing EGF^{+}DMSO or EGF alone, demonstrating the requirement for EGFR-facilitated cancer cell invasion. Similarly, invasion of both Mena^{INV}- and GFP-expressing cells was significantly reduced with needles containing α-CSF-1R as compared with cancer cell invasion toward needles containing EGF^{+}DMSO or EGF^{+}IgG control antibodies, indicating the necessity of EGF production and signal propagation by macrophages. These results are indeed consistent with the requirement for co-migrating macrophages in cancer cell migration. Finally, these results demonstrate the requirement for paracrine signaling between Mena^{INV}-expressing cancer cells and macrophages *in vivo*.

Both CSF-1 secretion and EGF binding to the EGFR by cancer cells are essential components of the carcinoma cell–macrophage paracrine loop (Wyckoff *et al* 2004, Patsialou *et al* 2009). Real-time PCR was performed to determine the relative mRNA expression of CSF-1 and EGFR in the Mena isoform-expressing cells lines in culture to investigate whether alterations in the expression of these signaling molecules may contribute to the differences detected in EGF-dependent *in vivo* invasion (Wyckoff *et al* 2004). As expected, Mena11a-expressing cells showed a

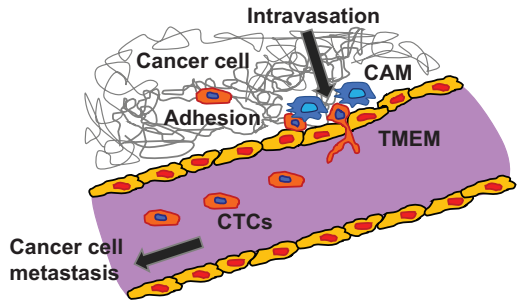

Figure 16.3. The formation of a tumor microenvironment for metastasis involves cancer associated macrophages.

four-fold decrease in CSF-1 expression compared to GFP-expressing cells, whereas CSF-1 expression in Mena- and Mena^{INV}-expressing cells was unaltered. Indeed, it has been reported that there is no difference in EGFR expression in cells expressing either Mena or Mena^{INV} compared to MTLn3 cells (Philippar *et al* 2008). However, cancer cells expressing Mena11a also showed no statistical difference in the expression of EGFR compared with GFP- and Mena^{INV}-expressing cells, indicating that altered receptor levels do not contribute to the altered EGF-dependent phenotypes observed in the different cell types. Thus, the inability of Mena11a-expressing cells to participate in macrophage-dependent invasion may be explained through the reduced CSF-1 expression, along with a reduction in direct response EGF.

In order to determine whether suppression of chemotaxis-dependent invasion of Mena11a-expressing cancer cells resulted from differences in the ability of these cancer cells to co-migrate with macrophages, a 3D invasion assay was performed that measured macrophage-dependent co-migration of cancer cells with macrophages in 3D collagen matrices (Goswami *et al* 2005). While addition of macrophages to GFP-expressing cancer cells significantly elevated 3D invasion, the addition of macrophages to Mena11a-expressing cells did not significantly increase cancer cell invasion. This result is consistent with the decreased response to EGF and the decreased CSF-1 expression levels in Mena11a-expressing cells that are below the threshold needed to stimulate pro-invasive macrophage behavior.

Mena^{INV}-expressing carcinoma cell streaming requires macrophages and paracrine signaling

The increased invasion of Mena^{INV}-expressing carcinoma cells depends on paracrine loop signaling with macrophages and the paracrine loop has been found to be required for cancer cell migration in mammary tumors (Wyckoff *et al* 2004). In particular, it has been hypothesized that the paracrine loop also promotes carcinoma cell streaming *in vivo*. Using intravital imaging, it has been observed that multiple carcinoma cells moved in streams among host cell shadows previously identified as immune cells and macrophages (Wyckoff *et al* 2000b, Wyckoff *et al* 2007). Intravenous injection of Texas Red dextran during intravital imaging indeed identified the presence of the host cell shadows in cancer cell streams as macrophages, because macrophages uniquely pinocytose dextran delivered intravenously into mammary tumors (Wyckoff *et al* 2007).

In order to investigate whether streaming required macrophages, mice were treated with clodronate liposomes 48 h prior to intravital imaging to decrease the level of functional macrophages (Hernandez *et al* 2009). As expected, a 70% decrease in the number of Texas Red dextran-labeled macrophages was observed in animals treated with clodronate liposomes compared with those treated with PBS liposomes (control). Intravital imaging of primary tumors revealed that there were 90% fewer streaming cells in mice treated with clodronate liposomes as compared to controls, confirming the involvement of the macrophage–cancer cell paracrine loop in streaming. Moreover, to analyze the involvement of the paracrine loop in streaming, mice were treated with Erlotinib two hours prior to intravital imaging

in order to block the EGFR on cancer cells (Zerbe *et al* 2008) or with a CSF-1R antibody four hours prior to intravital imaging to block the CSF-1R on macrophages (Wyckoff *et al* 2007). Intravital imaging of primary tumors demonstrated that there were 65% fewer streaming cells in mice treated with Erlotinib and 80% fewer streaming cells in mice treated with α-CSF-1R as compared with tumors.

Expression of Mena^{INV} in cancer cells increases transendothelial migration

Due to the data showing increased streaming and invasion in Mena^{INV}-expressing cells, it has been hypothesized that expression of Mena^{INV} might also increase intravasation and finally cancer metastasis. Following intravenous injection of Texas Red dextran, intravital imaging of Mena^{INV}-expressing tumors showed that cancer cell streaming was indeed directed toward blood vessels. Then the intravasation efficiency of tumors expressing Mena^{INV} and Mena11a was quantified using intravital imaging of photoconverted cancer cells adjacent to blood vessels to determine the percentage of cancer cells intravasating over a 24 h period (Kedrin *et al* 2008). The quantification of the alterations in the photoconverted tumor area 24 h after photoconversion showed that 95% of Mena11a-expressing cells remained in the converted area as compared with 75% of carcinoma cells expressing Mena^{INV}. Additionally, the cancer cell blood burden to measure intravasation *in vivo* was evaluated (Wyckoff *et al* 2000b). Mice with Mena^{INV}-expressing tumors had a four-fold increase in the number of carcinoma cells in circulation compared with mice with GFP- or Mena11a-expressing tumors. In line with this, Mena11a-expressing xenograft mice had similar numbers of circulating carcinoma cells compared to GFP-expressing xenograft mice.

As previous studies showed that interaction between cancer cells and perivascular macrophages is required for intravasation (Wyckoff *et al* 2007) and that enhanced Mena^{INV} cell streaming and invasion are paracrine-dependent, it has been hypothesized that increased intravasation in Mena^{INV}-expressing cells might also be paracrine-dependent. In order to determine the minimum requirements for macrophage-assisted intravasation, a subluminal-to-luminal transendothelial migration assay was used, in which the presence of macrophages could be varied to determine their need for carcinoma cell intravasation. Interestingly, less than 0.5% of cancer cells traversed the endothelium in the absence of macrophages, regardless of Mena isoform expression, indicating that Mena isoforms function only in the presence of macrophages. The addition of macrophages did not enhance transendothelial migration for cells expressing GFP or Mena11a. Importantly, in the presence of macrophages, 54% of Mena^{INV}-expressing cells traversed the endothelium, which is a 200-fold increase in transendothelial migration compared to all other cell types.

In order to investigate the paracrine dependence of intravasation *in vivo*, the number of circulating cancer cells was determined following functional impairment of macrophages achieved by treatment of mice with clodronate liposomes or CSF-1R-blocking antibody, or impairment of cancer cells by treatment with Erlotinib. Indeed, the tumors formed from injection of Mena^{INV}-expressing cells revealed a significant reduction in circulating cancer cells following treatment with clodronate liposomes, CCSF1R-blocking antibody and Erlotinib as compared to controls (Roussos *et al* 2011a).

Mena^{INV}, but not Mena11a, increases intravasation, dissemination and metastasis

In order to reveal the mechanistic consequence of enhanced transendothelial migration and intravasation by Mena^{INV} expression or the suppression of invasion by Mena11a expression, the ability of these cells to extravasate, disseminate and metastasize to the lung was investigated. Thus, an experimental metastasis assay was used in order to measure of extravasation of cancer cells (Xue *et al* 2006). Then micrometastases in the lungs were counted after intravenous injection of GFP-, Mena11a- or Mena^{INV}-expressing cells. However, the metastatic burden was similar for all cell lines. Previous studies have shown that MTLn3 cancer cells forced to express Mena^{INV} show a significant enhancement to metastases (Philippar *et al* 2008). Thus, it was investigated whether dissemination of single cancer cells to the lung, a step preceding growth of macrometastases, was also affected in xenograft mice derived from injection of cells expressing Mena^{INV} or Mena11a. Mice with Mena^{INV} xenografts showed significantly increased cancer cell dissemination to the lungs compared with animals bearing either Mena11a- or GFP-expressing tumors. Finally, mice with Mena11a xenografts had half as many cells in the lungs as mice bearing GFP-expressing tumors.

As Mena^{INV} expression is known to increase cancer cell dissemination and it is known that that Mena11a expression reduces cancer cell dissemination, it has been hypothesized that the Mena isoform expression may similarly modulate the final step in metastatic progression: the occurrence of spontaneous metastasis. Spontaneous metastases to the lungs have been detected in mice with mammary tumors at either three or four weeks after mammary gland injection of GFP-, Mena11a- and Mena^{INV}-expressing cells. In particular, expression of Mena^{INV} increased the incidence of metastasis compared with expression of GFP and Mena11a, whereas expression of Mena11a decreased metastases after three weeks of tumor growth. However, after four weeks of tumor growth, all primary tumors resulted in detectable metastases regardless of the Mena isoform expressed. In addition, Mena^{INV} expression induced metastatic spread to the lungs with a little effect on the primary tumor growth after three weeks or no effect on primary tumor growth after four weeks *in vivo* (Philippar *et al* 2008) or an effect on the cell growth *in vitro*. Hence, the differences in tumor metastasis occurring in tumors with different Mena isoform expression are not an indirect consequence of tumor growth. These data clearly suggest that the increased incidence of spontaneous metastasis revealed in Mena^{INV}-expressing tumors is due to metastatic events occurring prior to extravasation.

Enhanced Mena expression is correlated with metastasis in breast cancer patients (Di Modugno *et al* 2006). In particular, during invasion and migration of cancer cells the expression of Mena^{INV} increases (positive regulator), whereas the expression of Mena11a decreases (negative regulator) (Goswami *et al* 2009). Invasion, migration and intravasation have been identified as crucial metastasis steps that are affected by the expression of Mena^{INV}- and Mena11a-expressing cancer cells. A key characteristic of Mena^{INV}-expressing cells is their contribution to cell streaming and increased intravasation as a result of the pronounced elevation in transendothelial migration. Another important result is the effect of Mena^{INV} expression on cancer

cell sensitivity to macrophage-supplied EGF and the subsequent enhancement of paracrine-facilitated invasion. Taken together, these findings ultimately suggest that the EGF-dependent enhancement of invasion and intravasation in Mena^{INV}-expressing cancer cells ensures enhanced cancer cell dissemination and spontaneous metastasis to the lungs.

Conversely, it has been found that Mena11a-expressing cells do not show dramatically increased streaming and even fail to co-invade with macrophages, which indicates decreased paracrine signaling interaction. The decrease in CSF-1 expression in Mena11a-expressing cells leads to impaired paracrine signaling and deficits are observed in activities that depend on this paracrine signaling loop *in vivo*, such as streaming, invasion, transendothelial migration, cancer cell dissemination and spontaneous metastasis to the lungs.

During invasion, cancer cells are known to reduce their expression of Mena11a and initiate the production of the Mena^{INV} isoform (Goswami *et al* 2009). Moreover, it has been found that Mena11a expression is correlated with reduced EGF-induced *in vivo* invasion. In addition, it has been shown that Mena^{INV}-expressing migratory carcinoma cells are highly sensitive to EGF in their protrusion and chemotaxis activities, leading to significantly increased *in vivo* invasion. Finally, these activities can result in Mena^{INV}-expressing cell migration towards and in association with perivascular macrophages, leading to enhanced transendothelial migration and intravasation.

In addition to decreased EGF-induced *in vivo* invasion of Mena11a-expressing cells, these cells have been found to express less CSF-1 mRNA. Data from patients revealed that CSF-1 and its receptor play crucial roles during the progression of breast cancer (Kacinski *et al* 1991, Scholl *et al* 1994) and that CSF-1 and the CSF-1R are coexpressed in more than 50% of breast tumors (Kacinski 1997). An elevated circulating CSF-1 level has been proposed as an indicator of early metastatic relapse in patients with breast cancer, independent of the breast cancer subtype (Scholl *et al* 1994, Tamimi *et al* 2008, Beck *et al* 2009). Moreover, this suggests that lower levels of CSF-1 in Mena11a-expressing cells may lead to a reduced metastatic progression. The decreased invasion, intravasation and dissemination of Mena11a-expressing cells is consistent with the reduction in expression of CSF-1 and the decreased sensitivity to EGF, which seems to force these cells to participate in a paracrine signaling loop with macrophages.

A major finding is that the expression of Mena^{INV} supports a mode of coordinated cell migration not previously described, where cell migration is spatially and temporally coordinated between the cancer cells that are not connected by cell–cell junctions. This newly described mode of coordinated cell migration is called cell streaming. The streaming differs from previously described modes of coordinated cell migration, which require stable cell–cell junctions (Sahai 2005), while streaming cells require no cell–cell contacts and the migration velocities are 10–100 times more rapid. Previous studies have reported that *in vivo* MTLn3 cells express CSF-1 and EGFR, but express no CSF-1R on their cell surface and do not secrete EGF, while macrophages express CSF-1R and secrete EGF, but do not secrete CSF-1 or express EGFR on their cell surface (Goswami *et al* 2005). Thus, coordinated parts of the

paracrine signal transduction pathways are active in both cell types during cell invasion *in vivo* (Wyckoff *et al* 2004). Indeed, it has been demonstrated that streaming requires paracrine chemotaxis between carcinoma cells and macrophages. The ability of Mena^{INV}-expressing cells to protrude and perform chemotaxis with 25- to 50-fold lower concentrations of EGF compared to parental cancer cells, and to suppress cell turning in streams, leads to a pronounced contribution to the extraordinary coordination and maintenance of high velocity migration of cell streams *in vivo*. Moreover, it has been proposed that the increased sensitivity of Mena^{INV}-expressing cells to EGF in the EGF–CSF-1 paracrine loop is responsible for the enhancement in the streaming motility. This conclusion is further supported by the inhibition of streaming upon inhibition of the EGFR by Erlotinib or of CSF-1R by an anti-CCSF1R-blocking antibody.

Invasive cancer cells from PyMT mice displayed increased Mena^{INV} expression and decreased expression of Mena11a (Goswami *et al* 2009). Interestingly, studies using intravital imaging of mammary tumors in Mena-deficient PyMT mice revealed significantly reduced streaming motility of cancer cells, providing further evidence that Mena facilitates enhanced motility (Roussos *et al* 2010). Finally, mammary tumors derived from the human breast cancer cell line MDA-MB-231 contain a subpopulation of cancer cells that participate in macrophage–tumor cell paracrine-facilitated invasion (Patsialou *et al* 2009), and these invasive cancer cells have also been revealed to differentially up-regulate Mena^{INV} and down-regulate Mena11a (Goswami *et al* 2009). Taken together, these findings suggest that paracrine-facilitated carcinoma cell streaming is a generalized cellular feature that occurs in rat, mouse and human models of breast cancer and that it is a clear consequence of the differential regulation of the Mena isoforms.

In summary, the suppression of invasion and streaming by the inhibition of paracrine signaling between macrophages and cancer cells *in vivo* and by decreasing macrophage function in *vivo*, has established the crucial role of macrophages during the coordinated migration of Mena^{INV}-expressing cells (Wyckoff *et al* 2007, Hernandez *et al* 2009). It has also been demonstrated that macrophages are essential for the transendothelial migration of Mena^{INV}-expressing cancer cells. Indeed, these results are consistent with previous work demonstrating that paracrine signaling between cancer cells and macrophages, and the presence of perivascular macrophages in the primary tumor, are required for invasion and intravasation, respectively (Wyckoff *et al* 2004, Wyckoff *et al* 2007). In particular, these results support other previous work that suggested that Mena^{INV}- but not Mena11a-expressing cancer cells specifically contribute to the intravasation of breast cancer cells in humans by assisting cancer cells to assemble, leading to the formation of the macrophage-dependent intravasation compartment known as the TMEM (Robinson *et al* 2009, Roussos *et al* 2011b).

In vivo, it has been shown that Mena^{INV}-expressing cells invade towards very low concentrations of EGF in macrophage-dependent paracrine chemotaxis. However, *in vitro*, even low concentrations of EGF, such as that found in serum, lead to macrophage-independent 3D invasion of Mena^{INV}-expressing cells (Philippar *et al* 2008), while completion of transendothelial migration requires EGF secreted by

macrophages (Wyckoff *et al* 2004). The effects of Mena^{INV} expression on EGF-dependent processes lead to enhanced cell invasion, intravasation, dissemination and metastasis to the lungs. These data suggest that drugs directed specifically to the inhibition of Mena^{INV}-dependent increased EGF sensitivity will disrupt the paracrine interactions with macrophages required for metastasis and consequently result in the inhibition of metastasis in mammary tumors.

However, it is important to understand how the Mena isoforms differ functionally. The INV exon is inserted just after the EVH1 domain, which is primarily responsible for the subcellular localization of Ena/VASP proteins and interactions with several signaling proteins such as lamellipodin (Gertler *et al* 1996, Urbanelli *et al* 2006, Pula and Krause 2008). Thus, it is possible that the INV exon might influence the function of Mena^{INV} by regulating its EVH1-facilitated interactions (Niebuhr *et al* 1997, Boeda *et al* 2007). In contrast, the 11a exon is inserted within the EVH2 domain between the F-actin binding motif and the coiled-coil tetramerization site. Indeed, the F-actin binding is crucial for nearly all known Ena/VASP functions, such as the localization to the tips of lamellipods and the ability to drive filopod and lamellipod formation and extension (Gertler *et al* 1996, Loureiro *et al* 2002, Applewhite *et al* 2007). *In vitro*, F-actin binding is necessary for the anti-capping activity of Ena/VASP and can be disrupted by phosphorylation at nearby sites (Barzik *et al* 2005), as it is the case in F-actin bundling. Because 11a is inserted into the analogous region of Mena, it will be interesting to investigate whether the barbed end capture activity is affected. Due to the phosphorylation of the 11a insertion (Di Modugno *et al* 2006), it is likely that inclusion of the 11a exon provides a regulatory mechanism for Mena11a.

In summary, additional research is required to investigate the molecular and biochemical mechanisms of action of the Mena^{INV} and Mena11a isoforms and their potential as a prognostic marker for patient outcome and a therapeutic target for breast cancer metastasis.

Most cancer-related deaths result from cancer metastasis and thus it is important to increase knowledge of the molecular mechanisms of dissemination, including intra- and extravasation. Although the mechanisms of extravasation have been studied intensively *in vitro* and *in vivo*, the process of intravasation remains elusive. In particular, how cells such as macrophages in the tumor microenvironment facilitate cancer cell intravasation is still unknown. Using high-resolution imaging, it has been observed that macrophages provide increased cancer cell intravasation upon physical contact. In more detail, macrophage and cancer cell contact induce RhoA activity in cancer cells, triggering the assembly and exertion of actin-rich, degradative protrusions called invadopodia, enabling cancer cells to degrade and break through matrix confinements during cancer cell transendothelial migration (Roh-Johnson *et al* 2014). Interestingly, the macrophage-induced invadopodium formation and cancer cell intravasation also occurred in patient-derived cancer cells and *in vivo* models, suggesting a conserved mechanism of cancer cell intravasation. These results illustrate a novel heterotypic cell-contact-facilitated signaling role for RhoA and yield mechanistic insights into the capacity of cells such as macrophages within the tumor microenvironment to facilitate steps of the metastatic cascade (Roh-Johnson *et al* 2014).

In summary, understanding the mechanistic basis of specific steps within cancer metastasis is crucial for the identification of robust early prognostic markers. Knowing how cancer cells can leave the primary tumor and enter the vasculature (called intravasation) is a key step towards designing drug treatment strategies for the disease. Multiphoton-based intravital imaging of rodent mammary adenocarcinoma (Roussos *et al* 2011a) and human tumors has uniformly revealed that cancer cells and macrophages cooperate during several key steps of the metastatic cascade. The cell polarization and subsequent motility of invasive cancer cells toward the blood vessels seems to be dependent on a paracrine loop of cancer cell signaling with macrophages (Wyckoff *et al* 2004, 2007). Cancer cells respond to macrophage-secreted EGF and, in turn, macrophages respond to cancer-cell-secreted CSF-1. Moreover, it has also been demonstrated that macrophages play a role in cancer cell entry into the vasculature *in vitro* and *in vivo*. In line with this, treatment with drugs that eliminate the macrophage function results in a reduced number of circulating cancer cells (Roussos *et al* 2011a). As both the cancer cells' ability to migrate toward the blood vessel and the actual intravasation step are necessary for entry of cancer cells into circulation, precisely how macrophages regulate cancer cell intravasation remains elusive. Furthermore, several *in vitro* studies analyzing the mechanism of cancer cell intravasation have revealed that the presence of macrophages elevates cancer cell intravasation, while the mechanisms of this enhancement are still not well understood (Roussos *et al* 2011b, Zervantonakis *et al* 2012).

Intravital imaging of the tumor microenvironment has revealed that cancer cells intravasate into the vasculature at sites in close proximity to macrophages (Wyckoff *et al* 2004). Due to these observations, a microanatomic landmark composed of a perivascular macrophage in contact with a cancer cell at blood vessels has been identified and termed TMEM. In a case-controlled study of metastatic and non-metastatic breast cancers, TMEM density in breast tumors at initial resection was found to be associated with the risk of metastasis (Robinson *et al* 2009), suggesting that macrophages, cancer cells and endothelial cells cooperate in providing cancer cell entry into the vasculature. However, the cell biological mechanisms that exist between these three cell types during intravasation are still elusive. It has been suggested that they are dependent on the particular cellular mechanical properties of these cell types (Mierke *et al* 2011, Mierke 2014).

In order to gain a closer look at the molecular mechanisms of cancer cell transendothelial migration, *in vitro* experiments focusing on investigating cancer cell and endothelial cell behavior have been widely performed. Models of cancer cell extravasation or the exit of cancer cells from the vasculature are used frequently, because the apical surface of the endothelial cell monolayer can be accessed easily by plating endothelial monolayers and seeding cancer cells on top of these monolayers (Reymond *et al* 2012a, 2012b, Jin *et al* 2012, Haidari *et al* 2011, 2012). From these studies, an extensive view of how cancer cells affect the endothelial cell architecture has been built up and the specific steps of cancer cell adhesion and intercalation during transmigration have been revealed (Reymond *et al* 2012a, 2012b, Voura *et al* 1998a, 1998b). A common downstream mechanism of cancer cell transendothelial migration is the opening of the endothelial monolayer. In more detail, endothelial

cells lose their cell–cell adherence junctions and tight junctions and hence open gaps appear in the monolayer, allowing cancer cells to pass through. In addition, molecular pathways leading to adhesion dissolution have also been discovered (Voura *et al* 1998a, Stoletov *et al* 2007, Tremblay *et al* 2006). However, how cancer cells perform intravasation and whether intravasation and extravasation share mechanisms in common is less well understood. Cancer cells can withstand shear flow stresses in the blood vessels and, in addition, different cells are present at intravasation and extravasation sites; however, it can be predicted that different modes of cell–cell interactions between cancer cells and endothelial cells occur in the intravasation and extravasation steps of the metastatic cascade. Due to the fact that macrophages are recognized at sites of cancer cell intravasation *in vivo*, the link between the close association of macrophages and cancer cells should be characterized, as well as the subsequent cancer cell intravasation.

Using dissolution of endothelial cell–cell adhesion as a readout of cancer cell transendothelial migration, the relationship between macrophages and cancer cells can be assessed with an *in vitro* model of intravasation. In particular, the question of whether the association of macrophages and cancer cells reflects a cell biological mechanism promoting and increasing intravasation of cancer cells through the endothelial cell barrier should be addressed. High-resolution imaging has been used in combination with *in vitro* and *in vivo* models to investigate whether macrophages increase cancer cell intravasation and uncover the cell biological and signaling mechanisms regulating this process (Roh-Johnson *et al* 2014). Taken together, these results reveal that macrophages facilitate cancer cell intravasation by the activation of the RhoA signaling pathway, which promotes the formation of invadopodia, enabling the cancer cells to initiate intravasation by penetrating through the basement membrane of the vasculature.

16.4 Impact of Notch and Mena signaling during cancer cell and macrophage interaction

It has been demonstrated that the direct interaction of a cancer cell and macrophage results in the formation of the cancer cell invadopodium that is necessary for the transendothelial migration of cancer cells and hence this cannot be mimicked by adding macrophage-conditioned medium (Roh-Johnson *et al* 2014). Hence, direct contact between cancer cells and macrophages evokes a signal promoting the formation of invadopodia on cancer cells.

A major signal transduction pathway that is involved in cell contact-facilitated communication is the Notch signaling pathway. In addition to the crucial roles of Notch signaling in developmental processes, it has been suggested to have additional roles in various cancers, such as breast, lung and pancreatic cancers, as well as leukemia, where activation of Notch pathways can induce proliferation, impair differentiation and facilitate metastasis (Han *et al* 2011, Bolos *et al* 2013, Reedijk *et al* 2005, Andrieu *et al* 2016). In particular the disruption of the Notch signal transduction pathways can alter cell growth, cell fate, angiogenesis and apoptosis (synonymously termed programmed cell death). In cancer cells, the activation of

Notch after the formation of a homotypic cell contact induces the formation of an invadopodium under conditions of hypoxia (Diaz *et al* 2013). Hence the effect of the Notch signaling pathway has been analyzed on the tumor microenvironment of metastasis (TMEM) function. In particular the macrophage-dependent cancer cell invadopodium assembly and its relationship to Mena expression during transendothelial migration and cancer cell dissemination has been investigated.

Cancer cell dissemination through blood vessels in breast tumors essentially requires cancer cells to undergo transendothelial migration. Transendothelial migration has been reported to take place uniquely at TMEM sites where macrophages are in direct contact with cancer cells and endothelial cells (Harney *et al* 2015). However, it has been observed that cancer cells of certain cancer types can still undergo tranendothelial migration without the presence of macrophages (Mierke *et al* 2008, Mierke 2011). The molecular mechanism by which the direct contact between macrophages and cancer cells is mediated has been revealed and also how this causes the formation of an invadopodium and subsequently transendothelial migration. Indeed, it has been shown that these processes require the Notch1 receptor on the cancer cell and subsequently that the Notch1 signaling causes the $Mena^{INV}$ expression through the activation of its gene transcription. Moreover, Notch1 is necessary for transendothelial migration of cancer cells and the dissemination of cancer cells from the primary solid tumor. Indeed, these findings are in line with previous results showing that $Mena^{INV}$ expression is needed for transendothelial migration of cancer cells, the knockdown of $Mena^{INV}$ impairs the macrophage-facilitated transendothelial migration of cancer cells (Pignatelli *et al* 2014) and $Mena^{INV}$ overexpression evokes the assembly and function of an invadopodium, and finally transendothelial migration (Roussos *et al* 2011b).

In addition, the relative expression of $Mena^{INV}$ to that of Mena11a has been found to be associated with TMEM assembly, the reoccurrence of metastasis and subsequently the death of breast cancer patients (Pignatelli *et al* 2014, Forse *et al* 2015, Agarwal *et al* 2012). This is highly relevant as the TMEM is the starting point for the successful intravasation of cancer cells into the blood vessels in breast tumors and a high number of TMEM sites in a breast tumor seems to be correlated with a highly predictive risk of distant relapse of the tumor through the formation of secondary tumors (metastases) in patients (Robinson *et al* 2009, Rohan *et al* 2014). Indeed, it has been shown that these signals facilitate reduced expression of Mena11a (Shapiro *et al* 2011). However, the signals facilitating the induced expression of $Mena^{INV}$ have not yet been revealed and require further investigation. The expression of the $Mena^{INV}$ isoform has been reported to be mediated by the activation of Notch1 signaling pathways in cancer cells. These findings lead to the suggestion that there is a novel role for the Notch1 signaling processes in the regulation of the $Mena^{INV}$ expression and subsequently the transendothelial migration at TMEM sites. Moreover, the mechanistic insights into how the expression of $Mena^{INV}$ is upregulated in cancer cells through interaction with macrophages enlighten novel experimental research plans for the future exploration of how even different macrophage ligands can provide the activation of Notch signaling on cancer cells, creating distinct phenotypes associated with the process of tumor

metastasis (Pignatelli *et al* 2015). Hence these results are directly correlated to how the distinct Mena isoform expression and the number of TMEMs can help to prognose the risk of breast cancer relapse or even further malignant progression in patients (Robinson *et al* 2009, Rohan *et al* 2014, Karagiannis *et al* 2016, Forse *et al* 2015, Agarwal *et al* 2012, Karagiannis *et al* 2017). The molecular characterization of the Notch1 driven Mena^{INV} expression has revealed more insights into how biomarkers for cancer disease relapse or malignant progression can be designed, which may be used together with the identification of TMEM sites in order to improve the prediction of metastasis at distant metastatic sites for breast cancer patients and thereby their response to a certain treatment.

There is indeed high interest in targeting the Notch1 signal transduction pathway for the treatment of a wide variety of cancers, although it has also been demonstrated that chronic Notch inhibition causes detrimental secondary effects and even under certain circumstances enhanced development of vascular tumors (Han *et al* 2011, Ryeom 2011). Thus, future studies are required to reveal the detailed underlying mechanisms of Notch1 signaling in the progression of breast cancer, which may then shed light on the mechanism and thereby identify novel therapeutic targets or biomarkers within the Notch1 signaling cascade, which are suited for treatment of patients and have reduced side effects.

Taken together, it has been demonstrated that the macrophage-induced invadopodium is regulated by a Notch1/Mena^{INV} signal transduction pathway in the cancer cells due to a direct adhesive contact with macrophages. Moreover, this heterotypic cancer cell–macrophage interaction evokes the increased transcriptional expression of Mena^{INV}. Both, Notch1 and Mena^{INV} expression are necessary for the transendothelial migration of cancer cells and hence represent a required step for the intravasation of cancer cells. The impairment of Notch signaling using inhibitory drugs abolished the macrophage-based invadopodium formation *in vitro* and the dissemination of cancer cells from the primary solid tumor *in vivo*. Taken together, these findings enlightened a novel role for Notch1 signaling cascade in the regulation of the expression of Mena^{INV} and transendothelial migration of cancer cells and finally delivers significantly mechanistic insights, which can be important for the development of novel therapeutic inhibitors of cancer metastasis.

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