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Molecular and Cellular Mechanobiology

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Molecular and Cellular Mechanobiology

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Preface

At the request of the American Physiological Society, we have edited this book on *Molecular and Cellular Mechanobiology* as part of the "Perspectives in Physiology" series.

Mechanobiology is a rapidly developing scientific field at the interface of biology, medicine, and engineering that studies how physical forces, cell/tissue mechanics, and their interactions regulate homeostasis in health and pathophysiological changes in disease. Mechanobiology involves the sensing of mechanical cues by cells, the transduction of these cues into molecular signals, and the modulation of gene and protein expression and hence cellular functions. Mechanobiology is distinct from biomechanics in that the latter involves the active application of forces to cells for the elucidation of their physical properties.

This book covers the cutting-edge developments in mechanobiology, with the aim of providing the reader with a clear understanding of this frontier discipline at the molecular and cellular levels, encompassing the mechanosensors, transducers, and genetic and epigenetic regulation, as well as clinical applications.

Part I consists of three chapters on Mechanosensors. Chapter 1 (E.R. Moore and C.R. Jacobs) discusses the primary cilium as a mechanosensor that can modify its structure and composition to tune mechanosensitivity. This chapter presents the experimental studies and computational modeling of the molecular and mechanical bases of mechanosensing and uses animal models to explore cilium-based health complications. Chapter 2 (L.J. Chen, W.L. Wang, and J.J. Chiu) addresses the shear-responsive mechanosensors in the cell membrane, intercellular junctions, cytoplasm, and nucleus of vascular endothelial cells and presents a conceptual framework for understanding their regulation in response to hemodynamic forces in health and disease. Chapter 3 (D.E. Leckband) highlights a new class of force-sensitive cadherin-based adhesion complexes at intercellular junctions and elucidates a new force transduction mechanism that can impact cell mechanics and modulates such cell functions as barrier integrity and cell cycle control.

Part II consists of three chapters on Mechanotransducers. Chapter 4 (Y. Sun, Y. Shao, X. Xue, and J. Fu) presents the emerging roles of YAP/TAZ, which are transcription coactivators in the canonical Hippo signaling pathway, in

mechanobiology, addressing the different types of mechanical cues that mediate YAP/TAZ activities and their upstream mechanosensitive molecular machineries. Chapter 5 (C.A. McCulloch) focuses on the role of Rho GTPases in the translation of mechanical and chemical cues into the cellular responses that regulate cell, tissue, and organ structure and function, with special emphasis on their contribution to cell migration and responses to environmental forces. Chapter 6 (V. Swaminathan and C.M. Waterman) illuminates the role of cell adhesion in mechanobiology by using modern microscopy approaches to study integrin-based focal adhesions and discusses how these complex adhesion organelles are built and regulated, as well as the integration of the cell with its environment in mediating physiological functions.

Part III consists of three chapters on Epigenetic and Genetic Regulations in the Nucleus. Chapter 7 (Q. Peng, B. Cheng, S. Lu, S. Chien, and Y. Wang) introduces fluorescence resonance energy transfer (FRET) technologies to visualize in single cells the dynamic epigenetic regulations (particularly histone modifications and DNA methylations) related to mechanobiology in the nucleus, thus enabling the elucidation of functional responses to mechanical environments. Chapter 8 (D. Kelkhoff and T. Downing, S. Li) highlights the potential mechanisms through which mechanotransduction may lead to epigenetic modifications such as DNA methylation and histone methylation and acetylation, and the consequential long-term effects on phenotypic changes, including stem cell differentiation and cell reprogramming. Chapter 9 (J. Irianto, I.L. Ivanovska, J. Swift, and D.E. Discher) discusses the role of lamin in the mechano-responsiveness of nuclei and the signaling pathways that regulate lamin levels and cell fate in response to matrix mechanics and molecular cues. This chapter also discusses the importance of nuclear mechanics in niche anchorage and cell motility in development, hematopoietic differentiation, and cancer invasion. Chapter 10 (Y. Wang, E. Makhija, K. Damodaran, and G.V. Shivashankar) summarizes the physical and chemical connections between ECM and 3D chromosome organization that lead to modular gene regulation, and the role of nucleoskeleton-cytoskeleton linkage in nuclear mechanotransduction and the consequential remodeling of chromatin dynamics, epigenetic landscape, and 3D chromosome organization.

Following the coverage of the basic principles of mechanobiology in the first three parts, Part IV consists of three chapters on the clinical applications of mechanobiology to cardiovascular diseases and cancer. Chapter 11 (A.J. Putnam) focuses on the relationships between mechanical forces and cells of the cardiovascular system (including endothelial cells, smooth muscle cells, and cardiac myocytes), with an emphasis on translating fundamental mechanobiology insights into the control of cell fate for applications in cardiovascular regenerative medicine. Chapter 12 (A. Zhong and C.A. Simmons) reviews the influences of hemodynamic forces in valve development and the roles of shear stress, cyclic strain, and matrix mechanics in regulating the initiation and progression of calcific aortic valve disease, with the goals of identifying therapeutic targets for treating adult valve diseases and guiding the design of living tissue replacement valves. Chapter 13 (L. Fattet and J. Yang) describes how ECM stiffness contributes to tumorigenesis, through different roles

on tumor cells or stromal cells, at the primary tumor and at metastatic sites. This chapter also discusses the latest and most promising therapeutic approaches targeting or taking advantage of this newly defined implication of mechanoregulation in cancer progression.

We wish to thank the authors of the 13 chapters for their outstanding contributions that provide state-of-the-art information on this exciting field of molecular and cellular mechanobiology. We also thank Brian Halm of Springer Science for his administrative help in the preparation of this book.

This book has the following unique features:

- An integrative approach across different scales from molecular sensing to mechanotransduction, gene modulation, and physiological regulation of cellular functions, as well as application to pathophysiological states in disease.
- An integration of molecular and cellular physiology with the physics and engineering of biomechanics, thus providing a comprehensive understanding of the roles of physico-chemical microenvironment and intracellular responses in determining cellular function in health and disease.
- An interdisciplinary approach that takes into account the diverse backgrounds of readers. It is written to:

  Help physiologists and biologists interested in mechanobiology, but new to the field, to understand the impact of mechanobiology on physiological regulation in health and disease.

  Help engineers interested in physiology and life sciences, but lacking formal training, to gain insights into the molecular and cellular bases of fundamental biological processes related to engineering mechanics.

  Help clinicians to understand the roles of mechanobiology in the pathogenesis, diagnosis, treatment, and prevention of disease.

This book is suitable for a broad range of readers, including physiologists and other experimental biologists (including cell biologists, biophysicists, pharmacologists, pathologists, and others), bioengineers and other engineers (mechanical engineers, systems engineers, and others), clinical investigators, clinicians (including oncologists, cardiologists, and others), faculty, industry scientists, postdoctoral fellows, and graduate students.

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Part I
Mechanosensors

Chapter 1
The Primary Cilium as a Strain Amplifying Microdomain for Mechanotransduction at the Cell Membrane

Emily R. Moore and Christopher R. Jacobs

Abstract The primary cilium is an extracellular organelle that transduces mechanical signals into intracellular signaling cascades. The cilium is accompanied by a dense collection of stretch-activated channels and proteins involved in mechanotransduction, which form a highly sensitive microdomain at the cell surface that serves to amplify detected stimuli. The putative stress buildup at the base of the cilium activates components in this microdomain to trigger signaling via second messengers such as calcium and cyclic AMP. Defects in the structure of the cilium and mutations in ciliary proteins disrupt the cilium's ability to detect and transduce mechanical stimuli. This dysfunction at the cellular and molecular level translates to the organ and tissue level, resulting in disastrous diseases and syndromes spanning multiple organs. This chapter discusses the mechanisms for primary cilium-mediated mechanotransduction, the use of computational models to characterize cilium mechanics, modifications in cilium structure and composition to tune mechanosensitivity, and animal models created to explore cilium-based health complications.

Keywords Primary cilium • Mechanotransduction • Ift88 • Adenylyl cyclase 6 (AC6) • Polycystin (PC1, PC2) • Transient receptor potential channels (TRPV4) • Calcium • Cyclic AMP (cAMP) • Osteocyte • Ciliopathy

1.1 Introduction

Once believed to be a vestigial structure, the primary cilium is now regarded as a sensory organelle that occurs once per cell and projects through the cell membrane. In several cell types the primary cilium functions as a mechanosensor, detecting external mechanical stimuli and transducing these physical signals at the cell membrane to trigger intracellular signaling cascades. Generally, the primary cilium is thought to

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bend in a cantilever fashion in response to physical stimuli, such as fluid flow. The tension and compression generated along the cilium due to bending are believed to activate mechanosensitive proteins and open ion channels located in the ciliary and cellular membranes. This effect is amplified at the base of the cilium, where tensile and compressive strains are maximal. A dense cluster of mechanosensitive proteins and ion channels is present within the cell membrane near the base of the cilium, suggesting that ciliary mechanics facilitate cell membrane mechanosensing. For this reason, our lab investigates the primary cilium as a critical strain amplifying tool that significantly enhances mechanotransduction at the cell membrane.

Although mechanically stimulated ion channels are well characterized and studied, two important mysteries remain: how their sensitivity is regulated by extracellular signals and how the large membrane strains required for opening them are generated. Stretch-activated channels (SACs) undergo a conformational change when tension occurs in the surrounding cell membrane, pulling open to allow an influx of ions that trigger intracellular signaling cascades. The mechanical sensitivity of a SAC depends on the channel's ability to open or close and the duration of time it remains open (Sachs 2010; Charras et al. 2004). Since a SAC simply opens and closes, changes in sensitivity to external stimuli are likely mediated by the magnitude of the mean membrane tension that acts on the channel (Andersen et al. 1999; Sachs 2010) as opposed to modifications in the channel composition or structure (Markin and Martinac 1991; Markin and Sachs 2004). Lipid composition influences the affinity for channel opening (Goulian et al. 1998; Lundbaek and Andersen 1999), but it is unknown whether extracellular signals induce changes in the structure and composition of membrane lipids to modify membrane tension or if there is another mechanism for tuning ion channel mechanosensitivity. Additionally, there are non-mechanical extracellular signals that affect general cellular mechanosensitivity that have yet to be linked to ion channels. For example, estrogen treatment adjusts bone cell mechanosensitivity (Devlin and Lieberman 2007; Galea et al. 2013; Klein-Nulend et al. 2015) but has no known direct effect on ion channel structure or composition and is hypothesized to modify their mechanosensitivity via an alternative mechanism (Boyle and Kaczmarek 2012). The second question arises from an observation that, for a given external mechanical stimulus, there is often a discrepancy between the magnitude of tension generated in the plasma membrane and that required to open a SAC. For example, an 800 % radial membrane strain via micropipette aspiration is required to open 50 % of osteoblast SACs within the aspirated section, but a cellular strain of only 2.5 % via AFM microindentation is required to stimulate an influx of cytosolic calcium (Charras and Horton 2002; Charras et al. 2004). In fact, the reported strain required to open osteoblast SACs vastly exceeds the 3–5 % lytic strain for unsupported lipid bilayers (Sachs and Morris 1998), suggesting another cellular process is involved in amplifying strain to mediate ion channel opening.

An intriguing explanation for the disparity between external stimuli and ion channel mechanosensitivity is a subcellular structure that adapts itself to external mechanical stimuli and amplifies membrane tension. Primary cilia are excellent candidates for this role. In fact, the primary cilium may be uniquely responsible for sensing

small-scale changes in membrane strain (Mathieu et al. 2014). Investigators are currently examining strain buildup at the base of the cilium (Rydholm et al. 2010; Mathieu et al. 2014) but whether this correlates with strain in the plasma membrane is largely undetermined. One proposal is that, upon bending, small strains are amplified at the base of the cilium and stretch open ion channels in the cell membrane (Nauli et al. 2003; Rydholm et al. 2010; Mathieu et al. 2014). Because it can structurally adapt to external stimuli, it is also possible that the cilium modifies itself to modulate the signal received by membrane channels, without altering the structure and composition of the plasma membrane or the ion channels themselves. Revisiting the estrogen example, the primary cilium may account for the disparity between external stimuli and channel-mediated intracellular signaling since it is responsive to estrogen (Rambo and Szego 1983), critical to the estrous cycle (Johnson et al. 2008), and is known to modify bone cell mechanosensitivity (Malone et al. 2007; Anderson et al. 2008; Kwon et al. 2010; Temiyasathit et al. 2012; Lee et al. 2014). Furthermore, intracellular cytosolic calcium influxes are observed in both micropipette aspiration experiments conducted to open mechanosensitive calcium channels and fluid flow experiments where primary cilia are mechanically stimulated (Kirber et al. 2000; Zou et al. 2002; Charras et al. 2004; Su et al. 2013; Lee et al. 2015). Thus, our lab has pursued the idea that the primary cilium functions as an adaptable strain amplifier, sensing external mechanical stimuli and tuning membrane strain to generate an appropriate intracellular signaling response.

Despite the intimacy with which the cilium and ion channels interact, the ciliary membrane is continuous with but not a continuation of the cell membrane and it is structurally distinct in a way that enhances its mechanosensing capabilities. The body of the cilium is termed the axoneme and contains nine microtubule doublets surrounded by the ciliary membrane, which fuses with the cell's plasma membrane via a periciliary membrane at the base of the axoneme (Fig. 1.1). These three membranes differ in composition and serve distinct functions. The ciliary membrane is enriched with sterols, sphingolipids, and glycolipids (Tyler et al. 2009), all of which are known to encourage specific partitioning of sensory proteins involved in cell signaling (Giusto et al. 2010; Ohanian and Ohanian 2001; Dyer and Benjamins 1990). Additionally, the ciliary membrane contains a variety of sensory proteins and channels, many of which are unique to the cilium or present in significantly larger quantities compared to the plasma membrane (Nachury et al. 2010). The periciliary membrane selectively recruits ciliary resources by targeting vesicles, which fuse with this membrane and are actively transported to the cilium (Emmer et al. 2010). The three membranes are associated such that there is a ciliary pocket near the base of the axoneme. A domain called the transition zone lies within this pocket and forms a barrier to import proteins that are trafficked specifically to the cilium. Indeed, this selective diffusion barrier effectively separates the ciliary and plasma membranes (Hunnicutt et al. 1990; Vieira et al. 2006; Pazour and Bloodgood 2008). A small portion of these proteins are inherently mechanosensitive and an additional subset presumably enhances the cilium's mechanosensing capabilities (Yoder et al. 2002; Pazour et al. 2002; Nauli et al. 2003; Kwon et al. 2010; Lee et al. 2014). The high density of sensory

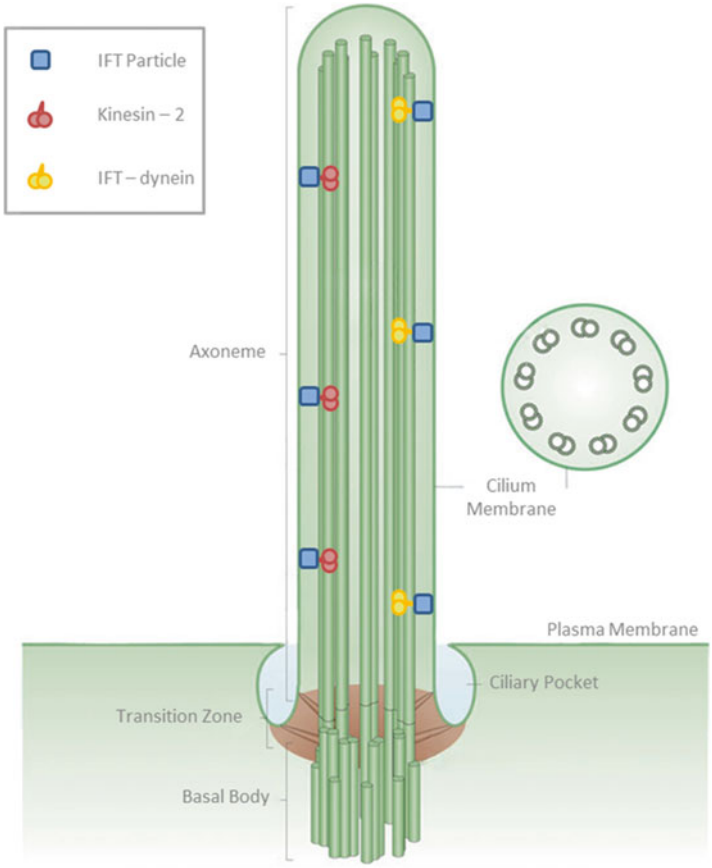

Fig. 1.1 Cartoon depicting primary cilium structure and intraflagellar transport. *Modified from* Ishikawa and Marshall 2011

proteins at the ciliary and periciliary membranes is what compositionally distinguishes the cilium from the rest of the cell, but allows for it to directly influence mechanosensing at the cell membrane.

The primary cilium requires intraflagellar transport (IFT) to maintain its structure and adapt to mechanical stimuli. Ciliogenesis, or the construction and lengthening of the axoneme, begins at the basal body, a collection of proteins that anchor the cilium to the cell (Marshall 2008). Intraflagellar transport motor proteins deliver protein complexes (IFT particles) from the ciliary base at the cell membrane to the distal tip, assembling and lengthening the axoneme (Fig. 1.1). Once the cilium is formed, motor proteins continue to supply IFT particles to maintain the presence of the cilium, which is not a temporally static structure. This phenomenon is best illustrated by cilium adjustment during the cell cycle, where it assembles around G1 or G0 phase and disassembles near mitosis in response to signaling cues (Plotnikova et al. 2009; Ishikawa and Marshall 2011; Goto et al. 2013). IFT motor proteins also

traffic deconstructed ciliary components away from the distal tip towards the ciliary base. In most instances, kinesin-2 and IFT-dynein are the motor proteins believed to drive anterograde and retrograde transport along the axoneme, respectively (Hao and Scholey 2009). Lengthening ceases as retrograde transport balances out the cargo delivered to the ciliary tip (Stephens 1997; Song and Dentler 2001) and the cilium disassembles when the rate of retrograde transport overwhelms anterograde delivery such that the quantity of IFT particles is insufficient to sustain the current length (Pan and Snell 2005; Marshall and Rosenbaum 2001). Thus, IFT transport is a bidirectional process whereby motor proteins work collectively as a feedback system to adapt ciliary length and composition (Lefebvre et al. 1995; Marshall and Rosenbaum 2001; Pan and Snell 2014). Although disparities in IFT rates are indicative of cilium length, there are multiple underlying mechanisms that influence cilium assembly and disassembly (Wren et al. 2013). For example, the outer microtubule doublets undergo posttranslational modifications, such as acetylation, that can influence cilium assembly (Thazhath et al. 2004; Pathak et al. 2007; Wloga et al. 2009). In other words, IFT does not independently dictate ciliary length but is adjusted based on cytoplasmic signaling cues (Wren et al. 2013; Pan and Snell 2014) and potentially ciliary adaptations (Besschetnova et al. 2010). The primary cilium's adaptability is crucial to its mechanosensing nature, as we will see later in the chapter.

1.2 Mechanisms of Primary Cilium-Mediated Mechanotransduction

The primary cilium is associated with a dense collection of mechanosensitive proteins and is thought to act as a sensory amplification microdomain when it deflects in response to mechanical stimulation, activating surrounding mechanosensors and transducing membrane strain into a biochemical response. The ciliary membrane, which is distinct from the cytoplasmic membrane, contains a wealth of mechanosensitive ion channels and transmembrane proteins that can be directly stimulated by strain due to ciliary bending. These densely clustered membrane proteins are trafficked from the cytosol and Golgi apparatus to the cilium via ciliary targeting sequences, which enable passage through the transition zone (Hsiao et al. 2012). In general, the cilium bends in response to a mechanical stimulus and transmits signals to the cell surface. Molecules termed second messengers then relay signals from the cell surface to the cytosol and nucleus to trigger intracellular signaling cascades. The polycystin-1/polycystin-2 (PC1/PC2) complex is a classic example of mechanosensitive proteins involved in primary cilium-mediated mechanotransduction (Nauli et al. 2003). PC1 is a large membrane protein that is believed to function as a cell surface receptor (Geng et al. 2006; Delmas et al. 2004) and PC2 is a stretch-activated calcium ion channel. Because PC1 is localized to the strain amplifying ciliary domain, changes conformation in response to mechanical stimulation, and is

coupled to a calcium ion channel, Nauli et al. hypothesized that this complex may be involved in primary cilium-mediated mechanotransduction. The authors isolated kidney cells from mice lacking *Pkd1*, the gene that encodes PC1, and found that PC2 failed to localize to the primary cilium. Furthermore, these mutant cells lacked the flow-induced calcium influx normally observed in kidney cells, although cilia formed normally (Nauli et al. 2003; Praetorius and Spring 2001). Nauli et al. therefore proposed that PC1's extensive extracellular domain detects cilium bending and acts as a stress sensor during fluid flow. The resultant change in PC1 conformation subsequently opens PC2 channels, triggering an influx of calcium ions into the cell. This finding led to the investigation of other mechanosensitive ion channels and proteins, such as Piezo1/2, transient receptor potential channels (i.e., TRPV4), and adenylyl cyclases (ACs), and further encouraged the exploration of second messengers in the biochemical aspects of ciliary mechanotransduction.

Calcium is perhaps the most ubiquitous and perplexing ciliary second messenger, due to its apparently distinct response in the cilium compared to the cytoplasm. Praetorius and Spring first identified the relationship between cilium bending and intracellular calcium release in kidney cells using fluorescence microscopy (Praetorius and Spring 2001). Cilia were bent via micropipette manipulation or fluid flow shear and increases in cellular calcium were monitored with a fluorescent calcium indicator. Regardless of how bending was initiated, a substantial increase in calcium was observed. This calcium response was lost when experiments were repeated in calcium-free media, indicating extracellular stores were responsible for the intracellular influx. The authors therefore hypothesized that an extracellular influx leads to calcium-induced intracellular calcium release and tested their assumption using thapsigargin, a chemical compound known to deplete intracellular calcium stores. In fact, the post-bending calcium response was significantly reduced in cells with diminished intracellular calcium. Treatment with gadolinium, an inhibitor of stretch-activated calcium channels, also abolished the observed calcium response. Furthermore, the authors measured membrane potential and observed a post-bending plasma membrane hyperpolarization that was consistent with activation of ion channels. Collectively, these results strongly suggest stretch-activated calcium channels are involved in mechanotransduction. Praetorius and Spring concluded that cilium bending opens calcium ion channels, causing an influx of extracellular calcium that triggers calcium release from intracellular stores and initiates signaling cascades within the cell. Liu et al. also observed coupling between extra- and intracellular calcium stores shortly after Praetorius and Spring released their findings (Liu et al. 2003). In a follow-up study, Praetorius and Spring confirmed that the cilium is solely responsible for the observed calcium response in mechanically stimulated cells (Praetorius and Spring 2003). They removed primary cilia from immortalized kidney epithelial cells using chloral hydrate treatment, a common cilia removal technique. The flow-induced calcium response was abolished in cells without cilia but regained when the cilia were allowed to recover, showing that the primary cilium is necessary for calcium signaling associated with membrane mechanotransduction.

Although existing fluorescent dyes are effective for measuring intracellular calcium release, cytoplasmic saturation tends to overwhelm local signals in subcellular compartments, such as the primary cilium. Su et al. developed a targeted genetically encoded calcium indicator (GECI) to study calcium dynamics exclusively in the primary cilium (Su et al. 2013). Their GECI contains a ciliary targeting sequence and a circularly permuted green fluorescent protein (GFP). Initially unbound, their GECI undergoes a conformational change upon binding calcium and circularizes the GFP so it fluoresces. Su et al. co-transfected cytosolic and ciliary GECIs into fibroblast-like cells in order to simultaneously detect changes in calcium within the cell and cilium, respectively. When cells were exposed to chemical stimuli, calcium increased first in the cytosol, followed by ciliary calcium influx approximately 6 s later. The authors also expressed their GECI in kidney cells and used epi-fluorescence coupled with a fluid flow system to detect an increase in ciliary calcium approximately 15 s after the initiation of flow. In both experiments the ciliary calcium flux traveled from the base to the distal tip and the authors concluded cytosolic intracellular stores were the source of ciliary calcium (Fig. 1.2). Conversely, Jin et al. observed calcium flux into the cilium first, followed by an increase in cytoplasmic calcium, using an integrated single-cell imaging technique to view pig kidney epithelial cells exposed to fluid flow (Jin et al. 2014). Intriguingly, these authors found that the method of stimulation, either mechanical or chemical, influenced the prioritization of calcium entry into the cilium or cytosol. Delling et al. have proposed that the interactions between cytosolic and ciliary calcium transfer are indeed controlled by an unidentified mechanism unknown system that determines calcium fate and is dependent on initial stimulation (Delling et al. 2013). Su et al. detected cytosolic calcium entry first in response to chemical stimulation, which may explain the discrepancy between their results and Jin et al.'s flow studies. Unfortunately, Su et al. did not measure the cytosolic calcium response when they visualized ciliary calcium influx in response to fluid flow, so a direct comparison is unavailable.

Studies conducted in our lab suggest that the observed calcium response not only varies with the method of initiation, but also differs across cell type. We recently designed a calcium indicator that exploits fluorescence resonance energy transfer (FRET) and utilized a flow system coupled with an epi-fluorescence microscope to simultaneously detect real-time changes in cytosolic and ciliary calcium flux in bone cells (Lee et al. 2015). In contrast to Su et al. and Jin et al.'s studies, calcium spikes in the cilium and cytoplasm of transfected osteocyte-like cells exposed to fluid flow occurred around the same time, with a slight majority of cells displaying ciliary influx just before a cytosolic response. We therefore hypothesized that extracellular and cytosolic stores are both potential sources of mechanically induced calcium signaling and explored them individually. To evaluate the role of intracellular calcium, we treated cells with thapsigargin. Interestingly, the ciliary and cytosolic calcium responses were both delayed, indicating intracellular stores may influence the timing of flow-induced calcium influx. In order to determine the membrane proteins involved in extracellular calcium entry, we used small interfering RNA (siRNA) to knockdown Piezo1, TRPV4, and PC2. The ciliary calcium spike was disrupted only in the TRPV4 knockdown, although all three were found to

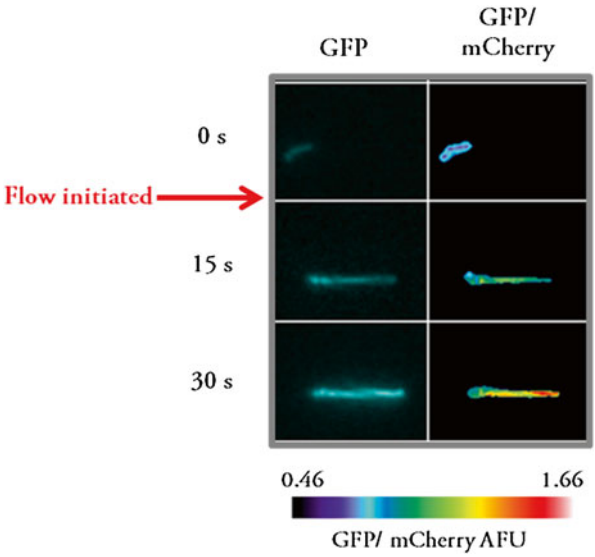

Fig. 1.2 Ciliary calcium detected with a GECI travels from the cilium base to the distal tip. Before flow is initiated, calcium is present at the base of the cilium. After 15 s of flow, a noticeable increase in calcium is detected and a maximum signal is observed after 30 s of flow. The fluorophore mCherry was used as a normalization factor for relative signal amplification of GFP. *Modified from* Su et al. 2013

localize to the cilium; however, the cytoplasmic calcium response was not affected by the TRPV4 knockdown, suggesting that it primarily affects calcium signaling within the cilium despite being present throughout the entire cell. Collectively, these data have three important implications. First, ciliary calcium influx relies on both intra- and extracellular stores and occurs either slightly before or at the same time as cytosolic influx. Second, TRPV4 is the ion channel most likely involved in primary cilium-mediated extracellular calcium entry in mechanically stimulated osteocytes, although other mechanosensitive proteins are present. Finally, kidney and bone cells appear to have distinct mechanisms for flow-induced calcium influxes, since calcium enters kidney cells via the PC1/PC2 complex. The discrepancy between timing of cytosolic and ciliary calcium peaks in the aforementioned studies likely lies within the method of stimulation and cell types used. Nevertheless, all studies have provided critical insight into the mechanism and potential sources of calcium signaling and established genetically encoded indicators as a valuable breakthrough in studying calcium interactions with the cilium.

Recently, a pathway involving another second messenger, cyclic AMP (cAMP), and its synthase, adenylyl cyclase (AC), has been proposed as a ciliary signaling mechanism. Masyuk et al. discovered that mechanically stimulated cholangiocyte primary cilia trigger intracellular signaling via both calcium and cAMP (Masyuk et al. 2006). Besschetnova et al. observed a similar phenomenon in kidney cells and both groups hypothesized that ACs, a family of proteins known to synthesize cAMP, were involved (Besschetnova et al. 2010; Masyuk et al. 2006). In 2007, our group identified primary cilia in osteoblast and osteocyte-like cell lines and confirmed they were required for osteogenic responses to fluid flow (Malone et al. 2007). We also found that intracellular calcium levels increased in response to fluid flow, similar to the calcium flux seen in kidney cells. However, when cilia were removed via chloral hydrate

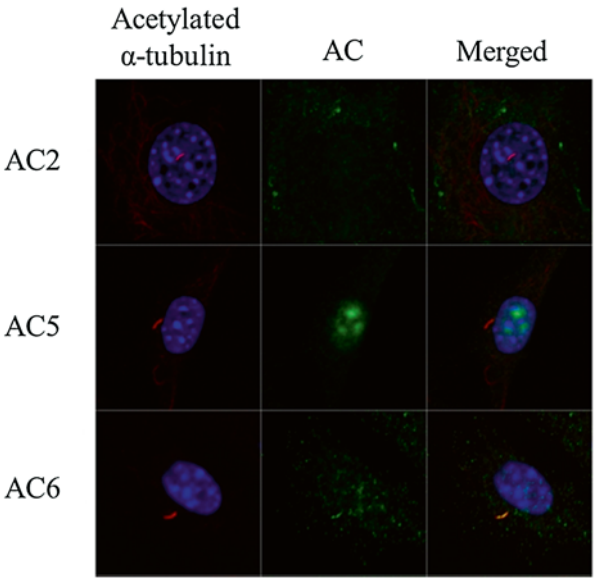

Fig. 1.3 Co-localization of adenylyl cyclase (AC) isoforms with the primary cilium, detected using immunocytochemistry with an acetylated α-tubulin antibody (*red*, nuclear stain in *blue*). Merging the cilium and AC stains indicates AC6 uniquely localizes to the cilium. Other isoforms, such as AC5 and AC2, are concentrated in the nucleus or faintly expressed in the cytoplasm, respectively. *Modified from* Kwon et al. 2010

treatment, calcium influx was maintained in stimulated bone cells although the osteogenic response was lost. Cell-to-cell interactions via gap junctions are known to facilitate cellular calcium influx, but we found that non-ciliated cells with inhibited gap junctions still displayed increased calcium levels in response to flow. This study therefore revealed that bone cell primary cilia are also capable of mediating mechanosensation independently of calcium, which was previously considered a critical component in ciliary signaling based on studies performed in kidney cells. Complementing our work with the calcium indicator, these results further suggest that the mechanisms of mechanotransduction in bone and kidney are distinct. In an attempt to address this discrepancy, we explored a calcium-independent mechanism that involves cyclic AMP and ACs (Kwon et al. 2010). Using immunocytochemistry with fluorescent indicators, we determined that adenylyl cyclase 6 (AC6) uniquely localizes to osteocyte cilia and was therefore the most likely candidate for a cAMP-mediated signaling pathway (Fig. 1.3). Interestingly, AC6 is the isoform also implicated by Besschetnova and Masyuk's groups (Besschetnova et al. 2010; Masyuk et al. 2006). Next, we exposed osteocyte-like cells to fluid flow and detected an immediate transient decrease in cAMP production as a result of mechanical stimulation. This response was lost in cells with siRNA-mediated knockdown of IFT88, a protein critical for ciliogenesis and maintenance of the axoneme, suggesting that primary cilia are necessary for the flow-induced decrease in cAMP. We similarly knocked down AC6 protein expression and found that cAMP levels were still comparable to cells that lacked cilia or weren't exposed to fluid flow. Furthermore, both knockdowns resulted in an attenuated osteogenic response, confirming that primary cilia and AC6 are critical for osteocyte mechanosensing. Finally, we treated cells with thapsigargin and gadolinium to investigate a potential connection between fluid flow-induced increase in calcium and decrease in

cAMP. cAMP levels decreased normally when intracellular calcium was depleted with thapsigargin, but did not decrease significantly when treated with gadolinium to inhibit extracellular calcium influx via stretch-activated channels. Collectively, these data suggest that the primary cilium is required for flow-induced bone cell mechanosensing and operates via a pathway mediated by AC6, cAMP, and extracellular calcium. In fact, AC6 is one of two AC isoforms that are inhibited by calcium (Hu et al. 2002; Mou et al. 2009). More specifically, calcium induces a conformational change when it binds, inactivating AC6 and halting the production of cAMP. This behavior is consistent with our observed decrease in cAMP, so it is possible that the post-flow osteogenic response in osteocytes is not entirely independent of calcium influx. Our lab is currently working to expose the relationship, if any, between calcium and AC6 as it pertains to primary cilium-mediated mechanotransduction in bone cells.

1.3 Modeling the Bending Mechanics of the Primary Cilium

In an effort to understand the effects of mechanical stimuli on ciliary mechanotransduction, models have been created to simulate the bending mechanics of primary cilia. The first model was developed by Schwartz et al. in 1997, which treated the cilium as a thin elastic cantilever beam (Schwartz et al. 1997). This approach is motivated by the beam-like deflections observed when cilia were exposed to fluid shear (Fig. 1.4). Schwartz et al. imaged kidney epithelial primary cilia bending in response to physiological fluid flow rates and characterized the fluid velocity surrounding bent cilia via polystyrene bead displacement. The deformed configurations were used to adjust model parameters in order to predict bending profiles, as well as measure the cilium's flexural rigidity. The results from this model revealed several important features that implicated the primary cilium as a mechanotransducer before it was classified as such less than a decade later (Praetorius and Spring 2001). First, Schwartz et al.'s flexural rigidity estimate of $1.4-1.6\times10^{-22}$ Nm^2 was high enough to suggest that primary cilia are not vestigial bodies, but designed to

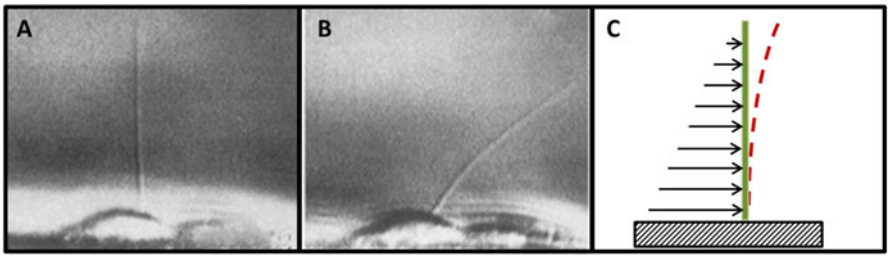

Fig. 1.4 The primary cilium (**a**) at rest, (**b**) bent when exposed to fluid shear, and (**c**) depicted as a cantilever beam in a free body diagram. In fluid flow experiments, the cilium at rest (*green*) deflects (*red*) in response to the distributed shear force (*black arrows*). *Modified from* Schwartz et al. 1997

withstand mechanical stimulation. Although this value is an order of magnitude less than that of motile cilia, they hypothesized the source of discrepancy lies within the missing central microtubules in the primary cilium's axoneme. Additionally, they found that the cilium base was firmly fixed to the cell membrane and the axoneme bent only in response to external stimuli, consistent with a mechanosensing role. Finally, the authors proposed that tension is generated at the convex face of the cilium as it bends and stretches the cell membrane, activating cytoskeletal transduction and/or stretch-activated channels in the plasma membrane. This was merely a prediction at the time, but we now know the cilium is associated with stretch-activated channels (Nauli et al. 2003; Gradilone et al. 2007). While their model did yield key findings, Schwartz et al. failed to account for large rotations they observed at the base of the cilium. A subsequent model by Liu et al. utilized a more precise fluid flow profile by solving the Stokes equation (Liu et al. 2003). Regardless of the model's limitations, the resulting insights from this experiment are profound and largely unchallenged.

More recent models have attempted to establish a link between membrane strain due to ciliary bending and subsequent intracellular calcium release, of which Rydholm et al.'s finite element model has been the most successful. Following Praetorius and Spring's experiments correlating the presence of cilia with calcium influx in kidney cells, Liu et al. built upon Schwartz et al.'s model to link the observed intracellular calcium response and the cilium's bending mechanics (Liu et al. 2003). More specifically, Liu et al. investigated whether the post-bending intracellular calcium influx was specifically mediated by the primary cilium or cell membrane stretching. The authors used their model to calculate the fluid shear force at the cell membrane and determined it was not large enough to elicit a calcium response; therefore, they hypothesized that the primary cilium functions as a flow sensor to trigger calcium release by amplifying strain in the plasma membrane to open stretch-activated channels and/or generate torque in the microtubules that anchor the cilium base to the cell membrane. In their fluid flow studies, Rydholm et al. went a step further and examined membrane stress at the cilium base and calcium release as functions of time. The authors visualized bending in real time by genetically encoding and trafficking GFP to kidney cell cilia. Interestingly, cilia bent more dramatically as fluid shear was increased except for a small region near the ciliary base, suggesting a difference in flexural rigidity and resulting stress concentration at the base of the axoneme. They also exposed cilia to different flow patterns and frequencies in order to characterize the timing of calcium release. Although the cilium bends immediately upon introduction of flow, calcium influx was not detected until approximately 20 s later. The delay in calcium release corresponded with the amount of time it took to detect a significant increase in stress at the base of the cilium. Furthermore, a calcium response was only observed in cells exposed to continuous flow longer than 30 s or several high frequency pulses, indicating that the amount of time the cilium remains bent is critical to calcium release. The authors therefore concluded that membrane stress at the ciliary base builds up in response to bending and eventually triggers the release of calcium stores when it reaches a critical level. However, there are some limitations of this study. First, the authors

modeled the ciliary-plasma membrane interface such that the cilium is directly fixed to the cell membrane. In reality, this interface involves a periciliary membrane, transition zone, and ciliary pocket. Although, the authors distinguished the ciliary membrane from the plasma membrane, they failed to consider the dense collection of ion channels and proteins concentrated at the cilium base. It is possible that the timing of their measured calcium response is influenced by the eventual activation of these mechanosensitive proteins, rather than dictated by a putative stress buildup at the ciliary base. Finally, more sophisticated tools to measure ciliary and cytosolic calcium have suggested that calcium influx occurs sooner than 20 s after flow. Despite these limitations, later studies by other groups have lent support to the notion of higher stress at the base of the cilium compared to the distal tip (Young et al. 2012; Mathieu et al. 2014; Mann et al. 2015). However, more work is required to properly correlate stress amplification at the cilium base with flow-induced calcium influx now that GECIs enable researchers to more sensitively detect ciliary and cytosolic calcium responses.

In an effort to characterize a variety of bending profiles and estimate ciliary flexural rigidity, our group built upon the aforementioned models to account for an initial contorted configuration and rotation at the base (Downs et al. 2014). We used high-speed confocal microscopy to visualize the three-dimensional bending behavior of cultured kidney cells containing cilia expressing GFP. Similar to previous authors, we observed large rotations at the base of the cilia in response to flow. Previous models were limited to cilia projecting linearly from the cell surface; however, we found that several cilia deviate from this position and may be slightly curved, angled, or lying flat along the cell surface when flow is initiated. We hypothesized that these characteristics largely influence flexural rigidity calculations and therefore proposed a large-rotation Euler–Bernoulli beam model with initial and boundary conditions that account for different starting positions and allowed for basal rotation. Our flow studies revealed bending shapes and post-flow arrangements that deviated from the characteristic deflections previously observed (Fig. 1.4b). For example, some cilia deflected linearly, likely due to more flexible anchorage at the cell body, and in others the axoneme exhibited minimal change in curvature, favoring rotation at the base. At higher shear values some cilia appeared kinked at the distal tip, suggesting a portion of the cilium may be invaginated in the cell membrane or shielded from flow via a glycocalyx barrier (Fig. 1.5b). Surprisingly, several cilia did not return to their pre-flow shape when fluid flow ceased, indicating that cilia experience plastic deformation and may adapt their mechanical properties in response to external stimuli (Fig. 1.5d). Overall, this study provided a more comprehensive depiction of cilium bending mechanics in fluid flow studies, but we have yet to explore how these different behaviors affect subsequent mechanotransduction. While the influence of specific bending profiles on mechanosensitivity remains unclear, mechanical models have provided great insight into the mechanosensing capabilities of primary cilia and have informed experimental designs.

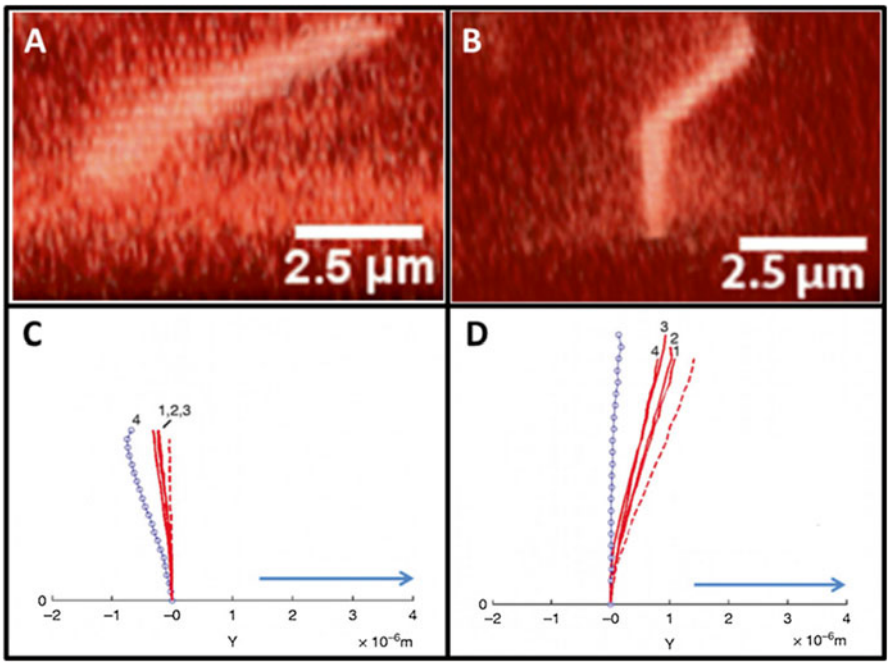

Fig. 1.5 A cilium bent similarly to those observed by Schwartz et al. (**a**) and a kinked cilium (**b**) in response to fluid shear. The numbers *1–4* indicate positions recorded at 30 s intervals after flow ceased (*solid red lines*), with *4* referring to the final position. Some cilia eventually returned to their pre-flow position (*purple line*, *circular points*) after deflecting (*red dashed line*) in response to fluid flow (*blue arrow*) (**c**). Other cilia did not return to their pre-flow position 2 min after flow ceased (**d**). *Modified from* Downs et al. 2014

1.4 Changes in Cilium Structure Alter Its Mechanosensing Capabilities

Improper anchoring to the cytoskeleton and disruptions in transport of key ciliary resources can prevent proper assembly and maintenance of the axoneme, thereby eliminating the cilium's ability to sense and transduce mechanical stimuli. By nature, mechanosensors have robust mechanical properties in order to withstand repeated mechanical stimulation. Two structural features of the cilium are critical to its mechanical integrity: proper anchoring and axoneme stability. It is no surprise that suitable anchoring is critical to mechanotransduction since the cilium bends like a cantilever beam in response to physical stimuli. The cilium is anchored to the cell via the basal body, which is tightly connected to the microtubules composing the axoneme (Fig. 1.2). Hierck et al. confirmed the importance of anchoring when they depolymerized the microtubule base of the cilium in primary embryonic cardiomyocytes and detected an attenuated response to fluid shear (Hierck et al. 2008). Conversely, they found that cells treated with agents to enhance microtubule integrity had elevated

expression of associated downstream genes and markers indicative of augmented signaling. Microtubule integrity also influences ciliogenesis, or formation and lengthening of the axoneme, whose existence is crucial to sensing the physical stimulus and potentially mediating strain amplification in the membrane. Schrøder et al. identified key microtubule proteins at the base of the cilium and noticed that microtubule anchorage to the basal body became disorganized when these proteins were knocked down. Ciliogenesis ceased in the knockdown cells, suggesting that adequate microtubule anchoring is required to guide delivery of ciliary proteins to the basal body (Schrøder et al. 2007, 2011). Perhaps the best known contributor to ciliogenesis is *Ift88*, a component of the IFT complex that is directly linked to cilia-related diseases and syndromes (Murcia et al. 2000; Pazour et al. 2000). Ciliary targeted proteins are carried along the axoneme via IFT-mediated transport upon arrival to the cilium. Cells deficient in IFT88 are unable to transport key ciliary proteins, often resulting in disassembly and improper function of the cilium, which eliminates its sensory capabilities. This IFT component is so critical that investigators commonly utilize siRNA-mediated knockdowns of IFT88 to disrupt the formation of cilia and study subsequent diminutions in mechanotransduction.

Defects in the transition zone and intraflagellar transport also impair ciliogenesis and have been directly linked to tissue-level diseases and disorders. The BBSome is a collection of proteins encoded by genes that, if mutated, result in Bardet–Biedl syndrome (BBS), a devastating genetic disorder we discuss at the end of the chapter. This protein complex localizes to the basal body, recognizes ciliary targeting sequences, and sorts ciliary proteins trafficked to the transition zone. If the BBSome fails to localize to the cilium or any protein associated with the complex is mutated, critical ciliary proteins that regulate formation and function are unable to enter the cilium (Hsiao et al. 2012). For example, Rab8, a protein crucial for vesicle formation, docking, and fusion at the ciliary membrane, cannot enter the cilium and promote axoneme extension without a functional BBSome (Nachury et al. 2007; Hsiao et al. 2009). The fact that a single mutation in any of the BBSome proteins directly causes serious health complications indicates the importance of a functional transition zone for axoneme maintenance and, subsequently, human well-being (Garcia-Gonzalo et al. 2011). In general, a significant body of cilia research is dedicated to studying the effects of structural modifications on cilium performance. These studies have established direct connections between structure and function of the cilium, highlighting the importance of microtubule anchorage, transition zone regulation, and axoneme formation and/ or maintenance in the cilium's ability to transduce mechanical stimuli.

The cilium's ability to adapt is crucial for adjusting its sensitivity to mechanical stimuli. In our cilium bending mechanics model discussed previously, we observed variation in the post-flow relaxation patterns of cilia exposed to fluid flow (Fig. 1.5) (Downs et al. 2014). We attributed this non-elastic behavior to a potential compensatory mechanism whereby the cilium actively remodels in order to decrease its sensitivity to succeeding mechanical stimuli. Consequently, we explored microtubular remodeling at the cilium base in response to flow as a possible mechanism for cilium adaptation (Espinha et al. 2014). Interestingly, kidney and bone cells exposed

to fluid shear had more microtubules at the ciliary base than cells incubated in static conditions. The number of anchoring microtubules positively correlated with increases in duration of flow and magnitude of shear, demonstrating that the cilium is capable of adjusting to a variety of flow conditions. We then inhibited cilia formation via siRNA-mediated knockdown of IFT88 and discovered the microtubular network did not change in non-ciliated cells exposed to flow. The axoneme is also thought to actively remodel in response to stimuli via common posttranslational microtubular modifications such as acetylation, phosphorylation, and polyglutamation (Westermann and Weber 2003). In fact, mechanical stimulation is known to increase microtubule acetylation, which correlates with increased binding of microtubule-associated proteins and subsequent microtubule stiffening (Geiger et al. 2009; Takemura et al. 1992; Felgner et al. 1997). Our lab recently observed that cilium bending stiffness increased in cells exposed to fluid flow compared to static controls. We then tested the role of increased acetylation in cilium stiffening and treated cells with tubacin or siRNA directed against histone deacetylase 6 (HDAC6). Both methods increase acetylation of α-tubulin by preventing catalysis of HDAC6, which deacetylates α-tubulin. Cilia were more resistant to bending in response to flow with both treatments, indicating acetylation is capable of stiffening and potentially mediating flexural rigidity of the cilium (Nguyen and Jacobs 2014a, b). Soppina et al. propose that acetylation of an α-tubulin Lysine residue (K40) induces a conformational change that enhances microtubule stability and subsequently affects ciliary bending (Soppina et al. 2012). It is important to note that calcium also influences microtubule stability since there is an influx into the cilium during mechanotransduction. O'Brien et al. noticed microtubules in solution depolymerized with increasing amounts of added calcium, so it is possible that physical stimuli could decrease axoneme stability (O'Brien et al. 1997). Indeed, Delaine-Smith et al. detected fewer and shorter cilia in osteoblast-like cells examined after 5 days of oscillatory fluid flow exposure compared to static controls (Delaine-Smith et al. 2014). Studies such as these have led investigators to believe that cilia alter their mechanical properties in order to selectively tune their sensitivity and subsequent amplification to current and successive mechanical stimuli.

Characteristics of the primary cilium, such as axoneme length, can be manipulated to recover or adjust mechanosensitivity. Cilia are capable of lengthening and shortening the axoneme due to the bidirectional nature of IFT (Marshall et al. 2005). In fact, cilium length is rarely fixed and frequently changes with respect to stage of the cell cycle and/or external stimuli. Readily available small molecules have recently become attractive candidates for modifying primary cilium length to investigate signaling mechanisms and effects in mechanosensitivity. For example, lithium, a drug typically used for treating mood disorders, is known to increase cilia length potentially by activating the adenylyl cyclase III (AC3)-cAMP pathway (Ou et al. 2009). Besschetnova et al. screened kidney and mesenchymal cells for bioactive compounds capable of blocking or activating the second messengers calcium and cAMP in hopes of adjusting cilium length (Besschetnova et al. 2010). The authors discovered cilia were shortened by small molecules that triggered intracellular calcium release or blocked cAMP production. Conversely, small

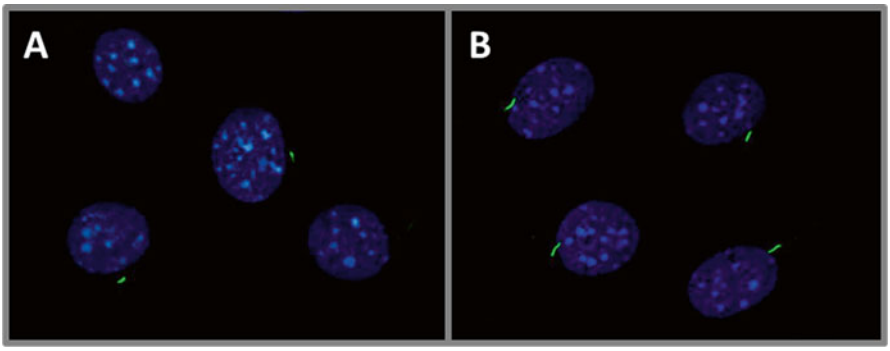

Fig. 1.6 Cells treated with fenoldopam (**b**) have longer cilia than untreated cells (**a**). *Modified from Spasic et al. 2014*

molecules that blocked calcium release or stimulated intracellular cAMP production caused the axoneme to double in length within 3 h of treatment. The authors treated cells with forskolin, which stimulates adenylyl cyclase to produce cAMP, and exposed them to fluid flow in order to correlate cilium length with mechanosensitivity. As expected, forskolin-treated cells had a significant increase in cAMP production and cilia length; however, cilia length and cAMP decreased when these cells were exposed to fluid shear. We have observed similar fluid flow-induced decreases in cAMP in osteocyte-like cells and, therefore, explored the effects of axoneme lengthening on osteocyte mechanosensitivity (Spasic and Jacobs 2014). Osteocytes treated with fenoldopam, a vasodilator prescribed for hypertension, had longer cilia and increased expression of osteogenic markers in response to fluid shear compared to controls. We then disrupted cilium length via siRNA-mediated knockdown of IFT88 to see if fenoldopam was capable of reversing cilia dysfunction. Indeed, cells lacking IFT88 treated with fenoldopam and exposed to fluid flow had longer cilia and increased osteogenic expression compared to untreated cells (Fig. 1.6). Collectively, these results indicate that increasing cilium length has the ability to not only enhance but recover mechanosensitivity in bone cells, making small molecules attractive possibilities as therapeutic treatments for cilia-related diseases.

1.5 Ciliopathies and Cilia-Based Abnormalities in Kidney and Bone

The term "ciliopathies" was created to describe a set of devastating disorders caused by defects in primary cilium function that affect a wide array of organs, especially the kidney. Ciliopathies include early embryonic death, abnormalities in embryonic patterning and symmetry, retinal degeneration, obesity and diabetes, skeletal defects, cystic diseases, and cancer (Satir et al. 2010). A variety of syndromes are

also linked to improper cilium assembly and/ or function, such as Bardet–Biedl, Joubert, and Alström syndromes (Satir et al. 2010) all of which are rare and affect numerous organs in the body. In fact, disruption of the primary cilium tends to create an assortment of simultaneous symptoms that span multiple organs since it is present in almost all mammalian cell types. For example, individuals with ciliopathies may exhibit a combination of eye abnormalities, impaired cognitive function, and/or behavioral problems in addition to health complications in the skeleton, kidney, muscle, lung, liver, bladder, and heart (Badano et al. 2006). The best known ciliopathy is polycystic kidney disease (PKD), a genetic disorder primarily characterized by fluid filled cysts formed in the kidney. Interestingly, prenatal humans with PKD tend to also have obvious skeletal abnormalities, such as polydactyly or club foot (Turco et al. 1993). Because this ciliopathy is a direct result of mutations in the genes that encode PC1 and PC2, *Pkd1* and *Pkd2*, respectively (Calvet and Grantham 2001; Stayner and Zhou 2001; Yoder et al. 2002), the role of PC1 and PC2 in primary cilium-mediated mechanotransduction has been a topic of great interest. The Oak Ridge Polycystic Kidney (ORPK) mouse model was developed in an attempt to fully investigate the pathophysiology of PKD in humans (Moyer et al. 1994). Moyer et al. introduced a transgene into an intron of the *Ift88* gene, which encodes for a key protein involved in IFT, to significantly reduce the expression of IFT88 and disrupt ciliogenesis without affecting embryonic survival. The mice subsequently developed PKD symptoms similar to those observed in humans. In fact, as more human diseases were linked to primary cilia, the ORPK mouse proved to display many more of these phenotypes in a way that closely resembled these abnormalities. The creation of this mouse model has, to a certain extent, motivated researchers to investigate primary cilium-mediated mechanotransduction in the context of kidney, although defects in ciliary mechanotransduction have devastating consequences in a variety of tissues.

Of late, investigators are utilizing novel mouse models to uncover the importance of primary cilium-mediated mechanotransduction in bone development and growth. Bone development refers to the initial patterning, lengthening, and thickening of the skeleton from embryo to adulthood. Alternatively, bone growth is a phenomenon whereby adults with fully developed skeletons are capable of growing additional bone. Animal models are designed to mimic instances where a gene of interest is mutated or lacking in a specific tissue (Hall et al. 2009). These models, termed genetic knockouts, can be lethal in the embryonic stage, so many genetic deletions are conditional, or limited to particular tissues, and/or induced at selected time points by introducing tamoxifen, an estrogen receptor antagonist that activates the knockouts. Investigators have tested the importance of cilia in a variety of tissues by disrupting *Ift88* and *Kif3a*, two genes that encode for key components of the IFT particle and a portion of the kinesin-II motor protein, respectively. Haycraft et al. cleverly used these knockouts to evaluate the role of periosteal primary cilia in embryonic skeletal development. They observed extensive polydactyly, stunted limb growth, and abnormal development of the bone collar, a section of periosteal bone vital to maintaining the shape of developing diaphyses, in mouse embryos with disrupted cilia, indicating that periosteal primary cilia are critical for proper

limb development (Haycraft et al. 2007). Periosteal cells continue to contribute to bone development through postnatal endochondral ossification, the process by which a cartilaginous shell is replaced with ossified bone to lengthen the limbs in young mice. Our lab therefore hypothesized that periosteal primary cilia are important in postnatal endochondral ossification and created a model to test the disruption of periosteal primary cilia after birth. Indeed, mutant pups treated with tamoxifen after birth had disorganized and underdeveloped growth plates, indicative of abnormal endochondral ossification (Moore et al. 2014, 2015). Embryonic and postnatal studies demonstrate that primary cilia are important in the developmental stages of bone; however, the cilium primarily operates as a chemosensor in these early stages of life. Conversely, the cilium may play a greater role as a mechanosensor in adult bone growth.

Mouse models have also been developed to study the effects of osteocyte, osteoblast, and periosteal progenitor primary cilia in mechanically stimulated adult bone growth. Osteocytes are mechanosensing cells embedded in the cortical bone and the primary cilium is a potential candidate for osteocyte cellular mechanosensing. Osteoblasts generate additional bone in response to mechanical loading and our lab postulates that osteoblastic activation is mediated by signaling from the osteocyte primary cilium. To test this hypothesis, we used an ulnar loading device to apply cyclic compression to mice forearms in order to stimulate cortical bone formation. Dynamic histomorphometry is a procedure whereby mice are injected with fluorescent dyes in order to label the mineralizing surfaces of their bones (Fig. 1.7). Mechanically induced bone growth can be quantified by injecting different colors at two separate time points and using computer software to measure standardized parameters, such as mineralizing surface, mineral apposition rate, and total bone formation (Dempster et al. 2013). We used these experimental techniques to evaluate bone growth in mice that contain osteoblasts and osteocytes lacking *Kif3a* (Temiyasathit et al. 2012). In fact, mice with disrupted osteoblast and osteocyte primary cilia grew less bone in response to mechanical loading than mice with functional cilia. We concluded that this effect is solely due to mechanotransduction, rather than defects in the bone formation per se, because the mutants and controls had comparable skeletal morphology before loading. Our lab has recently performed loading studies in mice with osteocyte primary cilia disrupted via *Ift88* deletion and also observed attenuated bone growth in mutants compared to controls (Nguyen and Jacobs 2014a, b). Interestingly, we found that functional osteoblast and osteocyte primary cilia are not necessary for normal bone development since there was no difference in the skeletal morphology of mutants and controls. Since periosteal primary cilia are critical to normal juvenile skeletal development and are recruited for fracture healing in adult mice (Kawanami et al. 2009), we investigated whether they were also involved in mechanically stimulated adult bone growth. Indeed, mutant mice injected with tamoxifen days before, during, and after mechanical loading had an attenuated response compared to mice with normal periosteal progenitor cilia, indicating that periosteal progenitor cilia are involved in generating bone at all stages of life (Chen et al. 2014). These studies show that bone cell primary cilia, regardless of the method or degree of impairment, are necessary for the

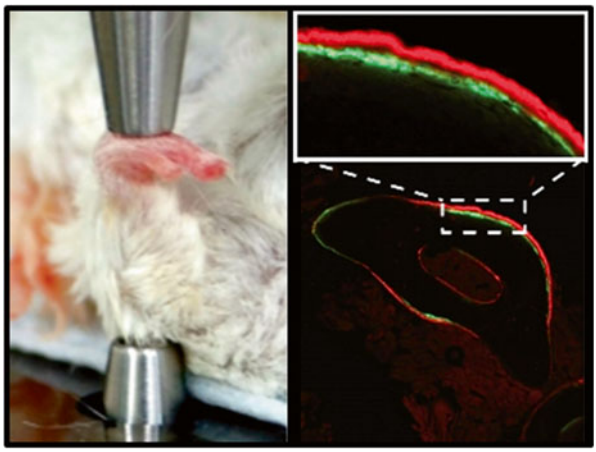

Fig. 1.7 A mouse forearm placed between the two platens of an ulnar loading device (*left*) and an example of how dynamic histomorphometry (*right*) is used to determine ulnar bone growth at the periosteal surface from 5 days (green fluorescent calcium chelator, calcein) to 12 days (red fluorescent calcium chelator, alizarin). *Modified from* Temiyasathit et al. 2012

adult skeleton to transduce mechanical stimuli into biochemical signals that enable it to adapt to loading. Interestingly, disrupting associated ciliary proteins can have a similar reduction in bone formation rate compared to completely interrupting cilia formation (Lee et al. 2014). We loaded the ulnae of mice containing a global knockout of AC6, such that periosteal progenitor, osteoblast, and osteocyte primary cilia all lack a known cilia-specific protein (Lee et al. 2014). The mutants formed significantly less bone in response to ulnar loading than mice with AC6, confirming the hypothesis from our in vitro studies that AC6 is important for bone cell primary cilium-mediated mechanotransduction and subsequent osteogenic responses (Kwon et al. 2010). Collectively, these studies are an excellent representation of the strides investigators have made to create sophisticated experimental techniques and they emphasize the value of in vivo mouse models for evaluating tissue-level effects of cellular mechanotransducers like the primary cilium. These models will be crucial for testing potential therapeutic treatments that restore primary cilium-mediated mechanotransduction and subsequent strain amplification in an attempt to offset the disastrous consequences of ciliopathies and cilia-based abnormalities.

1.6 Summary

Throughout this chapter, we have explored characteristics of the primary cilium that implicate its function as a strain amplifier for cell signaling. This solitary, extracellular organelle bends in response to mechanical stimuli and subsequently triggers intracellular biochemical signals via second messengers, such as calcium and cAMP. We saw that this cellular response is severely abrogated when cilia are rendered dysfunctional via loss of cytoskeletal anchorage, a reduction in axonemal structural integrity, disruptions in ciliogenesis, and/or depletion of key ciliary components. We also know the cilium is associated with a variety of stretch-activated

channels (PC2 and TRPV4) and proteins that facilitate mechanotransduction (PC1 and AC6) at the cell membrane. Computational models of cilium bending mechanics suggest that strain accumulates at the ciliary base, potentially stretching open these ion channels to activate intracellular signaling cascades. Interestingly, the cilium's structure and mechanical properties can be manipulated to tune its sensitivity to mechanical stimuli and resultant strain amplification in the cell membrane. This exciting discovery renders the primary cilium an attractive therapeutic target because its stiffness and/or length can be pharmacologically modified to alter cellular mechanosensitivity. The importance of the primary cilium's strain amplifying capability becomes apparent when we consider the vast array of developmental defects, ciliopathies, and diseases directly associated with this nearly ubiquitous organelle's inability to trigger cellular mechanotransduction. Fortunately, the scientific community has a wealth of experimental techniques, computational models, and mouse phenotypes available to further explore this fascinating sensory microdomain and uncover the underlying pathophysiology of a series of large-scale diseases and disorders.

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Chapter 2
Vascular Endothelial Mechanosensors in Response to Fluid Shear Stress

Li-Jing Chen, Wei-Li Wang, and Jeng-Jiann Chiu

Abstract The endothelium consists of a single layer of vascular endothelial cells (ECs) and serves as a selective barrier between the blood and arteries. ECs are constantly exposed to blood flow- and pulsatile blood pressure-induced hemodynamic forces. The cells are able to convert these mechanical stimuli into biochemical signals and then transmit the signals into the cell interior to affect cellular functions. These mechanical stimuli are detected by multiple mechanosensors in ECs that activate signaling pathways through their associated adaptor proteins, eventually leading to the maintenance of vascular homeostasis or the development of the pathogenesis of vascular disorders. These mechanosensors are distributed in different parts of the ECs, including the cell membrane, cell-to-cell junctions, the cytoplasm, and the nucleus. This review attempts to bring together recent findings on these mechanosensors and presents a conceptual framework for understanding the regulation of endothelial mechanosensors in response to hemodynamic forces. With verification by in vitro and in vivo evidence, endothelial mechanosensors have been demonstrated to contribute to health and disease by regulating physiological and pathophysiological processes in response to mechanical stimuli.

Keywords Endothelial cells • Shear stress • Mechanosensor • Mechanotransduction • Integrin

2.1 Introduction

The human body is constantly exposed to various types of mechanical forces, such as the stretching of skeletal muscle, the compression of cartilage and bone, and the hemodynamic forces on blood vessels (Butcher et al. 2009). As the monolayer is in direct contact with flowing blood, endothelial cells (ECs) are constantly exposed to

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blood flow- and pulsatile blood pressure-induced hemodynamic forces. These hemodynamic forces include shear stress and cyclic stretch. Fluid shear stress is the frictional force per *unit* area from flowing blood and acts on the ECs present on the luminal surface of the vessel (Chien 2007). Cyclic stretch arises due to the blood pressure and causes circumferential stretching of the vessel wall that affects both the ECs and the smooth muscle cells (SMCs) that surround the endothelium in the arteries. An increasing number of studies have indicated that hemodynamic forces regulate EC functions and vascular physiology and pathophysiology, thereby contributing to health and disease (Davies 2009; Hahn and Schwartz 2009; Li et al. 2005).

To accomplish these hemodynamic force-induced physiological and pathophysiological modulations, ECs must be able to initially sense these mechanical stimuli using mechanosensors. These mechanosensors then translate mechanical input into biochemical output that is transmitted into the interior of the cell, thereby initiating mechanoresponsive signaling pathways. These processes are known as mechanosensing and mechanotransduction (Fedorchak et al. 2014; Jaalouk and Lammerding 2009). Mechanosensors are defined as proteins or molecules (part of the cellular structure) that can receive the mechanical stimuli, translate these stimuli into a biochemical signal, and then transmit the signal into the interior of the cell. Generally, mechanosensing is initially dependent on the capacity of mechanical stimuli to induce conformational changes in the mechanosensor that lead to alterations of the association of the mechanosensor with its partner proteins or changes in the activity of these proteins.

Many different cellular proteins have been proposed to function as mechanosensors of hemodynamic forces in ECs. These mechanosensors are primarily located at the plasma membrane and include ion channels, G proteins and G protein-coupled receptors (GPCRs) (Gudi et al. 1996), endothelial glycocalyx (Florian et al. 2003), primary cilia (Pazour and Witman 2003), caveolae (Park et al. 2000; Yu et al. 2006), cell matrix receptors such as integrins (Tzima et al. 2001; Li et al. 1997), cell–cell adhesion junction proteins (Tzima et al. 2005), receptor tyrosine kinases (RTKs) (Jin et al. 2003; Shay-Salit et al. 2002), and bone morphogenetic protein receptors (BMPRs) (Zhou et al. 2012; 2013). The cytoskeleton and the nucleus were also reported to be mechanosensors in recent studies (Fedorchak et al. 2014; Helmke and Davies 2002; Tkachenko et al. 2013). In response to hemodynamic stimuli, these mechanosensors activate upstream signaling molecules through their associated adaptor proteins and mediate intracellular signaling through phosphorylation cascades, eventually leading to morphological and functional changes that maintain homeostasis. These changes include the regulation of gene expression, differentiation, proliferation, angiogenesis, and migration. Vascular cell dysfunction due to the impairment of these changes may lead to a pathophysiological state that contributes to the development of vascular disorders, such as atherosclerosis and hypertension (Hahn and Schwartz 2009).

This chapter provides an introduction to hemodynamic force-specific mechanosensors in ECs. We also provide in vitro and in vivo evidence of the importance of mechanosensors for the regulation of endothelial functions. In conclusion, we propose that these mechanosensors play initial and necessary roles in the hemodynamic force-modulated vascular biology and pathophysiology that contribute to health and disease states.

2.2 Membrane Molecules

2.2.1 Ion Channels

Although our understanding of shear stress-triggered endothelial signaling pathways has greatly increased over the past two decades, the mechanisms by which ECs sense shear stress remain largely unknown. Activation of shear stress-sensitive ion channels is among the fastest known endothelial responses to shear stress; hence, ion channels have been proposed to serve as endothelial mechanosensors. Ion channels are pore-forming membrane proteins whose functions include establishing a resting membrane potential and shaping action potentials and other electrical signals by gating the flow of ions across cell membranes (Doyle 2004). ECs express a bewildering variety of ion channels that promote the activation of ion fluxes. Among these ion fluxes, the transport of calcium (Ca^{2+}) across the cell membrane is involved in the early cellular response to shear stress. Ca^{2+} has been proposed to play a key role in signal transduction events, and an increase in the Ca^{2+} level stimulates the Ca^{2+}-dependent synthesis of vasodilators, such as nitric oxide (NO) and prostacyclin (Kuchan and Frangos 1994; Rubanyi et al. 1986; Falcone et al. 1993). In addition to Ca^{2+}, the flows of potassium (K^+), chloride (Cl^-), and sodium (Na^+) ions are rapidly elicited in ECs in response to shear stress (Nilius and Droogmans 2001). These shear stress-activated ion channels play a central role in the regulation of endothelial mechanoresponsive events (Gautam et al. 2006a).

2.2.1.1 Ca^{2+} Channel

In human ECs, the cation channel has been reported to be activated by shear stress, and Ca^{2+} is more permeable than other cations (Schwarz et al. 1992). In mammals, the gene encoding the Ca^{2+}-permeable cation channel has been identified as a *Drosophila* homologue of the transient receptor potential (TRP) gene. Several members of the TRP superfamily are expressed in humans and other species (Nilius et al. 2003). The TRP protein consists of six transmembrane segments, all of which constitute cation channels. TRP channels can be grouped into 7 subfamilies, including TRPC (C for canonical), TRPM (M for melastatin), TRPV (V for vanilloid), TRPA (A for ankyrin), TRPP (P for polycystic kidney disease), TRPML (ML for mucolipin), and TRPN (N for no mechanoreceptor potential C) (Pedersen et al. 2005). Functional TRP channels are tetrameric complexes formed by the heteromerization of TRP subunits crossing different TRP subfamilies. A variety of TRPs have been identified and demonstrated to serve as mechanosensors in ECs in response to shear stress. TRPP1 and TRPP2 are also known as polycystin-1 and polycystin-2. The C-terminal domain of TRPP1 can interact with the C-terminal domain of TRPP2, and this complex serves as a mechanosensor to activate the Ca^{2+} influx induced by shear stress (Nauli et al. 2008). Both TRPP1 and TRPP2 are localized to endothelial primary cilia and are required for mechanotransduction (Nauli et al. 2003;

AbouAlaiwi et al. 2009). Moreover, TRPP2 depletion results in the loss of shear stress-induced NO production in ECs. This finding suggests that TRPP1 serves as a mechanosensor to transduce the mechanical force to TRPP2, which allows the Ca^{2+} influx and leads to the activation of intracellular signaling pathways. TRPV4 (also named VR-OAC, VRL-2, OTRPC4, and TRP12) has moderately high Ca^{2+} permeability in ECs (Watanabe et al. 2002). Activation of TRPV4 by 4αPDD, the selective TRPV4 opener, increased Ca^{2+} entry into ECs and caused vasodilatation of the carotid artery in rats following intraluminal application of 4αPDD. Similar to 4αPDD, a shear stress of 3 dynes/cm^2 elicited vasodilation that could be blocked by a TRPV channel blocker (ruthenium red), a Ca^{2+} chelator (BAPTA-AM), and a NO synthase blocker (Nω-nitro-L-arginine). These results suggest that TRPV4 is activated by shear stress, causing Ca^{2+} entry and triggering NO-dependent vasodilatation (Kohler et al. 2006). Indeed, shear stress-induced vasodilation was lost in TRPV4-knockout mice (Hartmannsgruber et al. 2007). Du et al. demonstrated that TRPV4, TRPC1, and TRPP2 formed a heteromeric channel in rat mesenteric artery endothelial cells and that this heteromeric channel could induce vascular relaxation (Du et al. 2014). Pore-dead mutants for each of the TRP isoforms reduced the shear stress-induced Ca^{2+} current. These results suggest that TRP channels can assemble into heteromeric complexes that induce Ca^{2+} influx, resulting in vasodilation in response to shear stress. In addition to the TRP channels, TRPC3 and TRPM7 are expressed in ECs and are activated by shear stress. TRPC3 is activated by agonist-induced activation of plasma membrane GPCRs, synthetic diacylglycerols, and depletion of intracellular Ca^{2+} stores in some cell types (Birnbaumer et al. 1996; Montell 2001; Trebak et al. 2003). Knockdown of TRPC3 reduced Ca^{2+} influx and vasodilation in response to shear stress (Liu et al. 2006). TRMP7 is widely expressed in vascular SMCs and ECs (Runnels et al. 2001). The effect of TRPM7, which is heterologously expressed in human embryonic kidney (HEK) 293 cells, can be augmented by shear stress (Numata et al. 2007). Laminar shear stress also increases the plasma membrane translocation of TRPM7 to amplify its current amplitude (Oancea et al. 2006). In addition to the TRP channels, the P2X receptors are membrane ion channels that allow extracellular Ca^{2+} to enter and activate intracellular signaling pathways to evoke a variety of cellular responses (North 2002). Several P2X subtypes (P2X1-7) have been identified. P2X4 is the most abundantly expressed subtype of the P2X receptor in vascular ECs and is the major contributor to the shear stress-induced Ca^{2+} influx (Glass and Burnstock 2001; Ray et al. 2002; Yamamoto et al. 2000a, b). P2X4-deficient mice exhibit abnormal Ca^{2+} influx and NO production and lose their vasodilation capacity in response to shear stress (Yamamoto et al. 2006).

2.2.1.2 K^+ Channel

Inward-rectifying K^+ (Kir) channels were the first type of shear stress-activated ion channel reported in ECs and were identified using whole-cell patch-clamp recordings (Olesen et al. 1988). The Kirs are represented by the Kir2.1 family cloned from bovine aortic ECs (BAECs) (Forsyth et al. 1997). Overexpression of the Kir2.1

channels in either Xenopus oocytes or mammalian HEK293 cells results in a large shear stress-activated K^+ current in these cells (Hoger et al. 2002). In addition to the Kir2.1 channels, ATP-activated Kir6.2 currents were increased in rat pulmonary microvascular ECs and bovine pulmonary artery cells subjected to a shear stress of 10 dynes/cm^2 (Chatterjee et al. 2003). The role of Ca^{2+}-activated potassium channels (IK_{Ca}) in the shear stress-induced K^+ current in ECs has been studied. Application of a shear stress of 5 or 15 dynes/cm^2 upregulated IK_{Ca} expression, resulting in the enhancement of the whole cell K^+ current and increased membrane hyperpolarization (Brakemeier et al. 2003). Intraluminal administration of iberiotoxin, an inhibitor of high conductance K_{Ca} channels (BK_{Ca}), eliminated the shear stress-induced dilations of the arterials (Sun et al. 2001).

2.2.1.3 Cl^- Channels

Whole-cell patch-clamp recordings and measurements from fluorescent potentiometric dyes have demonstrated the presence of shear stress-sensitive outward-rectifying Cl^- channels in ECs (Barakat et al. 1999). Activation of outward-rectifying Cl^- channels by shear stress leads to membrane depolarization after membrane hyperpolarization due to the activation of shear stress-sensitive K^+ channels. K^+ and Cl^- channels can both immediately sense shear stress. However, the net electrochemical driving forces acting on Cl^- channels is larger than the forces acting on K^+ channels, and the dynamics of Cl^- current activation are slower than those of the K^+ current. This finding suggests that shear stress-sensitive Cl^- channels attain maximal activation slower than shear stress-sensitive K^+ channels. Lieu et al. demonstrated that a shear stress-induced hyperpolarizing current was carried in part by K^+, whereas the depolarizing current was carried in part by Cl^- (Lieu et al. 2004). A laminar shear stress of 1–10 dyns/cm^2 was able to active the hyperpolarizing current; however, the depolarizing current was less responsive to 1 dyns/cm^2 compared to 10 dyns/cm^2. These results indicate that shear stress-sensitive K^+ channels can respond to a wider range of shear stress for activation than Cl^- channels, which may be involved in sensing shear stress changes at a high shear stress of 10 dyns/cm^2. In addition to the magnitude of the shear stress, K^+ channels can sense both laminar and oscillatory flows, whereas Cl^- channels are only activated by laminar shear stress (Lieu et al. 2004). Previous studies demonstrated that laminar shear stress induced anti-inflammatory gene expression, whereas the oscillatory flow was associated with atherosclerotic development. These results imply that the shear stress-induced activation of the Cl^- channel may play an important role in the atheroprotective property of the endothelium (Gautam et al. 2006b). Human aortic ECs exposed to a stream of fluid through a pipette exhibit an increase in their intracellular calcium concentration ($[Ca^{2+}]_i$) that is caused by both the magnitude of the shear stress and the extracellular Ca^{2+} concentration (Nakao et al. 1999; Jow and Numann 1999). This finding suggests that the activation of the Cl^- current sufficiently changes the cell membrane potential to modulate the Ca^{2+} influx. However, the detailed mechanism underlying the shear stress induction of the Cl^- channel

remains unclear. The volume-regulated anion current (VRAC) is responsible for the regulation of osmolarity in a cell. Although shear stress does not induce VRAC currents in BAECs, it potentiates these currents in the presence of an osmotic challenge (Romanenko et al. 2002).

2.2.1.4 Na^+ Channels

Shear stress-increased Na^+ permeability has been reported in rat cardiac microvascular ECs (Moccia et al. 2000). Epithelial Na^+ channels (ENaCs) consist of three basic subunits: α, β, and γ. The α subunit of ENaCs forms the pore structure that allows Na^+ to permeate, whereas the β and γ subunits can interact with most of the ENaC regulators (Canessa et al. 1994). The α subunit of ENaCs is expressed in cultured human dermal microvascular ECs, human umbilical cord ECs (HUVEC), and rat ECs. Shear stress increased the open probability of ENaCs in ECs (Wang et al. 2009). There is evidence that shear stress-sensitive cation channels in HUVECs are permeable to not only Ca^{2+} but also Na^+ (Schwarz et al. 1992).

2.2.2 G Proteins and GPCRs

G proteins are known as guanine nucleotide-binding proteins. The heterotrimeric G proteins, which consist of α, β, and γ subunits, transduce signals from activated GPCRs into intercellular signaling molecules. The α subunit is a GTPase switch protein that alternates between an active status with bound GTP and an inactive status with bound GDP (Neves et al. 2002). G proteins are activated by shear stress and act as mediators of shear stress-induced EC responses, such as Ras activation and NO production (Ohno et al. 1993; Kuchan et al. 1994; Gudi et al. 2003). The G proteins are the one of the earliest known shear stress-induced cellular responses. Gudi and Frangos showed that G proteins were rapidly activated within 1 s in ECs subjected to shear stress. By immunoprecipitating AAGTP-labeled proteins with polyclonal anti-G_α antibodies, the G-protein subunits $G_{\alpha q/\alpha 11}$ and $G_{\alpha i3/\alpha o}$ were identified as activated by shear stress in a shear dose-dependent manner (Gudi et al. 1996, 1998). This shear stress-induced activation of G proteins was modulated by a shear stress-induced increase in membrane fluidity that was independent of cytoskeleton and cytosolic components (Gudi et al. 1998). Using time-resolved fluorescence microscopy and GPCR conformation-sensitive fluorescence resonance energy transfer (FRET) analysis, bradykinin B2 GPCR (BKRK2) was demonstrated to induce the conformational changes that led to the activation by shear stress at physiological shear stress values (≅15 dynes/cm^2). Conversely, a B2-selective antagonist blocked the shear stress-induced activation of BKRK2 (Chachisvilis et al. 2006). These specific features, including the cellular location, rapid activation, and force discrimination, strongly indicate that G proteins and GPCRs serve as primary mechanosensors of shear stress. Furthermore, recent studies demonstrated that BKRK2,

the G-protein subunits $G_{\alpha q/11}$, and PECAM-1 form a mechanosensitive complex in ECs in response to shear stress (Otte et al. 2009; Yeh et al. 2008; dela Paz et al. 2014). In primary human ECs, the oscillatory flow induced the rapid dissociation of the $G_{\alpha q/11}$–PECAM-1 complex within 30 s, and $G_{\alpha q/11}$ localized in the perinuclear region within 150 min. In a mouse study, co-localization of $G_{\alpha q/11}$ and PECAM-1 at the cell–cell junction in the atheroprotective areas of the mouse aorta was observed by immunohistochemical staining. In contrast, $G_{\alpha q/11}$ was absent from the junctions in atheroprone areas (Otte et al. 2009). These results indicate that the $G_{\alpha q/11}$–PECAM-1 complex may be involved in the regulation of atherosclerosis.

2.2.3 Endothelial Glycocalyx

The endothelial glycocalyx is a gel-like thin layer covering the luminal surface of ECs that interacts directly with hemodynamic forces (Fu and Tarbell 2013). The endothelial glycocalyx is 0.4–4 μm thick based on in vivo measurements; however, Ebong et al. used rapid freezing/freeze substitution transmission electron microscopy to show that the thickness of the endothelial glycocalyx in cultured ECs was 11 μm (Fu and Tarbell 2013; Ebong et al. 2011). These inconsistent results indicate that the integrity of the endothelial glycocalyx may be different following exposure to different factors, such as the different types of cells used in in vivo and in vitro studies or the measurement techniques employed. The endothelial glycocalyx can directly bind or selectively block some biomolecules to regulate the EC barrier function (Bernfield et al. 1999). Additionally, the endothelial glycocalyx has important functions in leukocyte recruitment and the inflammatory response (Mulivor and Lipowsky 2009). The endothelial glycocalyx is composed of proteoglycans, glycosaminoglycans (GAGs), glycoproteins, and plasma proteins (Weinbaum et al. 2007). The three major GAGs [heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronic acid (HA)] are found in the endothelial glycocalyx. HS is the most abundant, accounting for 50–90 % of the total GAGs. The transmembrane syndecans, membrane-bound glypicans, and basement matrix-associated perlecans are the three major protein core families of the heparan sulfate proteoglycans (HSPGs) found on ECs. The series of studies by J. Tarbell indicated that the endothelial glycocalyx acted as a mechanosensor. The abolishment of the endothelial glycocalyx by heparinase III impaired the shear stress-induced organization of the cytoskeleton and NO production (Florian et al. 2003; Thi et al. 2004). Several studies demonstrated that following the inhibition of the endothelial glycocalyx by heparinase III, ECs not only lost the capacity to sense shear stress and to modulate cell motility but also exhibited an increased proliferation rate in response to shear stress (Yao et al. 2007; Moon et al. 2005).

HS and associated proteoglycans have been extensively studied and have demonstrated their capacity to function as signal transduction molecules. The syndecans, including syndecan-1, -2, and -4, have three GAG attachment sites close to their N-termini and distal to their apical surfaces. Their cytoplasmic tails associate with

the cytoskeleton and a signaling protein, such as protein kinase C (PKC)-α or phosphatidylinositol-4,5-biphosphate (PIP2) (Florian et al. 2003; Weinbaum et al. 2007; Tarbell and Pahakis 2006). Koo et al. demonstrated that an atheroprotective flow increased the expression of the endothelial glycocalyx and syndecan-1 on the apical surface of ECs; however, this effect was decreased after EC exposure to the atheroprone flow. Silencing of syndecan-1 inhibited the shear stress-induced expression of the endothelial glycocalyx (Koo et al. 2013). Knockout of syndecan-1 abolished the shear stress-induced phosphorylation of Akt and paxillin and, in turn, the activation of integrin $\alpha_v\beta_3$ and gene expression, including Kruppel-like factors (KLFs)-2, -4, and -5, endothelial nitric oxide synthase (eNOS), and angiopoietin-2 (Voyvodic et al. 2014). Deletion of syndecan-4 in ECs inhibited the shear stress-induced alignment with the flow direction. Syndecan-4 knockdown activated the pro-inflammatory response and decreased atheroprotective flow-induced anti-inflammatory gene expression (i.e., KLF-2 and KLF-4), resulting in a significant increase in atherosclerotic lesions in normally resistant locations, such as the thoracic region (Baeyens et al. 2014). These results suggest that the syndecans play key roles in endothelial mechanosensing and are involved in hemodynamic force-modulated vascular pathophysiology.

2.2.4 Caveolae

Caveolae are cholesterol- and glycosphingolipid-rich, 50- to 100-nm vesicular invaginations of the plasma membrane that are found in many types of vascular cells, including ECs, SMCs, fibroblasts, and macrophages. Caveolae serve as transmembrane signaling microdomains for the transport of large and small molecules, such as the transcytosis of macromolecules and potocytosis of ions and folate. The chief structural proteins of caveolae are caveolins (Sowa 2012; Okamoto et al. 1998; Hansen and Nichols 2010). Three distinct caveolins have been identified: caveolin-1 (Cav-1), caveolin-2 (Cav-2), and caveolin-3 (Cav-3). Cav-1 and Cav-2 are the most abundantly expressed caveolins in ECs and fibroblasts, whereas Cav-3 expression is muscle-specific (Parton 1996). Caveolin is a 21- to 24-kDa membrane protein that binds directly to cholesterol and forms a scaffold. Many classes of signaling molecules can assemble on this scaffold to generate preassembled signaling complexes, resulting in the concentration of these signal molecules within a distinct region of the plasma membrane (Okamoto et al. 1998). These caveolin-interacting signaling molecules include the G protein α-subunit, Ras, Src family tyrosine kinases, eNOS, receptor tyrosine kinases, and PKC proteins. Caveolae have been demonstrated to be involved in shear stress-induced endothelial activation (Park et al. 2000; Yu et al. 2006; Fujioka et al. 2000; Sun et al. 2002; Radel and Rizzo 2005). Using recombinant glutathione S-transferase fusion proteins containing the epitopes of anti-cav-1 caveolin antibodies, Park et al. demonstrated that the scaffolding/oligomerization domain of cav-1 was critical for the regulation of the mechanosensitive activation of the extracellular signal-regulated kinase (ERK) (Park et al. 2000). Blocking Cav-1

resulted in the inhibition of the shear stress-induced activation of ERK. Shear stress induces an increase in Cav-1 density and the tyrosine phosphorylation of Cav-1 at the plasma membrane, along with its translocation from the Golgi to the plasma membrane (Fujioka et al. 2000; Sun et al. 2002). Moreover, the shear stress-induced translocation and tyrosine phosphorylation of Cav-1 is regulated by the shear stress-activated β1-integrin C-Src kinase (Csk) and the Src family kinases. Treatment with the β1-integrin-blocking antibody JB1A, a type 1 protein phosphatase, or Csk knockdown leads to the inhibition of the shear stress-induced Cav-1 phosphorylation in ECs. Immunoprecipitation and immunostaining revealed that Csk interacted with phosphorylated Cav-1 and integrins. These results suggest the detailed mechanism underlying the response of the β1-integrin-Cav-1-Csk complex to shear stress (Radel and Rizzo 2005). An in vivo study further supported the importance of Cav-1 in shear stress-induced arterial responses. Yu et al. demonstrated that the loss of Cav-1 impaired shear stress-induced vasodilation and eNOS activation and resulted in medial thickening. Cav-1 overexpression rescued these effects in Cav-1-knockout mice (Yu et al. 2006). This direct evidence indicates that Cav-1 may serve as a mechanosensor or mechanotransducer in the arterial response to shear stress.

2.2.5 RTKs

Shear stress rapidly activates several tyrosine kinases, including the Src family (Okuda et al. 1999), focal adhesion kinase (FAK) (Li et al. 1997), proline-rich tyrosine kinase (PyK2) (Tai et al. 2002), and vascular endothelial growth factor receptor-2 (VEGFR-2, also known as Flk-1/KDR) (Chen et al. 1999). VEGFR-2 can act as a mechanosensor in cell-to-cell junctions (Tzima et al. 2005; Shay-Salit et al. 2002; Osawa et al. 2002). Shear stress rapidly induces VEGFR-2 oligomerization, tyrosine phosphorylation, and association with different adaptor proteins, resulting in the transduction of signals and the activation of cellular functions. Several studies showed that shear stress transiently induced tyrosine phosphorylation of VEGFR-2, VEGFR-2-Shc association, or VEGFR-2-casitas B-lineage lymphoma (CDl) association, leading to the activation of ERK, c-Jun N-terminal kinases (JNK), or IkB kinase (IKK), respectively (Chen et al. 1999; Wang et al. 2004). This shear stress-induced activation of VEGFR-2 was not affected by treatment with an anti-VEGF antibody, suggesting that the shear stress-induced activation of VEGFR-2 was independent of its ligand. Jin et al. reported that shear stress induced the ligand-independent activation of VEGFR-2 and caused the recruitment of phosphatidylinositol 3-kinase (PI3K) and consequent Akt activation and NO production (Jin et al. 2003). Wang et al. showed that shear stress induced the activation of integrins to transactivate the association of VEGFR-2 and Cbl, leading to the tyrosine phosphorylation of Cbl and creating potential docking sites for downstream signaling molecules. These results indicate that integrin interacts with VEGFR-2 in response to shear stress (Wang et al. 2002).

2.2.6 BMPRs

BMPRs are a family of transmembrane serine/threonine kinases, including the type I receptors BMPR1A and BMPR1B and the type II receptor BMPR2, both of which are required for Smad signal transduction and transcriptional activity (Miyazono et al. 2005). In the canonical bone morphogenetic protein (BMP) pathway, the type I and II BMPRs do not associate, and Smad remains in the cytoplasm in the absence of BMP binding. Upon BMP binding, the type I and II BMPRs associate into dimers, leading to the phosphorylation of BMPR type I by BMPR type II. This phosphorylation of BMPR type I results in the phosphorylation of downstream Smads and their subsequent translocation into the nucleus. Different combinations of type I and II BMPRs are expressed in ECs, and their responses to the binding of various BMPs have been studied. For example, BMP2/4 induce the activation of BMPR1A or BMPR1B and BMPR2, leading to Smad1/5/8 phosphorylation and the regulation of cell proliferation, inflammation, and angiogenesis (Dyer et al. 2014). Ankeny et al. showed that Smad1/5 were highly activated in the calcified fibrosa endothelia of human aortic valves, which experience disturbed flow (Ankeny et al. 2011). These results suggest that Smad molecules may be regulated by oscillatory flow and contribute to shear stress-associated vascular disorders. Using a combination of in vitro/in vivo studies and clinical specimens, Zhou et al. demonstrated that BMPRs served as mechanosensors and that BMPR-specific Smad1/5 acted as important mechanosensitive molecules for vascular EC cycle progression in response to oscillatory flow (Zhou et al. 2012, 2013). This oscillatory flow induced the activation of BMPRII through its intracytoplasmic kinase domain to induce the BMPR1B-integrin $\alpha_v\beta_3$ association, resulting in Smad 1/5 phosphorylation and Smad1/5-Runx2 association (Zhou et al. 2013). This oscillatory flow-induced sustained activation of BMPR-specific Smad1/5 was mediated by the Shc/FAK/ERK pathway. This activation led to the activation of Runx2, mTOR, and p70S6K and the subsequent upregulation of cyclin A and the downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ and, hence, EC cell cycle progression (Fig. 2.1a). Pretreating these ECs with the BMP ligand inhibitor Noggin and BMP2/4-specific siRNAs could not abolish the oscillatory flow-induced activation of Smad1/5, indicating that the oscillatory flow-induced activation of BMPR-specific Smad1/5 was BMP ligand independent. The study of experimentally stenosed abdominal aortae in rats further substantiated that the activation of BMPR-specific Smad1/5 and cell cycle progression in vascular ECs in response to the oscillatory flow occurred in a manner similar to that observed in vitro. The force specificity of BMPR-specific Smad1/5 activation was induced in the EC layer of post-stenotic sites that experienced disturbed flow (Fig. 2.1b). However, Smad1/5 activation was not inhibited by intra-arterial injection of Noggin into the stenosed rat abdominal aortae. BrdU-positive cells were highly prevalent in the region of BMPR-specific Smad1/5 activation, and the percentage of BrdU-positive cells was reduced by a lentiviral Smad5-specific shRNA. These data suggest that BMPR-specific Smad1/5 may serve as promising hemodynamic-based targets for therapeutic intervention against EC dysfunction-associated vascular disorders, such as atherosclerosis.

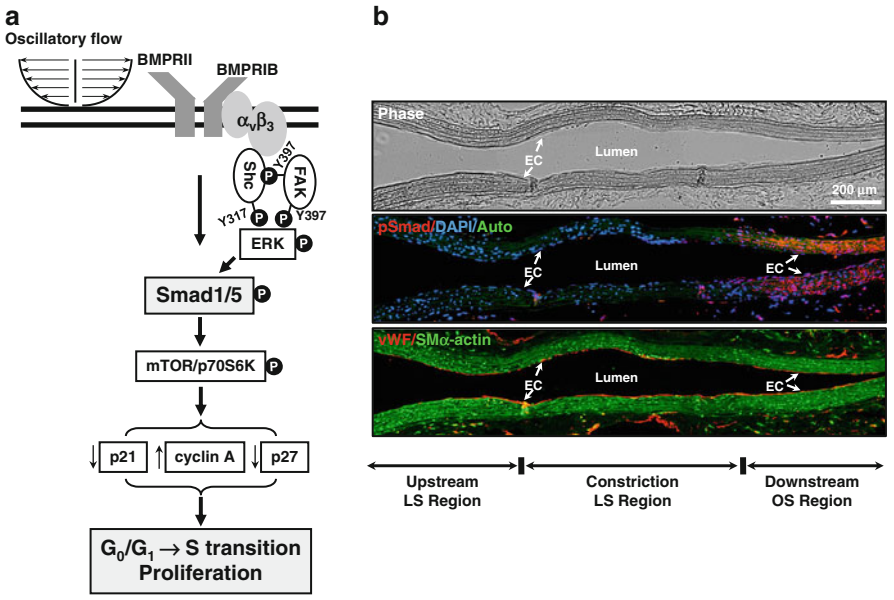

Fig. 2.1 Oscillatory flow induces EC cycle progression through the activation of BMPR-specific Smad1/5 in a BMP-independent manner. (**a**) Oscillatory flow induces BMPRII activation through its intracytoplasmic kinase domain to induce the BMPR1B-integrin $\alpha_v\beta_3$ association, resulting in Smad1/5 phosphorylation and its association with Runx2. Smad1/5 activation is mediated by the Shc/FAK/ERK pathway, leading to Runx2, mTOR, and p70S6K activation and the consequential upregulation of cyclin A and downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ in ECs. (**b**) The panoramic examination of Smad1/5 phosphorylation levels in stenosed rat abdominal aortae from the upstream through midpoint to downstream of the constriction. Auto, autofluorescence of the vessel wall. Pictures modified from Zhou et al. (2012, 2013)

2.3 Cell-to-Cell Junctions

Endothelial junctions are formed by tight junctions, vascular endothelial cadherin (VE-cadherin), and their intracellular components (Dejana 2004). ECs also express the platelet endothelial cell adhesion molecule (PECAM), which promotes hemophilic adhesion at sites of intercellular contact. The mechanosensory complex of cell-to-cell junctions is composed of PECAM-1, VE-cadherin, and VEGFR-2 (Tzima et al. 2005). This mechanosensory complex is necessary for the activation of shear stress-induced signal pathways.

2.3.1 PECAM-1

PECAM-1 (also known as CD31) is a glycoprotein with six Ig-like loops. It is a transmembrane protein with a short cytoplasmic domain that is expressed by ECs, platelets, and leukocytes (Newman et al. 1990). In EC monolayers, PECAM-1 is

located at intercellular junctions and utilizes hemophilic binding to neighboring ECs to form calcium-independent cell-to-cell adhesions (Ayalon et al. 1994). The cytoplasmic tail of PECAM-1 contains two tyrosine residues (Y663 and Y686), each of which is located in an immunoreceptor tyrosine-based inhibitory motif. These residues can be phosphorylated by Src family kinases, leading to binding to SH2-domain-containing protein tyrosine phosphatase-2 (SHP-2) domains (Jackson et al. 1997). PECAM-1 has been demonstrated to serve as a mechanosensor in ECs exposed to shear stress (Tzima et al. 2005; Osawa et al. 2002; Chiu et al. 2008). Shear stress induces the rapid phosphorylation of PECAM-1, resulting in SHP-2 binding, and mediates nuclear factor (NF)-kB and ERK/mitogen-activated protein kinase (MAPK) activation (Tzima et al. 2005; Osawa et al. 2002). Knockdown of PECAM-1 reduced the extent of lesions at the aortic arch and the aortic sinus, suggesting that PECAM-1 played an important role in the regulation of atherosclerotic progression (Goel et al. 2008; Stevens et al. 2008).

2.3.2 VE-Cadherin

VE-cadherin is an endothelial cell-specific main adhesive protein that is critical for the control of vascular permeability. VE-cadherin is mediated by transmembrane proteins that promote the homophilic interaction with neighboring ECs and form calcium-dependent cell-to-cell adhesion sites (Dejana et al. 1995). In the cytoplasmic domain, VE-cadherin forms complexes with α- and β-catenin, plakoglobin, vinculin, and cingulin that directly or indirectly bind to the actin cytoskeleton and signaling proteins, allowing the transfer of intracellular signals inside the ECs (Dejana 2004; Lampugnani et al. 1995). These cadherin complexes are actively involved in force-dependent junction remodeling. For example, vinculin has been demonstrated to not be necessary for cell-to-cell junction formation or maintenance but is needed for force-dependent junction remodeling to protect junctions from opening by leukocyte extravasation or angiogenic sprouting (Huveneers et al. 2012). Tolbert et al. demonstrated that Y1065 phosphorylation in vinculin regulated mechanical force-induced F-actin bundle formation (Tolbert et al. 2014). These results indicated that vinculin might serve as a mechanosensitive molecule in response to mechanical forces. Shear stress induced the alignment of ECs with the flow direction and altered the protein levels of VE-cadherin and its complexes; however, the partial disassembly of VE-cadherin was localized at EC junctions (Noria et al. 1999). These results imply that VE-cadherin controls the endothelial permeability barrier and is necessary for EC responses to shear stress. Although VE-cadherin is essential for the EC response to shear stress, it does not serve as a major mechanosensor for ECs in response to shear stress. PECAM-1 depletion abolished the shear stress-induced activation of PI3K-dependent events and Src family kinases. However, VE-cadherin depletion did not inhibit the activation of Src family kinases. A previous study showed that shear stress-induced activated VEGFR-2 formed a complex with VE-cadherin/β-catenin that was required for PI3K activation

(Shay-Salit et al. 2002; Carmeliet et al. 1999). These results suggest that VE-cadherin primarily functions as an adapter molecule in association with PECAM-1, whereas VEGFR-2 plays a role in transducing shear stress-dependent signals into ECs.

2.4 Cell Matrix Receptor

2.4.1 Integrins

Integrins are transmembrane receptors that function in cell adhesion to extracellular matrix (ECM) proteins. Integrins play important roles in cell–cell adhesion and communication with the ECM. Integrins are composed of α and β subunits that form 24 distinct heterodimers from a combination of 18 α and 8 β subunits (Hynes 2002). Each subunit has a large extracellular domain, a transmembrane-spanning region, and a short cytoplasmic domain. The extracellular domain typically binds to an Arg-Gly-Asp (RGD) sequence that is present in various ECM ligands, such as collagen, fibronectin, laminin, and vitronectin. The cytoplasmic domains of both the α and β subunits are the sites of interaction with cytoskeletal and signaling proteins, including talin, α-actinin, focal adhesion kinase (FAK), and c-Src. This important linkage modulates cell motility, cytoskeletal organization, signal transduction, and transcription via integrin activity (Hynes 1999). The unique structural features of integrins enable them to use bidirectional (outside-in and inside-out) signaling to integrate the intracellular and extracellular environments. In outside-in signaling, extracellular stimuli induce the intracellular signaling cascade via integrin activation. In inside-out signaling, intracellular signals trigger the cytoplasmic domains that induce the affinity of integrins for extracellular ligands (Schwartz and Ginsberg 2002; Takagi et al. 2002; Campbell and Humphries 2011). Evidence for integrins functioning as mechanosensors was provided by the finding that pulling integrins using RGD-coated microbeads or micropipettes resulted in cytoskeletal filament reorientation and nuclear distortion and redistribution along the axis of the applied forces (Maniotis et al. 1997). This evidence also provides a molecular connection between the integrin, cytoskeletal filaments, and nuclear scaffolds. Shear stress also induces FAK, MAPK, and IkB kinase (IKK) activation in a manner that can be attenuated by the anti-αvβ3 antibody LM609. These results show that the shear stress-induced activation of signaling molecules is integrin dependent (Li et al. 1997; Bhullar et al. 1998). The direct activation of integrin activity in ECs by shear stress was confirmed by immunostaining with the WOW-1 antibody, which specifically recognizes the activated integrin αvβ3. Shear stress rapidly activates the cluster of integrins into a high-affinity state and induces their association with the adaptor protein Shc (Tzima et al. 2001; Wang et al. 2002). FAK and Shc are major molecules that mediate the integrin-dependent activation of downstream MAPKs in response to shear stress (Shyy and Chien 1997, 2002). In the FAK-dependent pathway, FAK is autophosphorylated at Tyr397 and associates with the Src homology 2 (SH2) domain of c-Src, leading to paxillin and $p130^{CAS}$ phosphorylation by c-Src and the recruitment of various

adaptor proteins as a result of ERK activation (Geiger et al. 2009). Cav-1 and the Fyn tyrosine kinase are critical molecules in the Shc-dependent pathway. Cav-1 constitutively associates with the Src family member Fyn and interacts with the integrin α subunit within the lipid bilayer. Following integrin activation, Fyn is activated and binds to Shc, leading to the phosphorylation of Shc at Tyr317 and the recruitment of the adaptor protein Grb2. This sequence of events leads to the activation of the Ras-ERK pathway (Hynes 2002; Geiger et al. 2009).

2.5 Cytoskeleton and Nucleus

2.5.1 Cytoskeleton

Maniotis et al. demonstrated that mechanical tugging on the integrin receptors caused reorientation of the cytoskeletal filaments, nuclear distortion, and nucleolar redistribution along the axis of the applied tension field. These effects were specific for integrins and were mediated by the direct linkage between the cytoskeleton and the nucleus (Maniotis et al. 1997). The cytoskeleton is composed of three major types of protein filaments: microfilaments, intermediate filaments, and microtubules. Microfilaments are actin polymers, which are the most abundant molecules in the cell and form a continuous dynamic connection between cellular structures. The assembly of actin filaments is controlled by the Rho family of small GTP-binding proteins, including Rho, Rac, and cdc42, which dynamically remodel themselves in response to mechanical stimuli (Goehring and Grill 2013). Intermediate filaments contain eight protofilaments wound around each other in a ropelike structure. Intermediate filaments include class I, II, III, IV, and V proteins. The class III protein vimentin regulates the focal contact size to stabilize cell–matrix adhesion and is displaced by shear stress (Tsuruta and Jones 2003; Helmke et al. 2000). Recent studies suggested that the class V protein lamin also contributes to the mechanosensing pathway to propagate mechanical forces to the nuclear surface (Fedorchak et al. 2014; Alam et al. 2014; Osmanagic-Myers et al. 2015). Microtubules are assembled by protofilaments surrounding a hollow center, and the microtubule organizing center (MTOC) mediates the nucleation of the tubulins into microtubules. Under stimulation by shear stress, the MTOC is located posterior to the nucleus and helps to orient EC polarity (Masuda and Fujiwara 1993; Tzima et al. 2003). The cilia and flagella are composed of microtubules, and primary cilia have been identified as an important mechanosensor (Pazour and Witman 2003). These filaments themselves are interconnected and are also linked to membrane proteins throughout the cell. The linkages between external cellular contacts, adhesion receptors, and the cytoskeleton serve as mechanotransmitters for bidirectional communication between the interior and exterior of the cell. Furthermore, the cytoskeleton is “hardwired” to propagate mechanical forces to the nuclear surface, leading to nuclear deformation and gene expression (Thomas et al. 2002; Ingber 1997, 2003a, b).

2.5.2 Primary Cilia

There are two types of cilia in eukaryotic cells: motile cilia and nonmotile cilia (primary cilia). Primary cilia function as sensory organelles in response to chemical and physical stimuli and regulate tissue morphogenesis (Pazour and Witman 2003). In human aortic ECs, primary cilia were first observed by electron microscopy (Bystrevskaya et al. 1992). Single endothelial primary cilia protrude 1–5 μm from the apical surface and consist of a 9+0 bundle core of microtubule doubles. They extend from the basal body of the cell, where they connect to the cytoskeleton microtubules in the cytoplasm (Egorova et al. 2012). Using immunostaining with acetyl-α-tubulin, Iomini et al. (2004) found that endothelial primary cilia were disassembled by laminar shear stress at 15 dynes/cm^2. Nauli et al. (2008) demonstrated that cytosolic calcium and eNOS were induced in ECs by shear stress at 1.1 dynes/cm^2. Knockdown of endothelial primary cilia resulted in an inability to transmit the shear stress stimulus into intracellular calcium signaling and NO synthesis. Additionally, the loss of primary cilia promoted a shear stress-induced endothelial-to-mesenchymal transition (EMT) in ECs and a fibroblast-like phenotype (Egorova et al. 2011). The in vivo zebrafish model demonstrated that endothelial primary cilia could sense extraordinarily low shear stress in a polycystin-dependent manner and transduce the shear stress stimulus into intracellular calcium signaling (Goetz et al. 2014). These results demonstrated that endothelial primary cilia served as mechanosensors and modulated cellular functions. In vivo observations from C57BL/6 and apolipoprotein-E-deficient mice demonstrated that endothelial primary cilia were found in increased numbers in the area of atherosclerotic predilection where the shear was low and oscillatory, whereas endothelial primary cilia were absent in the areas of the atheroprotective region where the shear had a high and uniform direction (Van der Heiden et al. 2008). Thus, it is exciting to postulate that endothelial primary cilia may contribute to the formation of atherosclerosis.

2.5.3 Nucleus

The nucleus is encapsulated by the nuclear envelope, a double lipid membrane composed of the inner and outer nuclear membranes. Lamins are subjacent to the inner membrane of the nuclear envelope and tether the nucleus to the surrounding cytoskeleton via linker of nucleoskeleton and cytoskeleton (LINC) complexes. The LINC complexes are composed of SUN (Sad1p, UNC-84) domain proteins that span the inner membrane and KASH (Klarsicht/ANC-1/Syne Homology) domain proteins on the outer membrane (Crisp et al. 2006). The nucleus is connected to the rest of the cell through the LINC complexes. Mechanosensory complexes are known to transduce mechanical stimuli from the extracellular environment to the inside of the nucleus, where these stimuli are converted into biochemical signals and consequently result in gene expression and cellular functions. Whether the capacity of the

nucleus to directly sense mechanical stimuli from the extracellular environment is dependent on the cellular geography and unique features of the mechanotransensing pathway requires further investigation. The nucleus was suggested to function as an intracellular mechanical force-bearing organelle in studies by Deguchi et al. and Dahl et al. (Deguchi et al. 2005; Dahl et al. 2005). Indeed, EC nuclei were significantly elongated after exposure to shear stress at 20 dynes/cm^2 for 24 h. The micropipette aspiration technique on the isolated nuclei revealed that the elastic modulus of the shear stress-induced elongated nuclei was significantly higher compared to the control nuclei. These results suggested that the structure of the nuclei could be directly remodeled by the shear stress. Tkachenko et al. demonstrated that shear stress rapidly displaced the EC nuclei forward to downstream of the flow direction, resulting in a planar cell polarity and relocation of the MTOC by cytoskeletal motors (Tkachenko et al. 2013). The distance of the displaced nuclei by shear stress was 8 μm on average within 5 s; this rapid movement of the nuclei was unlikely to be caused by intracellular signaling events. These results suggest that EC nuclei may serve as mechanosensory organelles and that the shear stress-induced displacement of the nuclei contributes to the triggering of intracellular signaling events and eventually the relocation of the MTOC (Tzima et al. 2003; McCue et al. 2006).

Recent studies indicated that several nuclear envelope proteins were involved in the mechanosensing pathway, including nuclear lamins, emerin, and the LINC complexes (Fedorchak et al. 2014). The A-type lamins are the major nuclear mechanosensors of the mechanosensing pathway from the ECM into the nucleus (outside-in) and are key molecules of the nucleocytoskeletal coupling machinery. Poh et al. demonstrated local dynamic forces based on the matrix elasticity of transmitted forces from the ECM into the nuclear body; these forces were transmitted to the nuclear envelope via the F-actin/LINC/nuclear lamina structural pathways. Moreover, the forces triggered the dissociation of the SMN protein and coilin from the nuclear body. Lamin A knockout abolished the force-induced dissociation of SMN and coilin from the nuclear body, indicating an essential role for lamin A in the transmission of force to the nuclear body (Poh et al. 2012). The cells not only adjusted to the increase of mechanical forces by remodeling the structure of the nucleus and lamin A but also increased the level of lamin A in response to mechanical forces (Swift et al. 2013). Matrix elasticity directs the lineage specification of human bone marrow-derived mesenchymal stem cells (MSCs) in culture towards bone, fat, or other tissue types. A soft matrix promotes increased adipogenesis and decreased lamin A expression. Overexpression of lamin A in MSCs on the soft matrix led to an increase in osteogenesis, suggesting that lamin A enhanced matrix elasticity-directed differentiation. These studies showed that lamin A served as a nuclear mechanosensor for both the outside-in signal and the inside-out signal in the mechanosensing pathway. Transmission of mechanical forces into the nucleus requires the mechanical connection of the nucleus to the cytoskeleton; these connections are mediated by the LINC complexes. The LINC complexes consist of SUM domain proteins and KASH domain proteins, and intact LINC complexes are required for nuclear position, cell polarization, and force propagation (Jaalouk and Lammerding 2009; Lombardi and Lammerding 2011). There are five SUM domain

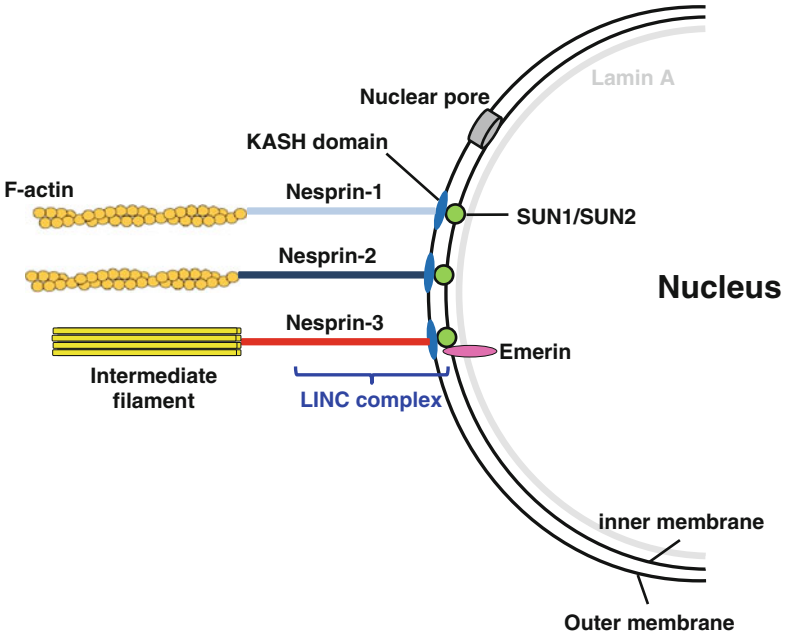

Fig. 2.2 The mechanosensing system in the endothelial nucleus. LINC complexes include the SUN (Sad1p, UNC-84) domain proteins that span the inner membrane and the KASH (Klarsicht/ANC-1/Syne Homology, nesprins 1–4, LRMP, and KASH5) domain proteins on the outer membrane. Nesprin-1 and nesprin-2 connect F-actin, whereas Nesprin-3 connects the intermediate filament to the outer nuclear membrane

proteins and six KASH domain proteins (nesprins 1–4, LRMP, and KASH5) in mammals, although only SUN1 and SUM2 have been demonstrated to associate with the nuclear lamina and nesprin 1–3 by directly binding to the cytoskeleton (Fig. 2.2) (Jaalouk and Lammerding 2009; Wang et al. 2012; Sosa et al. 2012). Disruption of these LINC complexes results in impaired cell motility, shear stress-induced cell polarization, defects in nuclear positioning and centrosome orientation, and disrupted perinuclear organization of actin and vimentin. The LINC complexes have been suggested to play prominent roles in force transmission between the nucleus and the cytoskeleton (Chancellor et al. 2010; Chambliss et al. 2013; Morgan et al. 2011; Lombardi et al. 2011). Interestingly, depletion of nesprin-1 caused an increase in the nuclear height, focal adhesion assembly, and traction in ECs. Actomyosin tension has been suggested to be balanced by the nucleus due to mechanical links mediated by nesprin-1. In the absence of these connections, actomyosin forces are assumed to be balanced by an additional number of focal adhesions, resulting in a decrease in cell motility. This evidence implies that nesprin-1 functions as a nuclear mechanosensor by linking mechanical force transmission between the nucleus and the cytoskeleton.

In addition to the nuclear envelope proteins, transmembrane actin-associated nuclear (TAN) lines, which are assembled from nesprin-2G and SUN-2, act as linkers between the actin bundles on the top of the nuclear surface and the nucleus. TAN lines are anchored by lamin A and allow the forces generated by the actin cytoskeleton to be transmitted to the nuclear envelope, consequently resulting in the movement of the nucleus. Super-resolution microscopy revealed that the structure of the TAN lines across the nuclear membrane was similar to the focal adhesions that crossed the cellular membrane. It is likely that the TAN lines are composed of additional cytoplasmic and nucleoplasmic proteins (Luxton et al. 2011). The perinuclear actin cap (actin cap) is composed of thick, parallel, and highly contractile actomyosin filament bundles that are anchored to the apical surface of the nucleus by LINC complexes and terminate at actin cap-associated focal adhesions (ACAFAs) at the basal surface of the adherent cell (Kim et al. 2013). Chambliss et al. showed that the conventional basal stress fiber reformed and organized in response to fluid shear stress at 0.5 dyne/cm^2. The actin cap formed at shear stress 50 times lower (as low as 0.01 dyne/cm^2 within 5 min) than the formation of the conventional basal stress fiber. This evidence suggests that the actin cap plays a key role in the fast and efficient transmission of mechanical forces from the ECM to the nucleus (Chambliss et al. 2013).

2.6 Summary and Conclusion

Most endothelial mechanosensors are located at the plasma membrane and serve as primary sensors in response to shear stress (Fig. 2.3). The cytoskeleton and the nucleus act as secondary sensors to accomplish the mechanotransduction. These mechanosensors activate upstream signaling molecules and mediate intracellular signaling through phosphorylation cascades via their associated adaptor proteins or elicited signaling proteins, eventually leading to the maintenance of endothelial homeostasis (Table 2.1). In this manuscript, we listed the endothelial mechanosensors and mechanosensing systems involved in the EC response to shear stress. Although these mechanosensors were identified one decade ago, the detailed mechanisms of the effects of ion channels in response to shear stress remain unclear. Ion channels are fast mechanoresponsive molecules and may play a role in the regulation of atherosclerosis. Due to the limitations of technical approaches and in vivo studies, little is known about which ion channels have the most important functional impacts on vascular physiology/pathophysiology in health and disease. Recent studies indicated novel mechanosensing in the nucleus. Several nuclear proteins, such as lamin A and the LINC complexes, have been identified as molecules that serve as nuclear mechanosensors and regulate signaling pathways in the nucleus. The mechanisms by which these nuclear mechanosensors regulate gene expression by transmitting signals into the nucleus and inducing a nuclear conformational remain unclear. These issues deserve further investigation.

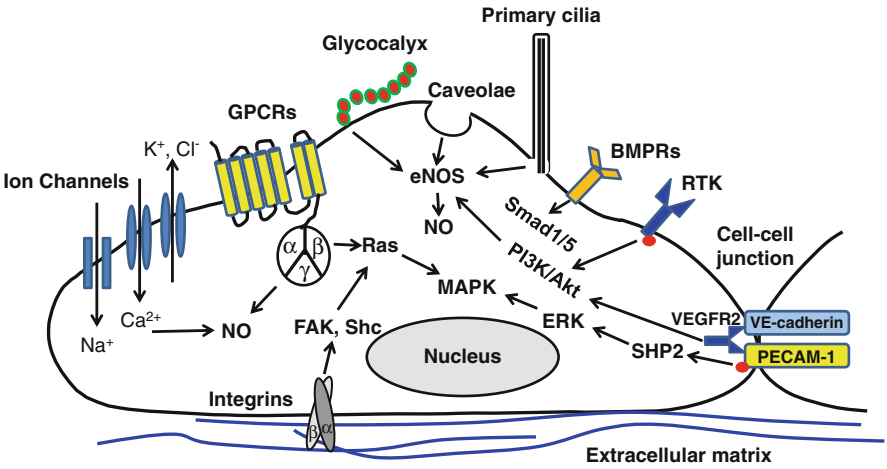

Fig. 2.3 The endothelial primary mechanosensors in response to shear stress. In ECs, shear stress is sensed by the primary mechanosensor on the membrane, including the ion channels, GPCRs, glycocalyx, caveolae, primary cilia, RTKs, BMPRs, mechanosensory complexes of cell-to-cell junctions (PECAM-1, VE-cadherin, and VEGFR-2), and integrin. The shear stress-induced activity of ion channels results in a Ca^{2+} influx and leads to NO production. G proteins are rapidly activated within 1 s by shear stress, leading to Ras activation and NO production. Activation of the glycocalyx and primary cilia results in NO production. Shear stress induces the phosphorylation and activation of caveolae and triggers NO production and ERK activation. The oscillatory flow induces the activation of BMPR-specific Smad1/5. The activation of RTKs and mechanosensory complexes of the cell-to-cell junction by shear stress induces PI3K/Akt activation, leading to NO production. PECAM-1 is phosphorylated by shear stress, resulting in SHP-2 recruitment and ERK activation. Activated integrins associate with the adaptor protein Shc and FAK and mediate the integrin-dependent activation of downstream MAPKs

There is evidence indicating that different mechanosensors can interact with one another to transmit mechanical stimuli into the cell interior. Integrins and the ECM are highly interactive, and this interaction causes integrin activation. Computational models predict that the glycocalyx largely mediates the interaction of integrins and the ECM, suggesting that the glycocalyx may be a key regulator of integrin functions (Paszek et al. 2009). Cilia sense mechanical forces through polycystins and trigger intracellular calcium signaling and nitric oxide synthesis. Recent studies showed that the ECM and integrins surrounded the primary cilia, implying that integrins might be involved in shear stress-induced cilia activation (McGlashan et al. 2006; Drummond 1812). The oscillatory flow induces the association of BMPR1B-integrin $\alpha_v\beta_3$ with the intracytoplasmic kinase domain of BMPRII, resulting in the activation of BMPRII and phosphorylation of Smad1/5. PECAM-1 can interact with VE-cadherin and VEGFR-2 to form a mechanosensory complex that transduces shear stress-dependent signals into cells. The GPCRs, G-protein subunits $G_{\alpha q/11}$, and PECAM-1 form a mechanosensory complex in response to shear stress. The shear stress-induced activation of integrins is required for the involvement of VEGFR-2 and Cav-1 to transduce signaling pathways. These results indicate that integrins are

Table 2.1 Mechanosensor proteins elicited by shear stress in ECs

Location	Mechanosensor	Elicited molecules	Reference
Membrane	Ion channels	Ca^2+, K^+, Na^+, Cl^-, NO	Kohler et al. (2006), Hartmannsgruber et al. (2007), North (2002), Olesen et al. (1988), Lieu et al. (2004), Wang et al. (2009)
	G proteins/GPCRs	Ras, NO	Ohno et al. (1993), Kuchan et al. (1994), Gudi et al. (2003)
	Glycocalyx	PKC-α, PIP2, NO	Weinbaum et al. (2007), Thi et al. (2004), Tarbell and Pahakis (2006)
	Caveolae	G protein α-subunit, Ras, Src family tyrosine kinases, eNOS, PKC, ERK	Park et al. (2000), Yu et al. (2006), Fujioka et al. (2000), Sun et al. (2002), Radel and Rizzo (2005)
	RTK	ERK, JNK, IKK, Akt, NO	Jin et al. (2003), Chen et al. (1999), Wang et al. (2004)
	BMPRs	Smad1/5, mTOR, p70S6K	Zhou et al. (2012), Zhou et al. (2013)
	Primary cilia	NO, Ca^{2+}	Egorova et al. (2011), Goetz et al. (2014)
Cell junction	PECAM-1	SHP2, NF-kB, ERK, MAPK	Tzima et al. (2005), Osawa et al. (2002), Jackson et al. (1997)
	VE-cadherin	α/β-Catenin, plakoglobin, vinculin, PI3K	Shay-Salit et al. (2002), Tolbert et al. (2014), Carmeliet et al. (1999)
Matrix receptor	Integrin	FAK, Shc, Src, paxillin and $p130^{CAS}$, Cav-1, Fyn, ERK, Ras	Hynes (2002), Shyy and Chien (2002), Shyy and Chien (1997), Geiger et al. (2009)
Cytoplasm	Cytoskeleton	Rho, Rac, cdc42, MTOC	Goehring and Grill (2013), Masuda and Fujiwara (1993), Tzima et al. (2003)
Nucleus	LINC complexes	F-actin, MTOC	Tkachenko et al. (2013), Chancellor et al. (2010), Chambliss et al. (2013), Morgan et al. (2011), Lombardi et al. (2011)

able to associate with other mechanosensors to transduce mechanical stimuli into the cell interior. Nuclear envelope proteins, such as nuclear lamins, emerin, and LINC complexes, and cytoskeleton molecules, such as TAN lines and the actin cap, are involved in the nuclear mechanosensing systems. These findings indicate that multiple endothelial mechanosensors work together to accomplish mechanosensing and mechanotransduction rather than a single mechanosensor. Indeed, a single endothelial mechanosensor is unlikely to exist.

In vivo studies revealed that these mechanosensors are involved in atherosclerotic formation. PECAM-1 and cilia were suggested to contribute to atherosclerotic progression. PECAM-1 was required for the activation of NF-kB and the downstream inflammatory responses induced by shear stress. PECAM-1 knockdown

reduced lesion formation. Single-nucleotide polymorphisms in the human PECAM-1 gene revealed links to early atherosclerosis and cardiovascular diseases because these polymorphisms influenced the tyrosine phosphorylation of PECAM-1 and leukocyte transmigration (Elrayess et al. 2003, 2004). Primary cilia were located in increased numbers in the atheroprone region and were disrupted by high shear stress. Moreover, primary cilia are unlikely to play a role in the atheroprotective region, which is characterized by high shear stress and a uniform flow. These results strongly imply the correlation of atherosclerotic formation with PECAM-1 and cilia. Whether other mechanosensors in addition to PECAM-1 and cilia, such as ion channels and nuclear mechanosensing systems, are involved in atherosclerosis deserves further investigation.

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Chapter 3
Cadherins in Mechanotransduction

D.E. Leckband

Abstract Intercellular adhesions are crucial mechanical and signaling hubs that mechanically integrate cells within tissues and transduce force perturbations generated by endogenous contractility and exogenous forces from the surrounding environment. Recent findings identified a new class of force-sensing adhesive complexes at intercellular junctions, and elucidated a new force transduction mechanism that can impact cell mechanics and instruct such cell functions as barrier integrity and cell cycle control. This chapter highlights the major, recent findings demonstrating that cadherin-based adhesion complexes, which are the principal adhesive proteins at cell-cell junctions, are force transducers. I focus on the methods used to investigate force transduction on length scales, ranging from atomistic simulations to tissue functions and development. These approaches revealed a key force transducer at these adhesive junctions, as well as one mechanism of cadherin-based force transduction. Although this mechanism shares some elements of mechanotransduction at cell-ECM adhesions, it is distinct from other integrin-based force sensing.

Keywords Cadherin • Mechanotransduction • Alpha catenin • Vinculin • Tension sensor • Magnetic twisting cytometry • Traction force microscopy

3.1 Introduction

Multicellular organisms integrate mechanical and chemical cues at the cellular level to govern processes including, for example, embryonic development, atherosclerosis, and tumor progression. For over a decade, investigations of mechanotransduction focused largely on mechanical signaling by integrin-based cell-extracellular matrix (ECM) adhesions (Choquet et al. 1997; Engler et al. 2006; Vogel and Sheetz 2006; Geiger et al. 2009; Pelham and Wang 1997; Wang et al. 1993), extracellular matrix remodeling, and ion channels. It is now well established that cell-cell

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adhesion complexes are also mechanosensors (Ladoux et al. 2010; le Duc et al. 2010; Liu et al. 2010; Yonemura et al. 2010).

Several examples suggest the importance of intercellular forces in tissue morphogenesis and developmental biology. Anisotropic, temporally controlled actomyosin contraction, coupled to intercellular adhesions, drives the remodeling of cell adhesions during the apical constriction and elongation of embryos through cell intercalation (Bertet et al. 2004; Rauzi et al. 2010; Rauzi et al. 2008; Sawyer et al. 2009; Martin et al. 2010). Similarly, contraction of the wing hinge during fly development regulates planar cell polarity (PCP) and cell intercalation during Drosophila wing elongation (Aigouy et al. 2010). Wnt-driven PCP signaling regulates putative cadherin-dependent tissue tension to regulate extracellular matrix, ECM organization (Dzamba et al. 2009). Furthermore, differences in myosin-dependent cortical tension directs cell sorting in the germ layer of embryos (Krieg et al. 2008), whereas coordinated actomyosin-based tension at the anterior-posterior boundary maintains tissue segmentation later in development (Sawyer et al. 2010; Monier et al. 2010; Landsberg et al. 2009). The central role of mechanically perturbed cell-cell adhesion complexes in epithelial sheets (Vaezi et al. 2002; Danjo and Gipson 1998) emphasize the importance of these junctions for proper transmission of actomyosin-based forces during tissue stratification. In two-dimensional cell culture models, increases in endogenous mechanical force at E-cadherin-mediated cell-cell contacts were implicated in the phenotypic transformation of epithelial cells by oncogenic growth factors such as Neu (Chausovsky et al. 1998), HGF (de Rooij et al. 2005), and TGFβ (Gomez et al. 2010), although the force at the cell-cell junctions was not determined.

Although the studies described above strongly suggested the existence of intercellular mechanotransduction machinery, observed cell morphology, subcellular architecture, or the composition of intercellular junctions are insufficient to unambiguously demonstrate a mechanosensory role for specific cell-cell adhesion proteins. Knowledge of the interplay of force and adhesion biochemistry was, until relatively recently, based on two-dimensional cell-culture models with a particular emphasis on cell-extracellular matrix adhesions (Geiger et al. 2009). Determining forces at cell-cell junctions and their impact on adhesion protein functions has been more challenging, in part due to limited available tools for interrogating cell-cell junctions. A number of biophysical and biochemical advances described briefly in this chapter established that intercellular adhesion complexes are not merely passive mechanical force conduits, but also active force transducers that modulate cell physiology in response to a variety of mechanical stimuli. Such approaches also enabled the establishment of the initial mechanisms of intercellular force transduction in a few cases.

The major adhesive proteins at all intercellular junctions are the transmembrane proteins cadherins. The cadherin superfamily is large, but classical type I and type II cadherins are the most extensively studied. Classical cadherins, including neural (N-), epithelial (E-), and vascular endothelial (VE-) cadherins have similar overall folds, but their sequences differ and their expression in different tissues is tightly regulated (Gumbiner 2005; Shapiro and Weis 2009). The classical cadherins are transmembrane proteins with an extracellular region embedding the adhesive function, a single pass alpha helical transmembrane domain and a cytoplasmic

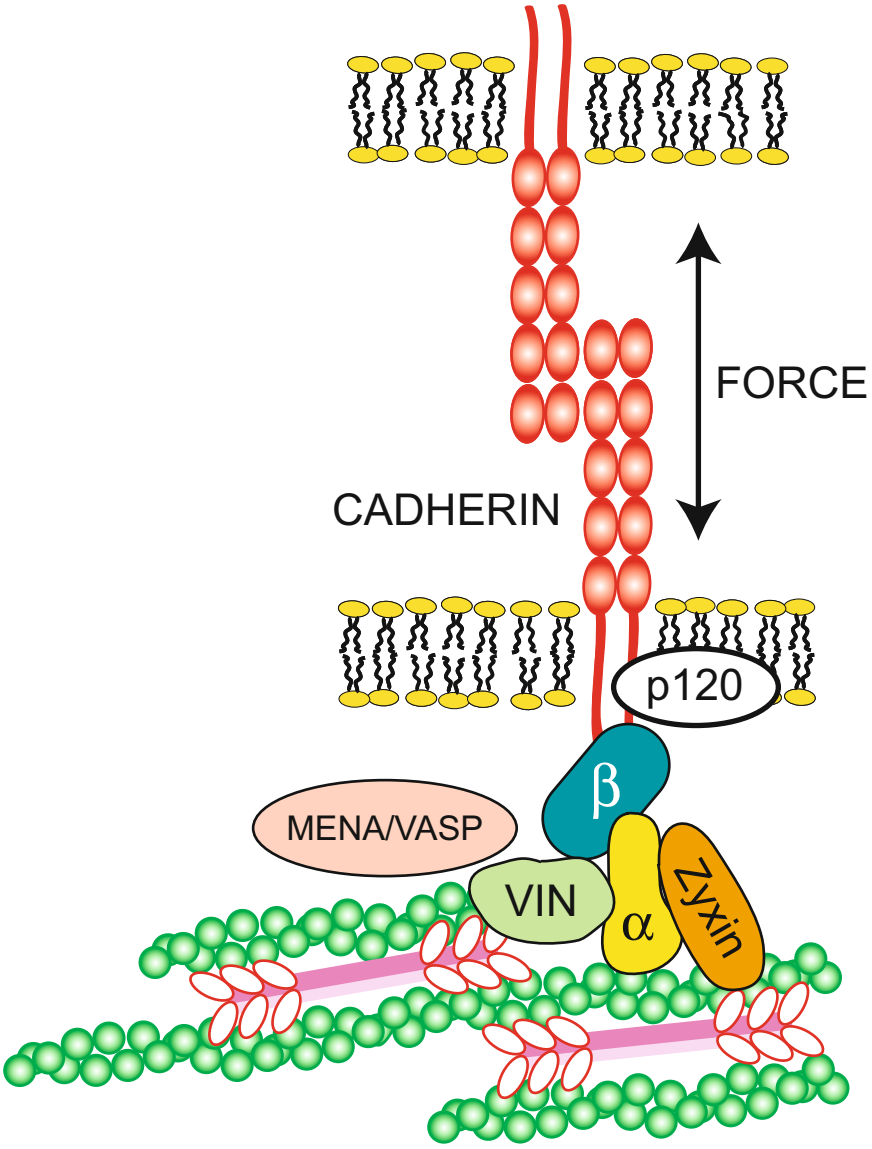

Fig. 3.1 Architecture of classical cadherin adhesion complexes. Classical cadherins are transmembrane proteins that bind to the extracellular domains of opposing cadherins on an adjacent cell. β-Catenin (β) binds the cadherin cytodomain and the actin binding protein α-catenin (α). α-Catenin binds vinculin in stressed junctions. Other actin binding proteins found at stressed cadherin complexes include zyxin and MENA/VASP, among others

domain (Fig. 3.1). Cadherins are mechanically coupled to actin microfilaments by α- and β-catenins (Shapiro and Weis 2009). β-Catenin binds the cadherin cytoplasmic domain and the actin binding protein α-catenin couples β-catenin and actin (Imamura et al. 1999; Buckley et al. 2014) (Fig. 3.1).

Cadherin adhesions at intercellular junctions intrinsically convey mechanical information between cells, by resisting forces generated by endogenous contractile forces or by exogenous forces from, for example, fluid shear stress, tissue rigidity, or compressive and tensile forces. Cadherin ligation alone can trigger biochemical signaling (reviewed in Yap and Kovacs 2003; Wheelock and Johnson 2003), but cadherin complexes also connect the cytoskeletons of adjacent cells, and are ideally positioned to transduce fluctuations in mechanical forces between cells due to cytoskeletal contractility or deformations. Changes in cytoskeletal organization and force generation are crucial in embryonic development and morphogenesis (Lecuit and Le Goff 2007; Paluch and Heisenberg 2009), in tissue repair, and in pathologies such as cancer (Kumar and Weaver 2009). Whereas cadherins were previously regarded as passive structural elements in force transmission between adjacent cells, it is now well established that cadherin complexes actively sense mechanical perturbations and transduce proportional biochemical responses that instruct cell behavior. This specific cadherin function is called mechanotransduction, and its mechanisms and emerging relevance in tissue development, physiology, and disease are the subject of this chapter.

3.2 Cadherin-Mediated Intercellular Junctions Are Under Tension

Cadherin adhesions are under tension, as evidenced by the recoil of junction-associated actin fibers, following laser severing (Cavey et al. 2008). In addition to this qualitative demonstration, the junctional tension was estimated using more quantitative methods.

Intercellular tension (force/length) was estimated from the balance of cell traction forces, calculated based on the deformations of elastomeric pillars or deformable hydrogels that are generated by cells adhering to these substrata (Fig. 3.2a, b). In force balance calculations, which typically involve clusters of fewer than five to seven cells (Liu et al. 2010; Ng et al. 2014; Maruthamuthu et al. 2011; Mertz et al. 2013), the tension between two cells is estimated from the excess force determined by the sum of the traction force contribution orthogonal to the junction. In isolated cells, the traction forces sum to zero, but between two adhering cells, an (nonzero) excess force is balanced by the tension (force/length) on cell-cell junctions (Liu et al. 2010; Ng et al. 2014; Maruthamuthu et al. 2011; Mertz et al. 2013).

Fig. 3.2 (continued) F9 epithelial cells. Controls with cells treated with the actin disrupters Latrunculin and Cytochalasin D ablated the response. Inactivating E-cadherin with EGTA ablated the response, as did the use of beads coated with PLL. (**h**) Illustration of the dual pipette system used to investigate the influence of force at cadherin-mediated, cell–cell contacts on intercellular adhesion strength. Suction pressure applied to the adhering cells held by pipettes increase the force on the cell–cell junction. Panels (**a**–**c**), reproduced with permission from (Liu et al. 2010; Maruthamuthu et al. 2011)

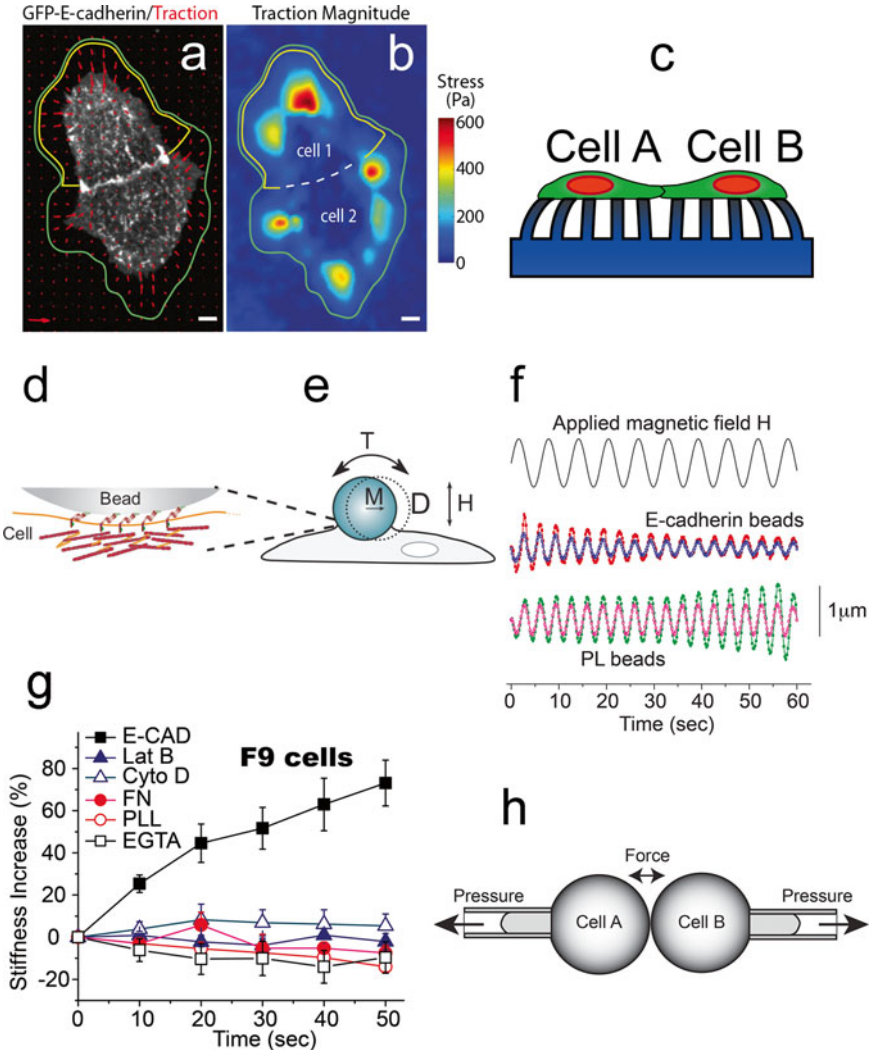

Fig. 3.2 Methods used to investigate the influence of force on cadherin-mediated intercellular adhesions. (**a**, **b**) Traction force measurements reveal the distributions of traction forces exerted by cells on elastomeric gels (*red arrows*). The cell is outlined in *green*, and the junction is indicated by the *white arrows*. The force vectors are used to calculate maps of substrate strain (**b**). (**c**) Cells on elastomeric pillars deform substrates in proportion to the bending modulus of the pillars and the force exerted by cells through adhesion receptors. (**d**–**f**) Magnetic Twisting Cytometry (MTC) measurements. (**d**) Ferromagnetic beads coated with ligand (cadherin) adhere to cells via cell surface (cadherin) receptors. (**e**) An oscillating magnetic field (*H*) orthogonal to the bead magnetization (M) induces a twisting torque (*T*) that displaces the beads by an amount *D*. (**f**) The oscillating field *H* and corresponding torque induced a decrease in the amplitude of E-cadherin-coated bead displacements with twisting time (*middle*). Control measurements (*bottom*) with poly-L-lysine (PLL)-coated beads did not induce changes in bead displacements. (**g**) Force-activated change in the stiffness of junctions between E-cadherin-coated beads (E-CAD) and E-cadherin expressing

Using such force balance calculations, Maruthamuthu and Gardel (Maruthamuthu et al. 2011) estimated that, on deformable hydrogels, epithelial cells in a cluster exert nano Newton forces on cadherin-mediated intercellular junctions. Additionally, factors that increase cell-ECM traction forces or myosin II-dependent contractility correspondingly increase the tension on cell-cell junctions (Liu et al. 2010; Maruthamuthu et al. 2011). Using deformable micropillar arrays to quantify traction forces (Fig. 3.2c), Liu et al. estimated the stress (force/area) between endothelial cells adhered to deformable elastomeric pillars (Liu et al. 2010). Alternatively, studies showed that cells exerted traction forces on cadherin-based adhesions to cadherin-coated micropillars. Somewhat surprisingly, the estimated forces between myoblasts and N-cadherin-coated elastomeric pillars (Ganz et al. 2006) were of the same order of magnitude as at cadherin-based junctions between two MDCK epithelial cells (Maruthamuthu et al. 2011).

The estimated tension at cell-cell junctions does not give the force on the cadherin bonds and could be influenced by other proteins at intercellular contacts. Engineered cadherin tension sensors, based on fluorescence resonance energy transfer (FRET), enabled measurements of population-averaged forces on cadherin receptors at cell-cell contacts. Tension sensors for both vascular-endothelial cadherin (VE-Cad) and epithelial cadherin (E-Cad) incorporated a tension reporter motif in the cadherin cytoplasmic domain (Borghi et al. 2012; Conway et al. 2013). The calibrated force-sensing motif used was a linker of flexible, spider silk protein flanked by donor and acceptor fluorescent proteins (Grashoff et al. 2010). This motif exhibits fluorescence changes as the elastic silk protein extends under tension, to separate the donor and acceptor in proportion to the applied force. FRET signals from cadherins incorporating these force sensing modules suggested that the average force on cadherin receptors is ~4 pN (Borghi et al. 2012; Conway et al. 2013). The actual forces may be larger because the dynamic range of the force measured with current sensors is limited by both the silk protein mechanics and the donor/acceptor characteristics. Also, the FRET measurements average signals from all proteins in the junction, including both bound and unbound proteins. Nevertheless, the biosensors are valuable tools for estimating the force on cadherin adhesions and changes in response to perturbations that had previously been inferred from average junction stress (force/area) or actin organization.

3.3 Cadherin Complexes Are Force Transducers

Diverse experimental approaches demonstrated that cadherin complexes are also force transducers that actuate biochemical and mechanical changes in cells, in response to force fluctuations. Common mechanical perturbations used include (1) altered endogenous actomyosin-dependent contractile force, (2) traction forces generated by adhesion receptors on substrates with different rigidity, (3) pulling on two adhering cells, and (4) cell responses to direct mechanical stimulation (exogenous

force) (Lele et al. 2007). Examples of cell responses to such mechanical perturbations include subcellular junction remodeling (Liu et al. 2010), altered mechanical properties such as intercellular adhesion and cell traction (Ladoux et al. 2010; Thomas et al. 2013; Tabdili et al. 2012), increased junction stiffness (le Duc et al. 2010), or altered cell polarity (Weber et al. 2012).

3.3.1 *Direct Mechanical Perturbations Actuate Cell Stiffening and Cytoskeletal Remodeling*

Direct evidence for cadherin-based mechanotransduction came from studies in which force was applied directly to cadherin receptors, using sensitive force probes that stimulate, but do not necessarily break bonds (Huang et al. 2007). Although reductionistic, this approach perturbs specific receptors without potential complicating influences from other junction proteins.

Cadherin receptors were directly perturbed, using magnetic beads coated with cadherin ectodomains (or other proteins) (le Duc et al. 2010). Beads coated with ligands to cell surface receptors were subject either to oscillating magnetic fields, as in magnetic twisting cytometry (MTC) (Fig. 3.2d, e), or to a static field by use of permanent magnets that induce a step change in force on the bead.

In MTC studies (Wang et al. 1993), an acute oscillatory twisting torque was applied to cadherin-mediated bead-cell junctions, on timescales of a few seconds to several minutes (Fig. 3.2d–f). An oscillating field H produces a torque T on the magnetic bead to generate a shear force (force/area) on the cell surface (Fig. 3.2e) that displaces the bead. Bead displacements D reflect the viscoelasticity of bead-cell-cytoskeletal connections (Fig. 3.2d), and active cell responses (mechanotransduction) typically result in altered displacement amplitudes with the twisting time (Fig. 3.2f). The measured specific cell modulus $G = T/D$ is a function of the storage G' (elastic) and loss G'' (viscous) moduli. In studies of cadherin-mediated force transduction, the viscous contribution to bead movements was negligible at the oscillating frequencies and field strengths used, such that changes in the specific modulus reflected changes in the elastic modulus of bead-cell adhesions.

In MTC measurements with E-cadherin-coated 4.5 μm ferromagnetic beads bound to the surface of E-cadherin expressing cells (Fig. 3.2d), the modulated shear force on cadherin bonds induced a rapid decrease in bead displacements (Fig. 3.2f, center), within 5 s of bead twisting (le Duc et al. 2010). This corresponds to an increase in the junction stiffness, relative to unperturbed cells (Fig. 3.2g). Several controls (Fig. 3.2g) confirmed the E-cadherin specificity of the mechanotransduction response. Typical of force sensing (Vogel and Sheetz 2006), the stiffness change increased with the applied bond shear. Studies further showed that the E-cadherin-mediated adaptive stiffening requires an organized actomyosin cytoskeleton (Fig. 3.2g), and quantitative immunofluorescence imaging of the region immediately surrounding the perturbed beads revealed actin accumulation

at mechanically perturbed cadherin adhesions (Barry et al. 2014). The actin-dependent, force-activated stiffening response was exhibited by N-, E-, and VE-cadherin, and is consistent across the cell types investigated (Tabdili et al. 2012; Barry et al. 2014; Barry et al. 2015). The underlying mechanism is addressed in later sections of this chapter.

The above findings revealed actomyosin-dependent processes proximal to cadherin adhesions, but a unique case of mechanotransduction was reported for the classical cleavage stage C-cadherin in *Xenopus* cells (Weber et al. 2012). Using a constant magnetic field, Weber et al. used C-cadherin-coated beads to tug on C-cadherin receptors on a single cell in a cohort of collectively migrating mesendoderm cells from Xenopus embryos. Tugging on a single cell in the cohort triggered the abrupt, coordinated reversal in cell polarity, the accumulation of keratin filaments near the pulled contact, and change in migration away from the applied force (Weber et al. 2012). In mesendoderm cells, plakoglobin (γ-catenin) was the identified physical linker between C-cadherin and keratin filaments (Weber et al. 2012). Plakoglobin itself, however, was only recruited to C-cadherin junctions after pulling. Exactly how force on the C-cadherin receptor was initially sensed remains unclear. Nevertheless, this latter study suggested that cadherin-based force transduction mechanisms may vary between cadherins and could depend on the cell type.

Cadherin junctions also remodel in response to endogenous tugging forces. In endothelial cells, endogenous, myosin II-dependent contractile forces exerted on junctions between endothelial cell doublets were estimated from analyses of deflections of patterned micropillars (see Fig. 3.2c). The activation of actomyosin contractility in the two cells by different chemical and genetic manipulations (Liu et al. 2010) increased the junctional tension and triggered a corresponding increase in the junction area. The latter behavior was phenotypically similar to force-dependent growth of focal adhesions, and reinforced the notion of the presence of force-sensing machinery at endothelial cell junctions.

In dual micropipette measurements (Fig. 3.2h), pulling on two adhering cells increased the intercellular cohesion, which in turn correlated with observed actomyosin remodeling at the junctions (Thomas et al. 2013). The latter adhesion strengthening could be due to cadherin-dependent force transduction cascades, or it could result from a variety of other mechanisms such as inside-out signaling, cadherin recruitment, or reinforced cytoskeletal linkages. However, the similarities of the subcellular changes with behavior observed in both micropillar and MTC studies suggested that the force-triggered, junction remodeling was due to similar mechanisms, in all three contexts.

3.3.2 Cadherin-Based Adhesions Sense Substratum Rigidity

The ability of cells to sense substrate rigidity is another signature of mechanotransduction (Vogel and Sheetz 2006). Measured cell spreading area and traction forces exerted on N-cadherin adhesions to N-cadherin-coated micropillars depended on

the bending moduli of the pillars, and also suggested cadherin-based force sensing. Both muscle cells on N-cadherin-based adhesions (Ladoux et al. 2010) and epithelial cells on E-cadherin-coated substrates generated higher traction forces on stiffer substrata (Tabdili et al. 2012). Through an undefined positive feedback loop, endogenous contractile forces exerted on cadherin adhesions increased in proportion to extracellular matrix rigidity, and demonstrated the force transducing activity of cadherin complexes.

3.4 Proposed Mechanism of Cadherin-Based Mechanosensing

Force transduction at cadherin adhesions was attributed to the cytosolic protein, α-catenin, within cadherin complexes (Fig. 3.1). The cytodomain of classical cadherins forms a stoichiometric complex with β-catenin, which in turn binds the actin-binding protein α-catenin (Fig. 3.1). α-Catenin is the central protein that is postulated to link E-cadherin-associated β-catenin to F-actin (Desai et al. 2013) (Fig. 3.1). Initial pathbreaking studies used an epitope-specific, anti-α-catenin antibody to detect conformational changes in α-catenin at junctions, following myosin II activation and increased junction tension (Yonemura et al. 2010). The postulate was that α-catenin is autoinhibited by an intramolecular interaction, which masked the central effector domains (see Fig. 3.3a). Increased junctional tension would relieve the autoinhibition, by inducing a conformational change in α-catenin (Yonemura et al. 2010), to expose the vinculin binding site (VBS) (V in Fig. 3.3b). This exposed VBS would in turn recruit vinculin (see Fig. 3.3b) to stressed cell-cell junctions, as an immediate molecular event in mechanotransduction (Yonemura et al. 2010; Yonemura 2011). This model was initially supported by myosin II-dependent epitope exposure, as well as by the loss of force-dependent vinculin recruitment, upon removal of the putative inhibitory domain (I in Fig. 3.3a, b) (Yonemura et al. 2010).

Crystal structures of different α-catenin fragments obtained under different conditions, and in combination with other proteins, revealed that the core force-sensitive region comprises three, 4-helix bundles (denoted MI–MIII) (Fig. 3.3c) that appear to be stabilized by a cluster of salt-bridges between charged amino acids (Rangarajan and Izard 2013; Ishiyama and Ikura 2012; Ishiyama et al. 2013) (Fig. 3.3c). The interface between two helices in the MI domain encrypts the vinculin site. Molecular dynamics simulations revealed that this salt-bridge network stabilizes the entire force-sensing module, and likely determines the force threshold needed to unfurl α-catenin and expose the vinculin site (Fig. 3.3e) (Li et al. 2015). The use of ionic interactions to regulate force sensitivity is an intriguing concept. The salt-bridge network appears to stabilize the autoinhibited state of α-catenin. This mechanism differs from that of the close homolog vinculin, which is autoinhibited by a high affinity interaction between its head and actin-binding tail domains (Ziegler et al. 2006), rather than by salt-bridges.

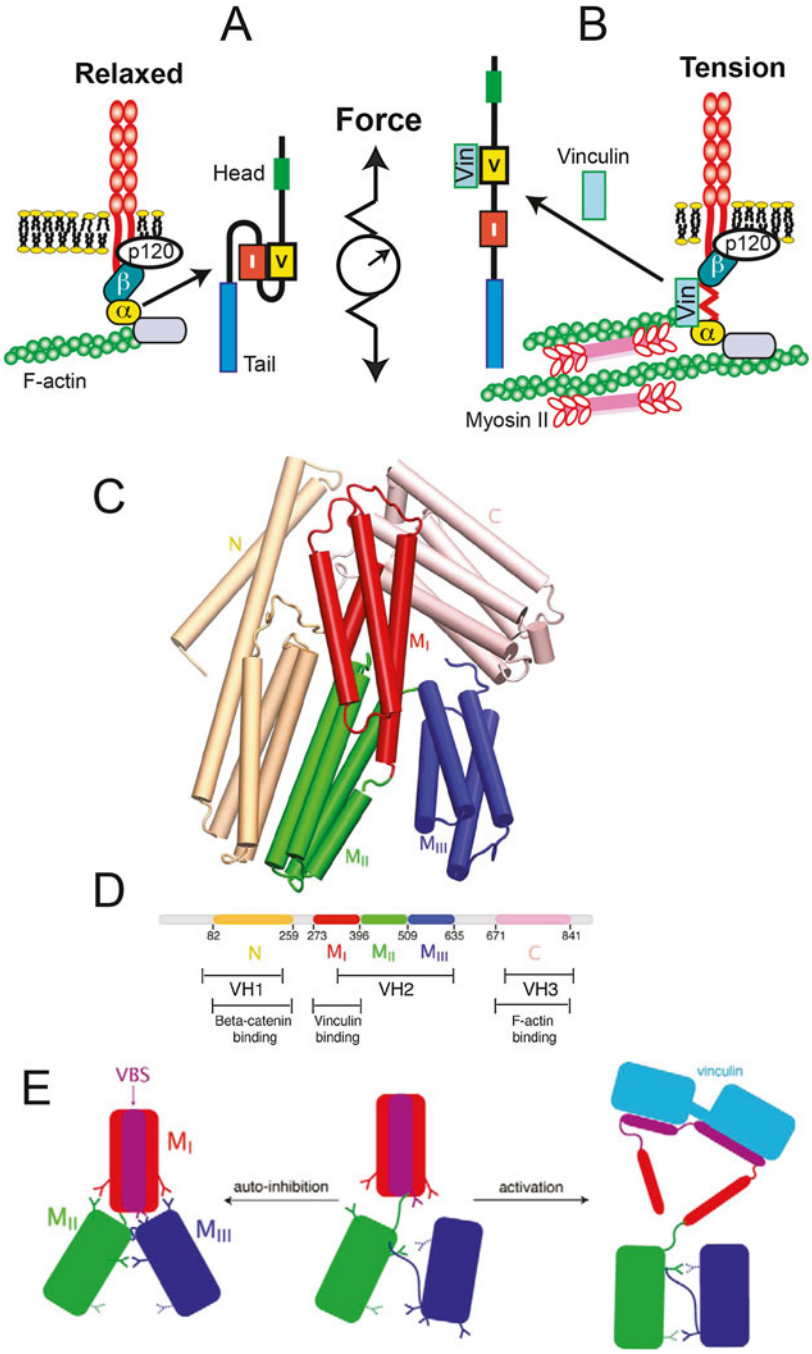

Fig. 3.3 α-Catenin force transducer at cadherin-mediated intercellular adhesions. (**a**, **b**) Initially proposed α-catenin force transduction model. (**a**) In the absence of tension, α-catenin in the complex adopts and autoinhibited conformation in which the vinculin binding site, V is encrypted by the inhibitory domain, I. (**b**) Under tension, α-catenin unfurls to expose the cryptic site that recruits

3.5 α-Catenin and Vinculin Are Crucial Elements of Cadherin-Mediated Force Transduction

3.5.1 α-Catenin and Its Vinculin-Binding-Site Are Essential for Force Transduction

Diverse experimental approaches confirmed that α-catenin and its vinculin binding site are required for vinculin and actin recruitment to cadherin-based adhesions, in response to both endogenous, myosin II-dependent contractility (Yonemura 2011; Huveneers et al. 2012) and exogenously applied force on cadherin adhesions (le Duc et al. 2010; Thomas et al. 2013; Barry et al. 2014; Twiss et al. 2012). In MTC measurements of epithelial cells in which α-catenin was knocked down or deficient, E-cadherin perturbations failed to actuate cell stiffening or actin accumulation, but rescuing cells with GFP-tagged α-catenin restored E-cadherin-based cell stiffening and associated cytoskeletal remodeling (Barry et al. 2014). Exchanging the VBS in α-catenin for the equivalent sequence in its homolog, vinculin, also abolished force transduction (Barry et al. 2014).

The proximal actin and vinculin recruitment induced by magnetic beads was analogous to the force-activated changes at bona fide cell-cell junctions in dual pipette measurements and between contractile cells on micropillars (Liu et al. 2010; Thomas et al. 2013). This similarity confirmed that the bead twisting results reflect biochemical events at bona fide intercellular adhesions.

The development of a FRET-based, α-catenin conformation sensor (Fig. 3.4a) confirmed that α-catenin undergoes rapid, force-activated conformation switching in live cells (Kim et al. 2015). The biosensor incorporated FRET donor/acceptor pairs flanking the MI–MIII force-sensing effector module within the full-length α-catenin. In response to various mechanical perturbations of cadherin adhesions, at both bead-cell and cell-cell junctions (see Fig. 3.4c), and on time scales from a few seconds to several minutes, the expressed α-catenin biosensor exhibited immediate, force-dependent conformational switching. The force activated conformation change correlated with vinculin and actin recruitment to junctions. Removing the VBS from the sensor abolished vinculin recruitment, but not the conformation switching.

The sensor exhibited immediate, reversible conformation switching at acutely stretched junctions between epithelial cells on deformable hydrogel substrata (Fig. 3.4b). A nanoprobe placed on the gel adjacent to cell clusters generated an

Fig. 3.3 (continued) vinculin other downstream effectors such as MENA/VASP (Fig. 3.1). (**c**) Model of the 3D structure of α-catenin. The central force-sensing region comprises three, 4-helix bundles MI (*red*), MII (*green*), and MIII (*blue*), flanked by the N-terminal domain (N) and by the C-terminal (C), cytoplasmic, actin binding domains. (**d**) Sequence map of α-catenin showing the relationship between the N-terminal, core force-sensing MI–MIII region, and C-terminal domains. (**e**) In the proposed model, clusters of salt bridges at the junctions between MI, MII, and MIII domains of the core force-sensing region appear to confer mechanical stability. Tension disrupts salt bridges, and induces a conformational change in MI that exposes the vinculin binding site, and MII and MII, which remain folded, reorient to form a more stable configuration. Figures reproduced from (Li et al. 2015; Leckband and de Rooij 2014)

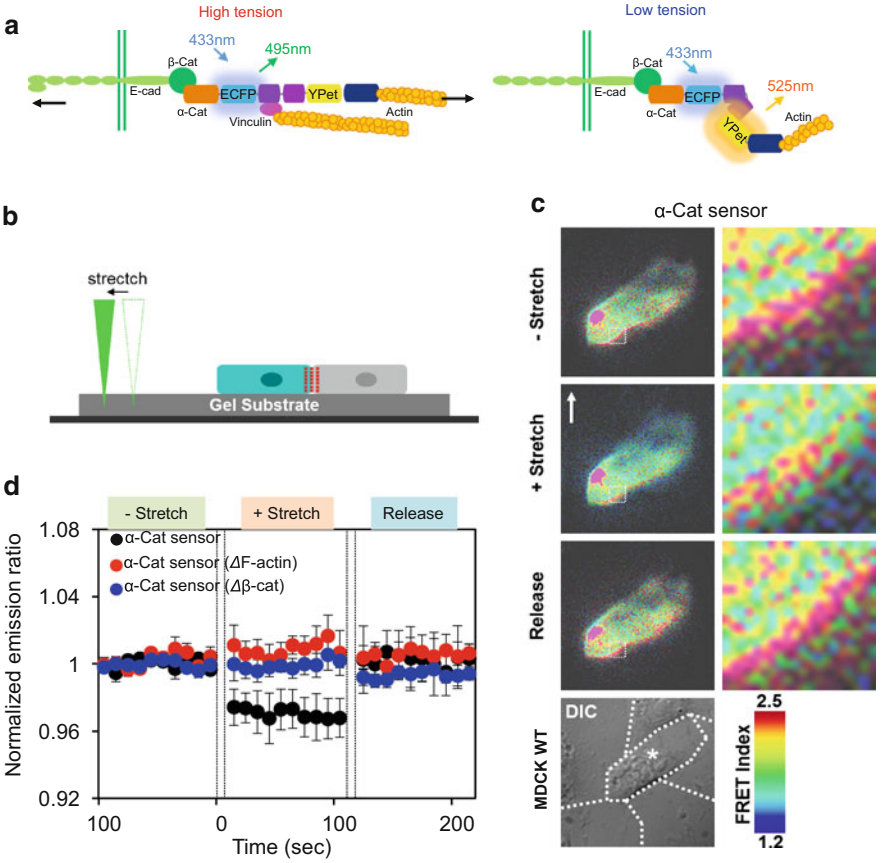

Fig. 3.4 Visualizing force-activated conformation switching by α-catenin. (**a**) FRET-based a-catenin conformation sensor incorporates enhanced cyan fluorescent protein (ECFP) at before (N-terminal to) the force-sensing module and YPet at the C-terminal end of the module. Under high force, the molecule extends to separate the donor and acceptor fluorophores. Excitation of the donor ECFP at 433 nm results in low FRET and donor emission at 495 nm. Under low tension, the molecule adopts the autoinhibited conformation, and exhibits high FRET and emission at 525 nm. (**b**) Nanoprobe substrate stretching device that acutely deforms the polyacrylamide gel on which cells are plated. The nanoprobe is indented into the gel adjacent to, but not touching cells, and stretches the gel laterally. (**c**) FRET images of the junction between two MDCK epithelial cells transfected with the α-catenin FRET sensor. Images show the junction before, during, and after stretching the gel. The lower panel is a DIC image of the cells. (**d**) FRET emission ratio measured at intercellular junctions as a function of time during the substrate stretching cycle. Substrate stretch is activated at $t=0$ s. *Black circles* indicate the intact sensor and *blue* and *red circles* indicate two controls. The *vertical dashed lines* indicate the time delay between activating/releasing the substrate stretch and image acquisition. Data show that the sensor undergoes an abrupt step change to a stable (open) conformation after substrate stretch, and recoils back to the initial state upon releasing substrate strain. Reproduced from (Kim et al. 2015)

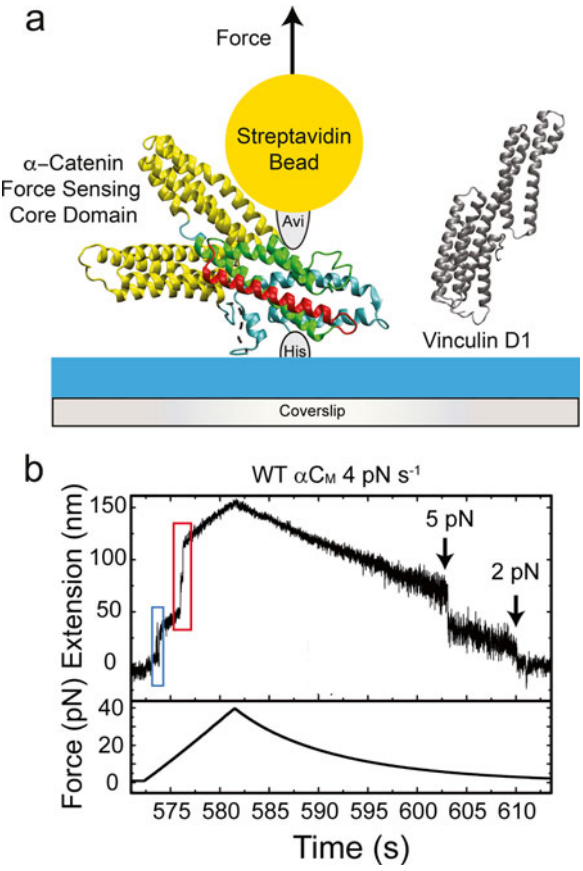

Fig. 3.5 Magnetic tweezers measurements reveal force-dependent unfolding and refolding of the α-catenin force-sensing module αC_M. (**a**) Experimental configuration used for magnetic tweezers studies of the forced unfolding of α-catenin. Biotinylated a-catenin was anchored to the substrate by an engineered hexahistidine tag. Streptavidin-coated magnetic beads then attached to the α-catenin and pulled on the protein. (**b**) Extension of the αC_M fragment in a representative force-extension-refolding cycle. During the stretch phase (force increase; lower panel) at a loading rate of 4 pN/s, there is an unfolding step at 5 pN (*blue box*), and two additional unfolding steps occurred at higher forces (*red box*). During the force-decrease (see lower panel), there are two refolding events: one at 5 pN and the other at 2 pN. The *inset* shows that the extension at 5 pN is reversible. Reproduced with permission from (Yao et al. 2014)

abrupt, step change in junction tension. FRET imaging of the cell-cell junctions during the stretch-release cycles (Fig. 3.4c) showed that the sensor exhibits immediate switching between conformation states with no further adaptation (Fig. 3.4d). This rapid, reversible switching with no adaptation demonstrates that, in vivo, α-catenin behaves as an elastic spring in series with cadherin bonds and actin (Fig. 3.4d).

Using sensitive magnetic tweezers (Fig. 3.5a) Yao et al. (2014) demonstrated directly that in vitro the putative force-sensing module of α-catenin unfolds

reversibly under piconewton forces. Increasing the force on the module resulted in an unfolding event at ~5 pN, and then two additional unfolding events at higher forces. The first, reversible unfolding event at ~5 pN results in an altered conformation that interacts with vinculin. The low level force required to activate α-catenin towards vinculin binding is comparable to average cadherin forces at epithelial junctions. Upon decreasing the force, the molecule refolded.

The reversible, force-activated α-catenin unfolding in vitro agrees with the behavior of the conformation sensor in vivo. Also, qualitative agreement between the forced unfolding trajectories in molecular dynamics simulations and features in the force-extension curves (Fig. 3.4b) suggests possible molecular events underlying the α-catenin unfolding mechanics (Li et al. 2015).

3.5.2 Vinculin and Cadherin-Based Force Transduction

Despite the importance of the MI domain of α-catenin (see Fig. 3.3) and its vinculin binding site, the role of cytosolic vinculin is less clear. Differential vinculin localization to focal adhesions and adherens junctions appears to be regulated by site-specific phosphorylation (Bays et al. 2014), but how phosphorylation targets vinculin to adherens junctions is unknown. Vinculin is also autoinhibited by high affinity binding between the head, which interacts with α-catenin and other proteins, and the actin-binding tail domain (Ziegler et al. 2006). Hence, the rate of vinculin recruitment to cadherin adhesions would be limited by the rate of alpha catenin activation. Indeed vinculin accumulation at re-annealing cell-cell junctions was over an order of magnitude slower than α-catenin conformation switching, but removing the inhibitory vinculin tail domain significantly enhanced the recruitment kinetics (Kim et al. 2015). This result supported the postulate that vinculin activation is a rate-determining step in its recruitment to stressed cadherin junctions. In vitro studies suggested that actin may co-activate vinculin and α-catenin and thereby enhance the kinetics (Choi et al. 2012), but this remains to be established in vivo.

Although deleting the VBS in α-catenin ablated the force-activated cell stiffening, knocking out vinculin in F9 epithelial cells only reduced force activated cell stiffening by ~40 % (le Duc et al. 2010). Vinculin may not, therefore, be the sole effector in cadherin mechanotransduction. The majority of studies have so far used vinculin as the principal marker for cadherin-based mechanotransduction, and therefore did not address additional mechanisms or roles of other cytosolic binding partners.

Despite these questions, vinculin is an important early effector in intercellular mechanotransduction in several documented cell contexts. It is required for the compaction of cell-cell junctions in breast cancer cells (Maddugoda et al. 2007), and the vinculin site in α-catenin is required for the assembly of apical adherens junctions between colon cancer cells (Imamura et al. 1999; Huveneers and de Rooij 2013; Watabe-Uchida et al. 1998). Besides enhancing cytoskeletal anchorage, in

epithelia, vinculin facilitates junction reinforcement, by recruiting Mena and VASP, which promote proximal actin polymerization (Leerberg et al. 2014).

3.6 Functional Consequences of Force-Activated Cytoskeletal Remodeling

The positive feedback through which force induces remodeling of intercellular junctions is a common feature of cadherin-based force transduction cascades, such as described in the previous sections. Changes observed in a diversity of assays include adhesion strengthening (Thomas et al. 2013), junction growth (Liu et al. 2010), altered junction stiffness (le Duc et al. 2010), and the dependence of cadherin-based traction forces (contractility) on substratum rigidity (Ladoux et al. 2010; le Duc et al. 2010). Such force-dependent changes can be protective as well as disruptive.

Inflammatory mediators, which activate RhoA and myosin II contractility, disrupt interendothelial junctions and increase vascular leak (Daneshjou et al. 2015). Increased RhoA activity associated with intimal stiffening in arteries and rigid substrates correlates with leakier interendothelial junctions (Huynh et al. 2011; Krishnan et al. 2011). Similarly, challenge with TGFβ or HGF, which increase RhoA activity increases epithelial cell scattering (de Rooij et al. 2005). One postulate is that proximal RhoA and downstream effectors disrupt junctions through biochemical changes that alter the phosphorylation status of junctional proteins (Behrens et al. 1993; Brady-Kalnay et al. 1995; Burden-Gulley and Brady-Kalnay 1999; Lilien and Balsamo 2005). However, analyses of force balances and actin in epithelial cells during cell scattering suggested that protrusive forces at the leading edge of cells reorient actin fibers perpendicular to distal cell-cell junctions (Maruthamuthu and Gardel 2014). This actin remodeling in turn redistributes force on cadherin contacts, in ways that may mechanically rupture cadherin-cadherin bonds. Clearly many questions remain regarding the role of force in junction assembly and stabilization, as well as how the distribution and magnitude of applied forces contribute to junction homeostasis.

3.7 Alternative Components and Possible Mechanisms

The modest effect of the vinculin knockout suggested that other proteins or mechanisms may also contribute to intercellular force transduction. Known proteins at cadherin adhesions that also bind α-catenin and regulate actomyosin dynamics and organization include formins (Kobielak et al. 2004), 1-afadin (Sawyer et al. 2009; Pokutta et al. 2002), and α-actinin (Kobielak and Fuchs 2004). These additional binders could further modulate the switching dynamics of α-catenin, which is an allosterically regulated protein (Ishiyama et al. 2013; Choi et al. 2012; Rangarajan

and Izard 2012). They could also be involved in other, secondary mechano-responses enabled by tension across cadherin junctions.

Vinculin and α-actinin bind overlapping regions of the MI domain of α-catenin (Kobielak and Fuchs 2004). α-Actinin-4 regulates actin-binding proteins that assemble and maintain F-actin at E-cadherin adhesions (Nieset et al. 1997; Tang and Brieher 2012), and could thus link cadherin mechanotransduction to F-actin polymerization. Formin1 binds the MI and MII domains of α-catenin (see Fig. 3.2d) (Kobielak et al. 2004). Formins are involved in force-dependent actin polymerization (Kozlov and Bershadsky 2004), and Formin1 could have a similar role in F-actin nucleation and polymerization. Specifically, Formin1 localizes to punctate cadherin junctions under tension, is implicated in adherens junction formation, and was shown to facilitate force-dependent actin polymerization (Kobielak and Fuchs 2004; Higashida et al. 2013).

The α-catenin binding protein AF-6 (Afadin in *Drosophila*) also binds the MII domain of α-catenin, and was required during apical constriction and germ band extension in *Drosophila* embryos (Sawyer et al. 2010; Sawyer et al. 2011). The latter processes both involve large increases in actomyosin contractility, and the absence of Afadin in *Drosophila* caused the actomyosin network to detach from cell-cell junctions during these processes (Sawyer et al. 2009; Sawyer et al. 2011). Thus, multiple proteins associated with α-catenin could be involved in force transduction in different cellular and mechanical contexts.

3.8 Intercellular Force Transduction Beyond α-Catenin

3.8.1 Force Transduction by Mechanically Linked Proteins

The mechanical chain formed by assembly of proteins linking cadherin adhesions to the cytoskeleton comprises a host of other proteins, such as β-catenin or other actin binders like EPLIN whose conformations and biochemical activity may also be force-modulated. For example, under low pN forces (~20–40 pN), isolated β-catenin stretches and refolds in discrete steps indicative of the unfolding of individual subdomains (Valbuena et al. 2012). Alternatively, although other proteins might not be involved in immediate force transduction, they could contribute to secondary, cadherin-dependent signaling. In the case of integrins, for example, Src activation was observed at sites of microtubule deformations distal from mechanically perturbed integrins (Na et al. 2008).

There are indications that force-dependent conformational regulation of F-actin and its binding proteins also contribute to mechanotransduction at cadherin adhesions. Zyxin exhibits localized, force-dependent recruitment to stressed F-actin, either at focal adhesions or within F-actin fibers (Hirata et al. 2008; Colombelli et al. 2009; Smith et al. 2010). Zyxin in turn recruits VASP, as well as possible additional proteins that regulate actin polymerization (Smith et al. 2010). Both Zyxin and VASP were implicated in the regulation of F-actin dynamics and organization

at cell-cell junctions (Leerberg et al. 2014; Nguyen et al. 2010; Scott et al. 2006; Sperry et al. 2010), and VASP also affects the force-dependent reinforcement of adhesion between VE-cadherin-coated beads and endothelial cells (Kris et al. 2008).

Beyond proximal actin perturbations, cadherin-linked actin filaments could also propagate mechanical forces across the cell to perturb distal, actin-coupled proteins such as integrins. Deemed "cytoplasmic coherence" in the case of integrins and myosin IIa (Cai et al. 2010), cadherins could similarly propagate force through the microfilament network by stress focusing (Hu et al. 2003), although this possibility has not been explored.

3.8.2 Force Activated Global Signaling Cascades

Investigations of cadherin-mediated force transduction have primarily focused on proximal cytoskeletal remodeling, but more extensive cytoskeletal organization could also propagate over large distances. The altered directional cell migration and front-rear asymmetry of keratin filaments induced by mechanically stimulating the C-cadherin complex in mesendoderm cells is an example (Weber et al. 2012). Differences in stress fiber morphology in cells on rigid versus soft cadherin-coated substrates suggest that cadherin mechanotransduction may regulate global actomyosin organization (Ladoux et al. 2010). Cardiomyocytes cultured on N-cadherin substrata with physiological rigidity exhibited stereotypical cytoskeletal organization, as in cardiac tissue (Chopra et al. 2011).

Cadherin-based mechanotransduction may also activate signal cascades with distant effects on both the immediately perturbed cells and surrounding tissue. Long-ranged effects could be transmitted through the cytoskeleton, by stress focusing (Hu et al. 2003), or by activating global, diffusible signaling proteins. For example, in endothelial cells, VE-cadherin-based mechanotransduction triggered global changes that altered focal adhesions and cell contractility, and resulted in the disruption of peripheral cadherin adhesions. Moreover, the VE-cadherin-specific disturbances propagated through the cell, across junctions, and through the monolayer to disrupt junctions between cells that did not share a boundary with the perturbed cell or its immediate neighbors (Barry et al. 2015). Force-independent, E-cadherin ligation to E-cadherin modified beads altered integrin-based traction forces (Jasaitis et al. 2012). In the latter studies, Src, phosphoinositide-3 kinase, myosin II, and ROCK inhibitors blocked the long-range intra- and intercellular perturbations. In addition, in epithelial cell cultures, the assembly of intercellular junctions correlated with focal adhesion remodeling (Mertz et al. 2013). These results anticipate the existence of other force-activated cadherin mechanotransduction signaling, beyond α-catenin conformation switching, that globally alter cell and tissue mechanics.

So far, no global (long-ranged), cadherin-based force transduction signal cascade has been identified, but parallels with mechanotransduction by the immunoglobulin family protein Ig CAM, platelet endothelial cell adhesion molecule one

(PECAM-1), may provide clues. Initial studies of endothelial flow sensing found that PECAM-1, VE-cadherin, and VEGFR2 at intercellular junctions were required for shear stress-dependent cytoskeletal alignment, but that PECAM-1 was the principle force sensor (Tzima et al. 2001; Tzima et al. 2005). Magnetic bead pulls on PECAM-1 revealed a global force-activated cascade in which PECAM-1 perturbations activate PI3K, in an as yet incompletely defined mechanism requiring VEGFR2 and VE-cadherin (Collins et al. 2012). PI3K is an upstream activator of integrins, which in turn activated RhoA through Rho guanine exchange factors LARG and RhoGEF H1 (Guilluy et al. 2011). Cell stiffening following PECAM-1 stimulation was attributed to the Rho-dependent increase in myosin II-dependent cell contractility (Conway et al. 2013). Whether cadherins activate a similar pathway remains to be established.

3.9 Cadherin Mechanotransduction in Development and Disease

Evidence supporting the importance of cadherin-based mechanotransduction in vivo still lags, but studies of model organisms suggest its importance in development and disease. For example, in fly development, forces converging at cadherin junctions influence cytoskeletal organization and cell shape, and α-catenin-dependent force transduction orchestrates junction-remodeling processes involved in tissue morphogenesis (Levayer and Lecuit 2013; Lecuit et al. 2011). α-Catenin is essential for morphogenetic processes during fly development that involve large changes in tension on junctions. Hyperplastic wing growth correlates with tension across the imaginal wing disk in the fly (Schluck et al. 2013). Recent findings showed that force on cadherin junctions and α-catenin activates the growth-regulatory Hippo pathway to in turn activate the nuclear localization of the transcriptional activator Yorkie (Yap/Taz in vertebrates) and cell proliferation (Rauskolb et al. 2014; Reddy and Irvine 2013).

In higher organisms, there are correlations, but thus far no clear evidence for causal links between cadherin-based mechanotransduction and physiology. Mechanically stretching cultured epithelial monolayers activated Yap1 to drive cell cycle entry (Benham-Pyle et al. 2015). In the cardiovascular system of mammals, heart development requires oscillations in blood flow and pressure that result in fluctuating tension at cell-cell junctions (Granados-Riveron and Brook 2012). Cardiomyocyte-specific vinculin knockouts correlated with developmental defects in specialized cell-cell junctions of the intercalated disk (Zemljic-Harpf et al. 2014). Cell contractility, which increases force on cell-cell junctions, induces endothelial barrier disruption in the microvasculature during leukocyte transmigration, and thrombin stimulated endothelial contractility contributes to increased paracellular permeability. Increased Rho activity associated with stiffened arteries also correlates with enhanced vascular leakage in vivo (Huynh et al. 2011). The finding that vinculin recruitment to VE-cadherin junctions protects against barrier disruption

under tension (Huveneers et al. 2012) suggests a role for cadherin mechanotransduction in endothelial homeostasis. Although PECAM-1 was the identified flow sensor at interendothelial junctions (Tzima et al. 2005), the association of VE-cadherin with VEGFR2 versus VEGFR3 determined the fluid shear stress at which cells preferentially aligned with fluid flow in arterial and lymphatic tissues, respectively (Baeyens et al. 2015; Coon et al. 2015).

3.10 Summary and Future Directions

This chapter highlights recent findings regarding a new mechanosensing mechanism at cadherin-dependent, intercellular junctions. These cadherin complexes sense force and trigger molecular cascades that remodel adhesions and alter the intercellular junction mechanics. Initial studies identified an α-catenin-dependent pathway, in which tension-dependent conformational changes in α-catenin actuate vinculin recruitment and actin remodeling at junctions. This mechanism has been verified with diverse experimental approaches and cell types. Beyond α-catenin, there are however, a number of additional, possible secondary mechanotransduction events that could also be activated as a consequence of cadherin adhesion and force fluctuations at cell-cell junctions. Finally, studies in model organisms are beginning to reveal the broader implications of intercellular force transduction, but the implications for development, tissue regeneration, and disease in mammals remain a wide open question.

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Part II
Transducers

Chapter 4
Emerging Roles of YAP/TAZ in Mechanobiology

Yubing Sun, Yue Shao, Xufeng Xue, and Jianping Fu

Abstract Understanding mechanotransduction is a major goal in the field of mechanobiology. YAP, and its paralog TAZ, are transcription coactivators at the core of the canonical Hippo signaling pathway. Recent studies have identified YAP/TAZ as both mechano-sensors and -transducers that respond to multiple extracellular mechanical signals and relay them to downstream transcriptional signals to regulate cell functions. In this chapter, we discuss how different types of mechanical cues, including the actin cytoskeleton, substrate rigidity, and external mechanical forces, mediate YAP/TAZ activities. We also discuss some possible mechanosensitive molecular machineries that function upstream of YAP/TAZ to control their mechanotransductive properties.

Keywords YAP • TAZ • Hippo • Mechanotransduction • The actin cytoskeleton • Rigidity sensing • Mechanical forces

Terms and Abbreviations

ECM	Extracellular matrix
HA	Hyaluronic acid
hESCs	Human embryonic stem cells
hMSCs	Human mesenchymal stem cells
Lats	Large tumor suppressor
ROCK	Rho-associated coiled-coil-containing protein kinase
TAZ	PDZ-binding motif
YAP	Yes associated protein

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Mechanotransduction	Biological processes whereby cells convert mechanical stimuli into intracellular biochemical responses
Focal adhesion (FA)	Adhesion sites for cell attachment to the ECM where intracellular actin filaments can link to ECM proteins through transmembrane proteins such as integrins.

4.1 Introduction

Mechanobiology research focuses on studying responses of mammalian cells or tissues to dynamic mechanical signals in the cell microenvironment and the underlying mechanotransductive mechanisms. RhoA/ROCK signaling has been identified as one of the major signaling pathways that interact with various mechanosensitive components (i.e., integrin, focal adhesion, actin cytoskeleton (CSK), myosin) to regulate cellular behaviors such as adhesion, spreading, and contractile forces (Sun et al. 2012; McBeath et al. 2004; Arnsdorf et al. 2009; Riveline et al. 2001). However, it remains largely elusive how, at the transcriptional level, extracellular mechanical signals regulate intracellular signaling cascades and gene expression. Recently, several prominent studies have identified YAP/TAZ, the core transcriptional components in the canonical Hippo signaling pathway, as nuclear mechano-sensors and -transducers to regular mechanosensitive cellular behaviors (Piccolo et al. 2014; Low et al. 2014; Hao et al. 2014; Dupont et al. 2011). Importantly, Hippo signaling is a highly conserved pathway, and has been demonstrated to crosstalk with many major signaling cascades in mammalian cells. Thus, it is important to understand functional roles of YAP/TAZ in mechanobiology to further extend our current molecular understanding of mechanotransduction.

YAP, and its paralog TAZ, are transcription coactivators at the core of Hippo signaling pathway (Zhao et al. 2007). They can be inactivated through Lats-dependent cytoplasmic sequestration and degradation, and function mainly through binding to TEAD family transcription factors (Zhao et al. 2007; Zhao et al. 2014). Hippo/YAP signaling has been shown to regulate many fundamental biological processes including proliferation (Heallen et al. 2011), tumorigenesis (Wang et al. 2014), epithelial-mesenchymal transition (EMT) (Diepenbruck et al. 2014), metabolism (Sorrentino et al. 2014), energy stress (DeRan et al. 2014), oxidative stress (Shao et al. 2014), polarity (Genevet and Tapon 2011), wound healing (Lee et al. 2014), senescence (Xie et al. 2013), anoikis (Zhao et al. 2012), differentiation (Zhao et al. 2011a), and reprogramming (Qin et al. 2012). Hippo/YAP signaling has also been shown important for homeostatic regulation of various tissues including intestinal epithelium (Imajo et al. 2015), airway epithelium (Zhao et al. 2014; Mahoney et al. 2014), skeletal and cardiac muscles (Wackerhage et al. 2014), skin (Quan et al. 2014), liver (Grijalva et al. 2014), and pancreas (George et al. 2012).

Given the central role of YAP/TAZ activities in mammalian cells and their functional relationship to extracellular mechanical signals, it is of great importance to understand how different types of mechanical cues regulate YAP/TAZ activities under different contexts. In this chapter, we provide a concise review of recent findings showing YAP/TAZ-mediated mechanotransductive processes. We describe how the actin CSK, substrate rigidity, and external forces regulate YAP/TAZ activities. Readers are also referred to other recent excellent reviews focusing on specifically YAP/TAZ signaling and their functional crosstalk with other major signaling pathways (Piccolo et al. 2014; Varelas 2014; Yu and Guan 2013; Barry and Camargo 2013).

4.2 Regulation of YAP/TAZ by Actin CSK

The actin CSK functions as a control center in cell signaling (DuFort et al. 2011). In particular, the actin CSK is integrated into the overall cell signaling network through its multifaceted functions, e.g., generation of intracellular contractility (DuFort et al. 2011; Maruthamuthu et al. 2010; Schwarz and Gardel 2012), control of cell morphology (McBeath et al. 2004; Wada et al. 2011), providing binding scaffold for various signaling proteins (Blanchoin et al. 2014), and, in its monomer form, a nucleocytoplasmic shuttling carrier for transcription factors (Ho et al. 2013). In recent studies, the actin CSK has been found of particular significance in regulating YAP/TAZ-mediated mechanotransduction.

Pointing to possible functional connections between the actin CSK and YAP/TAZ activities, early studies have shown that responses of YAP/TAZ to both cell shape and substrate rigidity are similar to their effects on the actin CSK architecture in mechanosensitive mammalian cells. Specifically, prominent, bundled stress fibers are observable in cells cultured on rigid substrates or grown with little geometric confinement, correlating with nuclear accumulations of YAP/TAZ. In contrast, cortical actin meshwork with diffusive actin filaments is usually observed in cells cultured on soft substrates or under strong physical confinement, correlating with cytoplasmic retention of YAP/TAZ (Dupont et al. 2011; Connelly et al. 2010; Sun et al. 2014a). Although such correlative observations have been made for some time, direct evidence showing the functional role of the actin CSK in YAP/TAZ-mediated mechanotransduction did not come until very recently.

The first concrete evidence came from studies using pharmacological perturbations targeting the actin CSK integrity. In mammalian cells treated with actin polymerization inhibitors, such as Latrunculin A and Cytochalasin D, YAP/TAZ were retained in the cytoplasm and remained inactive (Fig. 4.1a) (Wada et al. 2011). In contrast, treating cells with actin polymerization activator Jasplakinolide activated YAP/TAZ (Reddy et al. 2013). Researchers also utilized molecular and genetics tools to specifically examine the role of the actin CSK in YAP/TAZ-mediated mechanotransduction (Piccolo et al. 2014). Aragona et al. demonstrated that knockdown

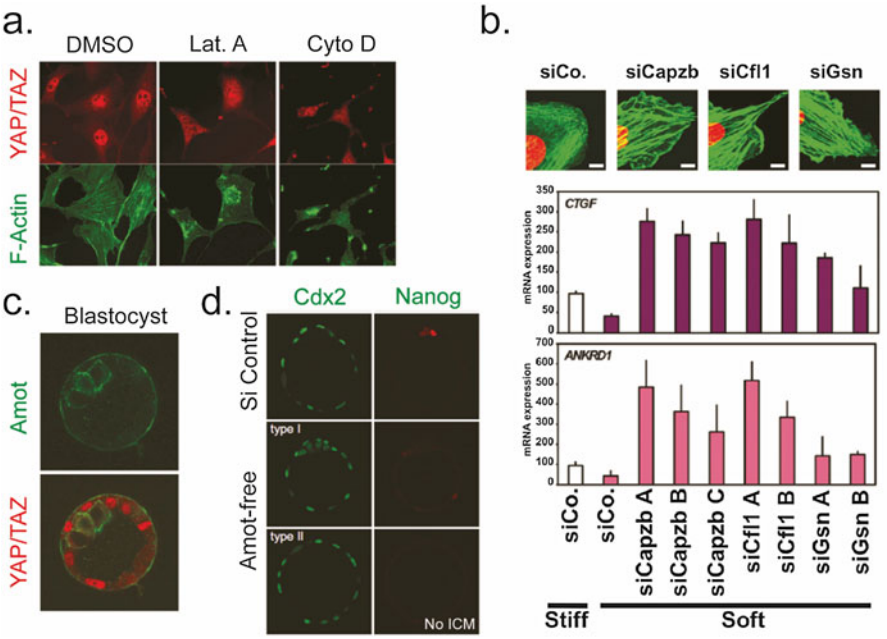

Fig. 4.1 (**a**) Immunofluorescence images showing actin disruption by Cytochalasin D (Cyto D) and Latrunculin A (Lat. A) could promote YAP/TAZ inactivation through cytoplasmic retention. Adapted with permission from Wada et al. (2011). Copyright 2011, the Company of Biologists Ltd. (**b**) (*top* panel) Immunofluorescence images showing enhanced actin polymerization upon knockdown of actin capping proteins cap-Z (siCapzb), cofilin (siCfl1), and gelsolin (siGsn). (*bottom* panel) Gene expression results showing the rescue of YAP/TAZ activity on soft substrate by knocking down actin capping proteins. Adapted with permission from Aragona et al. (2013). Copyright 2013, the Cell Press. (**c**) Immunofluorescence images showing the differential localization of Angiomotins (Amot) in mouse blastocyst, and the corresponding nuclear/cytoplasmic localization of YAP/TAZ. (**d**) Immunofluorescence images showing ectopic expression of Cdx2 in inner cell mass (ICM) cells upon Angiomotins knockdown, suggesting the physiologically apically localized Angiomotins are inactive, while the cell–cell junction localized Angiomotins in ICM cells are active. Adapted with permission from Hirate et al. (2013). Copyright 2013, the Cell Press

of actin capping proteins, such as cofilin, cap-Z, or gelsolin, was sufficient to override mechanosensitive control of cell proliferation and differentiation through YAP/TAZ overactivation (Fig. 4.1b) (Aragona et al. 2013). Similarly, Fernandez et al. demonstrated in *Drosophila* that actin capping proteins, e.g., cofilin and capulet, were required for suppressing Yorki (*Drosophila* homolog of YAP) activity during wing tissue development (Fernandez et al. 2011).

Although the role of the actin CSK in controlling YAP/TAZ-mediated mechanotransduction has now been established, it remains elusive how integrity and dynamics of the actin CSK are relayed to YAP/TAZ activities. Recent studies have suggested cell type specific molecular mechanisms may be in play. Here we briefly review a few models proposed recently to explain signaling events downstream of the actin CSK to control YAP/TAZ activities.

Lats1, which is the upstream kinase in the Hippo pathway that inactivates YAP/TAZ through phosphorylation, has recently been suggested as an actin-binding protein (Visser-Grieve et al. 2011). This raises an interesting possibility that the actin CSK may serve as a scaffold to sequester Lats1 from either interacting with upstream kinases such as Merlin, or inactivating downstream YAP/TAZ, leading to YAP/TAZ activation under conditions favoring prominent actin stress fibers, e.g., rigid substrates and unconstrained cell spreading (Shao et al. 2015). This model is consistent with early findings by Wada et al. that suppression of YAP/TAZ activities from disruption of the actin CSK would require Lats kinases (Wada et al. 2011). This hypothesis is also supported by recent findings that direct binding of Merlin with Wts (*Drosophila* homolog of Lats) became biochemically detectable only after treatments of cells with Latrunculin A or Rho GTPase inhibitor C3, which disrupted the actin CSK (Yin et al. 2013). However, there were also conflicting results reported recently suggesting that Lats knockdown was not sufficient to abolish YAP/TAZ sensitivity to pharmacological perturbations to the actin CSK, suggesting that there might be other mechanisms acting in parallel to transduce the CSK integrity to downstream YAP/TAZ activities (Dupont et al. 2011).

Another family of actin-binding proteins, the Angiomotin (AMOT) family proteins, can also regulate YAP/TAZ activities with direct interactions through conserved PPxY domains (Chan et al. 2011; Zhao et al. 2011b). Importantly, AMOT proteins are cytoplasmic proteins. Thus, AMOT proteins, in principle, can function as suppressors of YAP/TAZ through cytoplasmic retention, as shown in early overexpression studies (Zhao et al. 2011b). Interestingly, recent studies revealed that AMOT proteins could be sequestered by the actin CSK depending on their phosphorylation states. In particular, while wild-type, non-phosphorylated, or constitutively non-phosphorylatable AMOT proteins could bind to and be sequestered by the actin CSK, phosphorylated (or phosphor-mimic form of) AMOT proteins would remain in the cytoplasm, bind to and inactivate YAP/TAZ (Chan et al. 2013; Mana-Capelli et al. 2014; Dai et al. 2013). Such mechanism supports the switchable interaction between the actin CSK and AMOT proteins to relay mechanotransductive signals from the actin CSK to downstream YAP/TAZ activities.

Recent findings have specifically supported that cytoplasmic localization of AMOT proteins is important for functional regulation of YAP/TAZ activities (Hirate et al. 2013). Experiments in mouse embryos showed that AMOT-mediated YAP/TAZ cytoplasmic retention was switched off when AMOT proteins were predominantly associated with the apical actin CSK, which could occur in the trophectoderm (Fig. 4.1c) (Hirate et al. 2013; Leung and Zernicka-Goetz 2013). However, when AMOT proteins predominantly localized to the apical Crumbs complexes or adherent junctions, as occurred in the inner cell mass cells, the suppressive function of AMOT proteins was switched back on through direct binding between YAP/TAZ and AMOT proteins (Hirate et al. 2013; Leung and Zernicka-Goetz 2013; Varelas et al. 2010). Such subcellular position-dependent action of AMOT proteins suggests its important role in determining lineage choices between trophectoderm and inner cell mass through regulation of YAP/TAZ activities in early blastocysts (Fig. 4.1d).

4.3 YAP/TAZ Activities Mediated by Substrate Rigidity

As probably the most extensively studied mechanical cue in the cell microenvironment, substrate rigidity has been found to regulate YAP/TAZ activities in many cell systems. Dupont et al. first observed nuclear exclusion of YAP/TAZ in mammary epithelial cells (MECs), MDA-MB-231 breast cancer cells, and human mesenchymal stem cells (hMSCs) seeded on soft hydrogels and elastic micropost arrays (Fig. 4.2a) (Dupont et al. 2011). Dupont et al. further reported that such rigidity-dependent nucleocytoplasmic shuttling of YAP/TAZ was Lats independent, as MDA-MB-231 cells depleted with endogenous YAP/TAZ but expressing

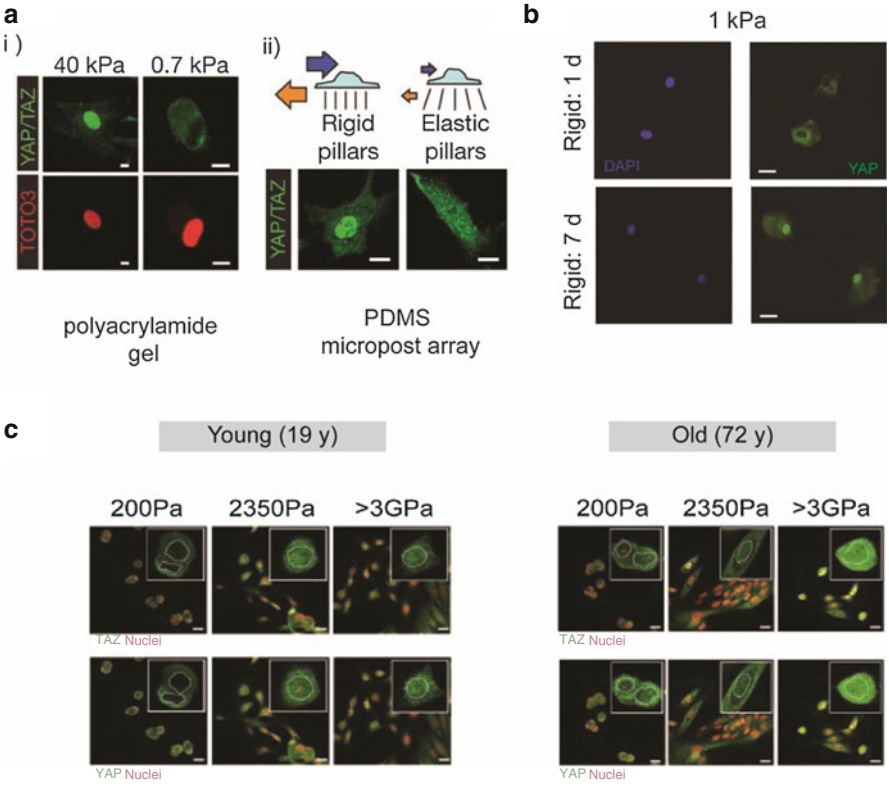

Fig. 4.2 (**a**) Confocal immunofluorescence images of YAP/TAZ and nuclei (TOTO3) in human mesenchymal stem cells (hMSCs) plated on (i) polyacrylamide hydrogels or on (ii) arrays of micropillars of different rigidities. Scale bars, 15 μm. Adapted from Dupont et al. (2011), Copyright 2011, the Nature Publishing Group. (**b**) YAP localization in hMSCs on soft hydrogels with 1 day of mechanical dosing on TCPS and 7 days of mechanical dosing on TCPS. Adapted from Yang et al. (2014). Copyright 2014, the Nature Publishing Group. (**c**) Immunofluorescence images of TAZ, YAP, and DAPI in HMEC from a young strain (*left*; 19 years) and an older strain (*right*; 72 years). Scale bar, 20 μm. Adapted from Pelissier et al. (2014)

Lats-insensitive 4SA[16] TAZ remained sensitive to substrate rigidity, similar to wild-type controls. Interestingly, TAZ expression was also downregulated in MECs on soft substrates. Together, these results suggest Hippo-independent mechanism(s) in play for controlling mechanosensitive YAP/TAZ localization and expression. Notably, Dupont et al. further demonstrated that the actin CSK tension and Rho activities were required for nuclear localization of YAP/TAZ in hMSCs. However, a conflicting observation was reported recently by Rauskolb et al. that in *Drosophila*, high CSK tension could promote Yorkie (*Drosophila* homologue of YAP) activity by facilitating recruitment of Warts (*Drosophila* homologue of Lats) through Jub (also known as Ajuba LIM protein) (Rauskolb et al. 2014). It remains to be fully determined the involvement of the Hippo pathway in regulating rigidity-dependent YAP/TAZ activities.

In addition to responding to mechanical cues, YAP/TAZ also function as mechanotransducers to mediate downstream long-term cell behaviors including proliferation and stem cell differentiation. Dupont et al. showed that YAP/TAZ knockdown promoted adipogenic differentiation of hMSCs on rigid substrates, thus mimicking the effect of seeding hMSCs on soft substrates. We and others recently observed that neural conversion of human pluripotent stem cells (hPSCs) was also mechanosensitive and depended on Hippo/YAP signaling (Sun et al. 2014b; Musah et al. 2014). Specifically, soft, elastic micropost arrays promoted neuroepithelial differentiation and cytoplasmic retention of YAP/TAZ in hPSCs. TAZ knockdown also induced neuroepithelial differentiation of hPSCs on tissue culture dishes (Varelas et al. 2008). Importantly, our data further suggested that mechanosensitive YAP/TAZ nucleocytoplasmic shuttling required Lats. Higher level of phosphorylated YAP at serine 127 was observed for hPSCs seeded on soft substrates. Furthermore, Lats knockdown attenuated the effect of soft substrates on promoting neuroepithelial induction of hPSCs. It should be noted that protein expression levels of YAP/TAZ in hPSCs was insensitive to substrate rigidity, excluding Lats-mediated ubiquitination and degradation of YAP/TAZ as a mechanotransductive mechanism in play in hPSCs.

Another recent work from Yang et al. highlighted YAP/TAZ activities as an intracellular mechanical rheostat to store information from past physical environments for epigenetic regulation of stem cell fates (Yang et al. 2014). Specifically, Yang et al. showed that for freshly isolated hMSCs pre-exposed to rigid microenvironment (i.e., tissue culture dishes) for 7 days, YAP remained activated even after hMSCs were passaged to and cultured on soft hydrogels for 3 days. In contrast, for hMSCs exposed to rigid tissue culture plates for only 1 day, YAP was excluded from the nucleus immediately after the cells were passaged to soft hydrogels (Fig. 4.2b). Yang et al. further applied a photodegradable hydrogel system to dynamically control substrate rigidity. Subcellular YAP localization was shown to be reversibly modulated by substrate rigidity only when hMSCs were cultured on rigid substrates for a short period of time (1 day). Longer exposure to rigid environment (>10 days) could prevent YAP from shuttling to the cytoplasm even when the phototunable hydrogel system was tuned to become softer. Strikingly, treating hMSCs with Latrunculin A did not prevent YAP from nuclear translocation for hMSCs cultured on rigid hydrogels, contradictory to what Dupont et al. reported previously. Importantly, nucleocytoplasmic shuttling of

YAP coincided with Runx2, an important transcription factor known to drive osteogenic differentiation of hMSCs, even though the functional connection between YAP and Runx2 remains to be fully examined.

Recent evidence also suggests that the effect of cellular aging and senescence on cellular mechanosensitivity may be connected to YAP/TAZ activities (Cheung et al. 2012; Cheung et al. 2013; Wu et al. 2011). For example, Pelissier et al. showed that human mammary multipotent progenitors (hMMPs) isolated from young adults preferentially differentiated into myoepithelial cells (MEPs) but not luminal epithelial cells (LEPs) when cultured on substrates with a physiologically relevant rigidity (2 kPa) compared to softer substrates (200 Pa) (Pelissier et al. 2014). In contrast, hMMPs obtained from older adults did not show such mechanosensitive differentiation preference when cultured on substrates covering the same rigidity range. Pelissier et al. further showed that in hMMPs isolated from young adults, nuclear localization of YAP/TAZ was associated with MEP phenotype and was required for mechanosensitive lineage decisions, as YAP/TAZ knockdown attenuated mechanosensitivity of hMMPs and promoted LEP differentiation regardless of substrate rigidity (Fig. 4.2c). However, in hMMPs obtained from older adults, nuclear translocation of YAP/TAZ could only be promoted by substrate rigidity beyond the physiological range (>3 GPa), and YAP/TAZ knockdown did not affect lineage bias on substrates with the physiological range of rigidities (200 Pa–2 KPa).

In addition to monitoring and transducing mechanical cues in the extracellular space, YAP/TAZ can also feedback to remodel extracellular matrix (ECM) through myosin-mediated CSK tension. Such mechanoregulation and feedback between mammalian cells and the ECM is of particular importance to tumor progression, as it has been well documented that increased matrix rigidity is often observed in solid tumors (Paszek et al. 2005). Calvo et al. observed that ECM stiffening and YAP activation could form a feed-forward self-reinforcing loop in cancer-associated fibroblasts (CAFs), playing an important role in cancer cell invasion and angiogenesis (Calvo et al. 2013). Compared with normal mammary fibroblasts (NFs), CAFs exhibited elevated YAP activity and more pronounced stress fibers and focal adhesions. Importantly, CAFs but not NFs induced strong contraction of collagen gels, leading to significantly stiffened gels. This matrix remodeling capability depended on YAP-mediated myosin light chain 2 (MLC2) activity. Activating YAP in NFs enhanced their matrix remodeling capability, and YAP knockdown or inhibition of ROCK/myosin signaling in CAFs blocked their matrix remodeling capability.

4.4 Force-Mediated YAP/TAZ Activities

Mechanical forces including shear stress, pulling and compression by neighboring cells, and mechanical stretch have all been shown to affect cellular behaviors such as the CSK reorganization, cell proliferation, and stem cells differentiation. Recent findings suggest that YAP/TAZ play pivotal roles in sensing these mechanical forces.

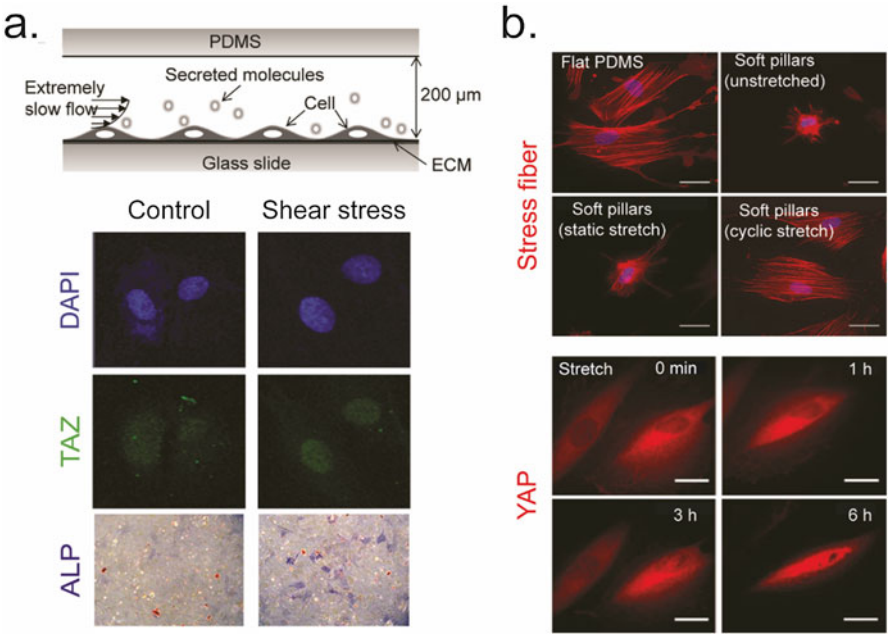

Fig. 4.3 (**a**) Shear stress induces TAZ nuclear localization and MSCs osteogenic differentiation. (*top* panel) Cells in the microchannel are exposed to an extremely slow interstitial level of flow. (*middle* panel) Immunofluorescence images showing the nuclear localization of TAZ under shear stress. (*bottom* panel) Alkaline phosphatase activity showing MSCs osteogenic differentiation under shear stress. Adapted from Kim et al. (2014). (**b**) Cyclic stretch of soft substrates induces spreading and growth. (*top* panel) Immunofluorescence images showing the effect of substrate rigidity and stretching type on cell spreading and stress fiber formation. (*bottom* panel) YAP-GFP (green fluorescent protein tagged Yes-associated protein) activity under cyclic stretch showing that YAP gradually localized into nucleus with cyclic stretch even on soft substrate. Adapted from Cui et al. (2015)

Interstitial flow describes slow extravascular flow in the extracellular matrix. Shear stress resulted from interstitial flow can regulate proliferation and differentiation of hMSCs. To study the effect of interstitial flow on cellular behaviors, Kim et al. recently developed an osmotic driven microfluidic chip to generate extremely slow flow (0.012–0.015 Pa) mimicking interstitial flow (Fig. 4.3a) (Kim et al. 2014). Using this microfluidic platform, Kim et al. observed that such interstitial flow could induce nuclear localization and transcriptional activity of TAZ and further promote hMSC osteogenic differentiation. Shear stress-induced osteogenic differentiation of TAZ-depleted hMSCs was significantly attenuated. Thus, TAZ appeared to be an important mediator for regulating interstitial flow-mediated hMSC osteogenic differentiation. Importantly, shear stress could not promote TAZ nuclear translocation and hMSC osteogenesis when RhoA/ROCK signaling was inhibited, indicating that RhoA/ROCK signaling was involved upstream in interstitial flow-mediated osteogenesis through regulation of TAZ activity. This observation was consistent with previous discussions about rigidity-mediated YAP/TAZ activities

through activation of Rho GTPase. Another recent study by Zhong et al. further investigated the effect of shear stress in a broader range (0.0009–0.1089 Pa) and reported similarly that shear stress could promote nuclear localization of YAP and hMSC osteogenesis (Zhong et al. 2013).

Mechanical stretch is another form of extracellular mechanical forces that can be sensed by mammalian cells. Stretch sensing is believed to involve integrin-mediated adhesion signaling that is directly linked to the CSK contractility and other mechanotransductive pathways downstream of rigidity sensing. Cui et al. applied both static and cyclic stretches to cells seeded on elastomeric micropost arrays integrated onto a stretchable membrane to separate effects of stretch from substrate rigidity (Cui et al. 2015; Mann et al. 2012). It is a common observation that fibroblasts seeded on soft substrates could not spread out or proliferate as compared to the cells seeded on rigid substrates including tissue culture plates (Fig. 4.3b). Interestingly, Cui et al. found that cyclic stretches (5 %), but not static stretches, at frequencies ranging from 0.01 to 10 Hz, resulted in significant increases in cell spreading, stress fiber formation, and cell proliferation in fibroblasts seeded on soft micropost arrays. Importantly, in fibroblasts seeded on soft micropost arrays, YAP translocated to the nucleus after 6 h of cyclic stretches and slowly relocated back to the cytoplasm after cessation of cell stretches. Knockdown of YAP inhibited cell spreading or stress fiber formation for fibroblasts seeded on soft micropost arrays under cyclic stretch conditions, suggesting the functional role of nucleocytoplasmic shuttling of YAP in regulating stretch-mediated cell behaviors.

A few recent studies have provided important insights into upstream molecular mechanisms relaying mechanical force sensing to YAP/TAZ signaling. Using an integrin α_v floxed mouse model, Kaneko et al. reported that integrin α_v functioned upstream of YAP/TAZ to regulate shear stress induced mechanosensitive responses of osteoblastic cells through the Src-p130Cas-JNK signaling axis (Kaneko et al. 2014). Mechanically gated ion channel Piezo1 is critically involved in sensing mechanical stretches and substrate rigidity (Pathak et al. 2014). Pathak et al. reported that in human neural stem cells (hNSCs), Piezo1 activity could influence whether hNSCs differentiate along a neuronal or astrocytic lineage (Pathak et al. 2014). Importantly, Pathak et al. reported that pharmacological inhibition or knockdown of Piezo1 in hNSCs evoked nuclear exclusion of YAP and led to suppressed neurogenesis and enhanced astrogenesis, suggesting possible functional links between Piezo1 and YAP (Pathak et al. 2014).

4.5 Summary

YAP/TAZ are important nuclear mechano-sensors and -transducers that have been shown involved in regulating many mechanosensitive cellular behaviors. Although evidence has started to accumulate to suggest functional roles of YAP/TAZ in mechanotransduction in many different cell systems, there are many important questions yet to be answered. It is not clear yet how different upstream mechanosensors (integrins,

focal adhesions, the actin CSK, mechanosensitive channels, etc.) communicate with YAP/TAZ signals and why in different systems, regulatory mechanisms upstream of YAP/TAZ might be completely different. In addition, as YAP/TAZ can directly interact with a broad range of transcription factors, it is critical to understand how mechanical signals, synergizing with biochemical cues, mediate crosstalk between YAP/TAZ and other classic signaling pathways to precisely control specific cell function. Furthermore, emerging evidence shows that senescence and mechanical dosing and memory can regulate YAP/TAZ activities independent of the actin CSK and RhoA/ROCK signaling. Elucidating novel, noncanonical YAP/TAZ regulatory mechanisms therefore represents another key challenge of the field. With better understanding of YAP/TAZ-mediated mechanotransduction, there will be great opportunities to identify novel drug targets to treat human diseases that are known to be associated with deregulated changes in mechanical properties of extracellular environments, making it possible to leverage knowledge in mechanobiology for therapeutic solutions.

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Chapter 5
Role of Rho GTPases in Mechanobiology

Christopher A. McCulloch

Abstract Mechanobiology comprises an emerging group of experimental approaches, data sets, and theories that provide insights into how cells sense, interpret, and respond to physical forces and to the mechanical properties of their immediate tissue or organ environments. Mechanobiology embraces several subfields that are now enabling molecular definition of the processes by which cells interrogate and respond to the mechanical properties and forces within their environments. One of the subfields of mechanobiology is mechanotransduction, an integrated series of processes by which cells translate information on local cell and tissue mechanical conditions into signals and responses that can help to maintain tissue homeostasis or that may contribute to disease. Indeed, disorders of mechanobiology in general, and mechanotransduction in particular, are now recognized for their potential in explaining the pathological mechanisms of a large number of high prevalence human diseases including heart failure, osteoarthritis, hearing disorders, and periodontitis. The Rho GTPases are an important group of signaling molecules that enable translation of mechanical and chemical cues into the cellular responses that regulate cell, tissue, and organ structure and function. In this chapter, the role of Rho GTPases in mechanobiology is considered with special emphasis on their contribution to cell migration and responses to environmental forces.

Keywords Cdc42 • Rac • Rho • GEFs • Actin • Extracellular matrix • Collagen • Mechanosensing • Focal adhesions

5.1 Introduction

Cells can respond to a wide variety of inputs that arise from their pericellular environments (Geiger and Bershadsky 2002). These inputs, which may manifest as a broad array of biochemical or mechanical cues, are interpreted by cells and promote subsequent modifications of cell behavior (Schwartz 2009). While cell responses to chemical cues from the surrounding environment have been studied in great depth

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(Etienne-Manneville and Hall 2002), responses to mechanical cues are less well defined, even though they play a central role in cell migration, stem cell development, wound healing, cancer cell metastasis, and hearing (Butcher et al. 2009; Lopez et al. 2008; Geiger et al. 2009; Vogel and Sheetz 2009; Ingber 2006). Cells can respond to mechanical signals that are generated by the same cell, as is observed in cell migration (Kole et al. 2005). Mechanical inputs into the generation of collective cell responses can also occur, as is observed in cell sheet migration (Das et al. 2015); these processes may be regulated by Rho GTPases (Zegers and Friedl 2014). Cells may also be subjected to external forces as observed in tissues in which force-generating cells stimulate target cells through intercellular or matrix adhesions; these processes may also involve Rho GTPases (Marjoram et al. 2014; Zhao et al. 2007). Taken together, the systems by which cells interpret mechanical signals and then translate these signals into new, downstream chemical and mechanical behaviors are known as mechanotransduction (Jaalouk and Lammerding 2009).

5.2 Cell Mechanics and Cell Function

Over the last decade there has been increased appreciation of how the mechanical properties of the extracellular matrix in concert with cell-generated tension interact to regulate cell and tissue function (Peyton et al. 2007). Indeed the mechanical properties of the pericellular microenvironment can drive specific cellular behaviors. Understanding the consequences of how mechanical alterations to cells and the extracellular matrix is relevant for our understanding of tissue homeostasis. Further, altered mechanics and resultant signaling processes may be related to, or play important roles in, the initiation of pathology. Some well-known examples include diminished compliance of blood vessels that is observed in atherosclerosis and intimal hyperplasia, and changes in the stiffness of developing tumors that facilitate tumor growth. Thus regulation of cytoskeletal tension through modifications of extracellular matrix mechanics may either directly or indirectly critically regulate cell function. Here we consider how Rho GTPases play central roles as molecular switches that regulate mechanobiology.

5.3 What Are Rho GTPases?

Rho GTPases are a group of small (~21 kDa) proteins that are a subfamily of the Ras superfamily of small guanine nucleotide binding proteins. Rho GTPases regulate the assembly and organization of cytoskeletal polymers, as well as providing essential control for cell cycle progression, cell migration, cell adhesion, the transformation of cells in malignancy, apoptosis, cell polarity, the invasion of tissues by cancer cells and metastatic processes (Jaffe and Hall 2005). Many of these processes involve the translation of mechanical cues arising from the extracellular matrix (Kutys and Yamada 2015) into downstream signals that regulate a wide variety of

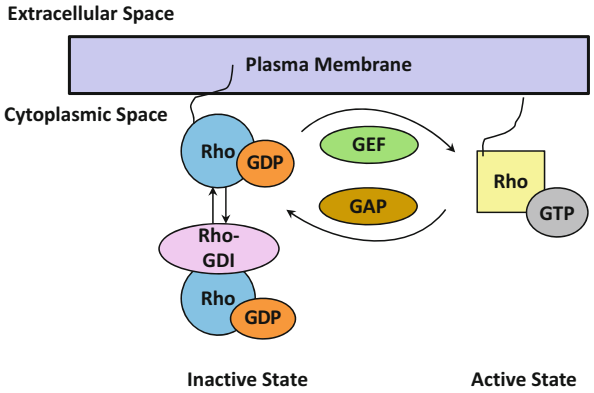

Fig. 5.1 This diagram was adapted from Fig. 1 of Huveneers and Danen (2009) and shows active and inactive forms of Rho and the involvement of guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP), and GDP dissociation inhibitors (GDI) adjacent to the cell membrane. These proteins regulate the catalytic activity of Rho by controlling the binding and dissociation of GTP and GDP from Rho GTPases

cell behavior, including the remodeling of the extracellular matrix itself (such as tractional reorganization of collagen) and the enhanced transit of cells through the extracellular matrix (facilitated, for example, by matrix proteolysis).

In simple terms, Rho GTPases transduce signals from cell surface receptors (such as integrins bound to matrix proteins (Marjoram et al. 2014)) into intracellular signaling pathways. While the nature and signaling pathways of chemically activated receptors are well described, the identity and signal processing of mechanoreceptors are much less well understood. It now seems likely that Rho GTPases can signal through both chemically activated and mechanically activated receptors. Notably, Rho GTPases are a family of proteins that alternate between an inactive, GDP-bound state form and an active, GTP-bound state (Fig. 5.1). After activation, Rho GTPases can interact with downstream effectors that can directly impact cell responses to mechanical inputs. While a large body of research has focussed on the role of Rho GTPases in the remodeling of cytoskeletal polymers (Ridley 2006), more recent work directly implicates Rho GTPases in mechanobiology in general and mechanotransduction in particular (Marjoram et al. 2014). These advances in our understanding of Rho GTPases in cell mechanics suggest considerable specificity of action depending on cell type, adhesion receptor type (Marjoram et al. 2014; Huveneers and Danen 2009) as well as indicating new avenues for treatment of diseases that involve disorders of mechanotransduction (Zhou et al. 2013).

5.4 Identification

Initial research on Rho GTPase genes started with studies of the marine snail *Aplysia californica Rho*. Arising from this research, the genes for human *rhoA, rhoB*, and *rhoC* were identified (Madaule and Axel 1985). After this work, investigations of

Rho1 and Rho2 proteins in yeast showed homologous human Rho isoforms (Madaule et al. 1987) and that Cdc42 in *S. cerevisiae* was important for budding and cell polarity (Johnson and Pringle 1990). Analyses of genome sequences indicate there are no fewer than 20 *rho* GTPases in many mammals and further diversity is provided by splice variants (Boureux et al. 2007). Evolutionary analysis has been instrumental in showing that Rac was likely the original Rho GTPase, and that Rho and Cdc42 were most likely created by subsequent gene duplication. In addition to the classical Rho GTPases, there are several atypical Rho family members, which include RhoH, Rnd1, Rnd2, and Rnd3; these proteins do not hydrolyse GTP and therefore constitutively bind GTP. Further, RhoBTB1 and RhoBTB2 are atypical Rho GTPases except they are much larger than other proteins in the Rho family and probably do not hydrolyse GTP (Aspenstrom et al. 2007; Riou et al. 2010).

5.5 Regulation

Rho GTPases are activated by several families of modifying proteins that include guanine nucleotide exchange factors, which are a large group of proteins that enable release of GDP and, as a result, promote GTP binding by Rho GTPases. This process is favored by the tenfold higher concentration of GTP than GDP that is usually present in the cytoplasm. When Rho GTPases bind GTP, their interactions with downstream effectors are enhanced, which then lead to new cellular responses. The catalytic activity of Rho GTPases is reduced by GTPase-activating proteins, which promote GTP hydrolysis and as a result favor the formation of the GDP-bound (inactive) conformation. A third general group of Rho regulators and one of the first to be discovered was a Rho GDPase Dissociation Inhibitor, a protein that can inhibit GDP dissociation from RhoA and RhoB (Ueda et al. 1990). Rho GDPase Dissociation Inhibitors promote dissociation of Rho proteins from cell membranes by binding to their C-terminal prenyl groups (Isomura et al. 1991; DerMardirossian and Bokoch 2005).

The first guanine nucleotide exchange factor that was identified for Rho GTPases was Dbl (Hart et al. 1991), a transforming protein that was cloned from a diffuse B-cell lymphoma. Dbl shares a region of amino acid sequence homology with the yeast protein, Cdc24, which acts upon the same signaling pathway as Cdc42 (Hart et al. 1991). Dbl and Cdc24 both act as guanine nucleotide exchange factors for Cdc42. The structural basis for the selective activation of Rho GTPases by Dbl exchange factors was examined by Sondek and colleagues (Snyder et al. 2002). They found that despite the similarity of amino acid sequences among various Rho GTPases, Dbl proteins can discriminate between Rho-family members. Structure-based mutagenesis of intersectin and Dbl exchange factors was used to identify the critical determinants that mediate GTP exchange activity for Cdc42, Rac1, and RhoA. A completely separate family of Rho guanine exchange factors, the DOCK proteins was identified later (Brugnera et al. 2002) and these include homologues of DOCK1/DOCK180 that act upstream of Rac (Cote and Vuori 2007). In the context

of mechanobiology, Burridge and coworkers identified a role for the Rho guanine exchange factors LARG and GEF-H1 in regulating the mechanical responses of cells to tensile forces applied through integrins (Guilluy et al. 2011). These guanine exchange factors were recruited to adhesion sites by tensile force. The activation of LARG involved the Src family tyrosine kinase Fyn while GEF-H1 activity was increased by FAK and Ras acting through ERK.

While guanine nucleotide exchange factors enhance the catalytic activity of Rho GTPases, Rho GTPase activating proteins inhibit catalytic activity. One of the first Rho A GTPase activating proteins to be identified was discovered through biochemical purification (Garrett et al. 1989) and subsequently the protein Bcr was reported as a GTPase activating protein (Diekmann et al. 1991). Several Rho GTPase activating proteins and Rho guanine exchange factors were identified in mammals based on examination of sequence homology (Tcherkezian and Lamarche-Vane 2007; Rossman et al. 2005). Further, the analysis of the structure of Rho GTPases along with increased understanding of the regulatory factors described above was important for defining regulation of Rho function (Rittinger et al. 1997; Worthylake et al. 2000; Scheffzek et al. 2000).

Many protein complexes that are involved in regulation of cell signaling contain Rho GTPase interactors. These interactors may include exchange factors, activating proteins, and downstream targets for Rho GTPases that are tightly sequestered in cells. For example, the Cdc42/Rac1-activated PAK kinases themselves bind to the Cdc42/Rac1 guanine exchange factor PIX (Manser et al. 1998). Further, the Cdc42-interacting protein N-WASP binds to and activates the Cdc42 guanine exchange factor intersectin (Hussain et al. 2001). The apparent sequestration of some Rho GTPases and their associated interacting proteins into complexes, some of which associate with cell adhesions (Guilluy et al. 2011; Lee et al. 2015; Toret et al. 2014), may provide cells with the ability to transduce mechanical signals through feedback systems. These systems may in turn integrate mechanical inputs and the organization and dynamics of the actin cytoskeleton into context-sensitive responses that tune cells to the mechanics of their surrounding environments and thereby optimize cell structure and adhesion.

5.6 Effectors

When activated, Rho GTPases interact with targets that act as downstream effectors in the signaling pathway. Some of the early effectors that were identified included a non-receptor tyrosine kinase (Manser et al. 1993) and the p21-activated kinase (Manser et al. 1994). RhoA targets that were identified later include the RhoA binding coiled-coil containing kinases, citron kinase, rhophilin, and rhotekin (Van Aelst and D'Souza-Schorey 1997; Jaffe and Hall 2005). Work by Treisman and colleagues is of particular interest in the context of downstream effectors of Rho GTPase signals leading to regulation of transcription. They showed that RhoA, Rac1, and Cdc42 can enhance the transcriptional activity of the serum response factor and Jun

transcription factors (Hill et al. 1995). Indeed one of the best understood pathways linking Rho GTPases and transcription involves the serum response factor, a transcription factor that is intimately involved in linking the expression of the mechanically sensitive protein, α-smooth muscle actin, to mechanical inputs (Mack et al. 2000; Wang et al. 2002).

Some mechanical signals can regulate transcription of genes with promoters that contain serum response factor binding sites, such as α-smooth muscle actin, an actin isoform that is expressed in mechanically active tissues such as the smooth muscle around blood vessels and in myofibroblasts. The transcriptional activation of the serum response factor is dependent in part on transcriptional coactivators, such as MRTF (myocardin-related transcription factor). The ability of MRTF to affect serum response factor activity is in turn dependent on the balance between the relative abundance of actin monomers and actin filaments in the cytoplasm of cells, which in cultured fibroblasts is known to be affected by application of exogenous tensile forces (Fig. 5.2) (Pender and McCulloch 1991). Since actin monomers bind to and reduce the nuclear import of MRTF, the relative abundance of actin monomers can control the transcriptional activity of the serum response factor (Miralles

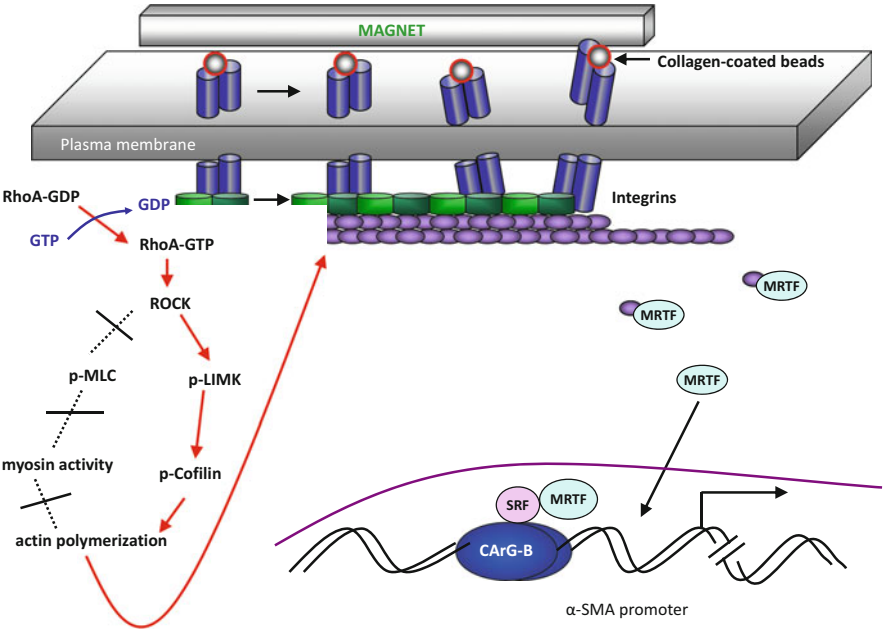

Fig. 5.2 Diagram illustrating application of a culture system for modeling tensile forces delivered through collagen-coated beads that pulls on collagen receptors. This stimulus activates the expression of the α-smooth muscle actin promoter through a signaling system involving activation of the small GTPase Rho, and the generation of downstream signaling through cofilin and the LIM kinase to affect actin assembly. The relative abundance of actin filaments in cells in turn regulates the trafficking of the myocardin-related transcription factor to the nucleus where it acts as a transcriptional coactivator on the CARG box of the α-smooth muscle actin promoter to enhance expression of this protein

et al. 2003). Activation of Rho GTPases can reduce the relative levels of actin monomers by enhancing actin assembly, thereby leading to nuclear translocation of MRTF and increased serum response factor-mediated transcription. Rho GTPases may also affect the activity of other transcription factors such as NFkB and Stat3, although the mechanisms that mediate these signals are not wholly defined (Jaffe and Hall 2005).

The activation of transcription by Rho GTPases may be involved in cell migration, a process that is important in development, wound healing, and metastasis. Cell migration involves extensive mechanical signals and the remodeling of matrix proteins as cells traverse connective tissues. Alterations of transcriptional activity of specific genes induced by Rho GTPases may be viewed as long-term responses that enable their role as rapid responders for initiating and organizing cell migration processes. Notably, in early work, Rac1 was shown to be required for expression and activation of matrix metalloproteinases, enzymes that degrade the matrix and that facilitate migration of cells through connective tissues (Kheradmand et al. 1998). In addition, MAL and serum response factor enhance the expression of genes that contribute to cancer cell invasion and metastasis in animal models (Medjkane et al. 2009).

5.7 Impact of Small GTPases on the Cytoskeleton

The organization and dynamic turnover of actin filaments has been at the core of many reports on mechanobiological phenomena (Pender and McCulloch 1991; Wang et al. 1993). One of the early observations that Rho GTPases could affect the organization of actin filaments was made in Vero cells treated with C3 transferase, an enzyme from Clostridium botulinum that modifies Rho isoforms (i.e., RhoA, RhoB, and RhoC) by ADP-ribosylation and that causes depletion of actin stress fibers (Chardin et al. 1989). While ADP ribosylation inhibits the function of Rho proteins, microinjection of RhoA protein promotes the formation of stress fibers (Paterson et al. 1990). The constitutively active form of Rho induces stress fibers (Fig. 5.3) and the generation of contractile forces (Paterson et al. 1990) while later experiments showed that constitutively active Rac1 promoted the formation of membrane ruffles and lamellipodia (Ridley et al. 1992).

A powerful experimental approach was developed and used in subsequent functional studies to examine how Rho GTPase activation may be involved in mechanotransduction. This approach arose from early reports showing that mutation of a critical amino acid in the GTP-binding site of Ras conferred dominant negative function (Feig and Cooper 1988). Through the use of specific mutations in Rho GTPases, subsequent work showed that it was possible to analyze how these proteins regulated actin filament structures (Ridley et al. 1992; Ridley and Hall 1992). Further, related genetic methods demonstrated that Cdc42 was associated with the extension of filopodia (Kozma et al. 1995; Nobes and Hall 1995). Subsequent screening of a large group of Rho GTPases showed that these proteins

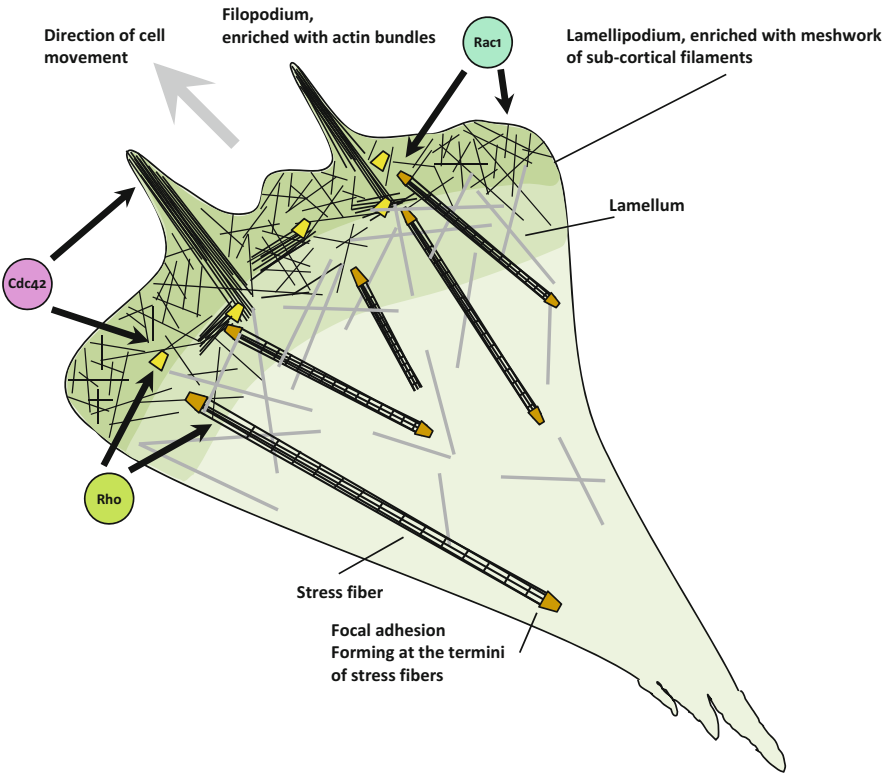

Fig. 5.3 Cartoon of spreading fibroblast on a stiff cell culture substrate showing direction of cell migration and morphologically discrete groups of actin filaments that are associated with stress fiber formation, filopodia formation, and the generation of lamellipodia. This figure was adapted from www.mechanobioinfo/modules/go-0041893

regulate the organization and assembly of actin filaments in endothelial cells in diverse ways (Aspenstrom et al. 2004). Collectively, these data underline the importance of Rho GTPases as critical sensory and response proteins in mechanotransduction involving the actin cytoskeleton.

The actin cytoskeleton is intimately linked to cell–cell and cell–extracellular matrix adhesion function and dynamics. Some targets of Rho GTPases have direct links to adhesion components as well as modulating adhesion-cytoskeleton dynamics. A recent review has considered in depth the role of Rho GTPases in mechanical signaling through various adhesion complexes (Marjoram et al. 2014). Indeed, regulation of cytoskeletal dynamics by Rho GTPases may explain some of their effects on membrane trafficking (Ridley 2006). Cdc42 is also critical for cell polarity, which in part reflects its ability to polarize the cytoskeleton and to regulate vesicle trafficking (Harris and Tepass 2010). More recent work shows that Rho GTPases affect microtubules, intermediate filaments, and septins in addition to the actin cytoskeleton, indicating a very broad role of these proteins in regulating cell shape and function (Braga 2002; Ito et al. 2005; Hall 2009).

5.8 Biosensors

Biochemical methods for assaying the activation of Rho GTPases were advanced through the discovery that the active form of these proteins would bind to specific domains of their downstream effectors, such as rhotekin (for Rho) or the PAK-binding domain for Rac. Further progress in biosensor development for Rho GTPases, such as the use of intracellular affinity labeling for active Rho (Zhao et al. 2007) or employing microscope adaptations of FRET and FLIM for in situ measurement of activation, has enabled new insights into the role of these proteins in the regulation of the actin cytoskeleton and in mechanobiology (Hinde et al. 2013; Donnelly et al. 2014). Biosensors can locally report the activities of Rac1 (Kraynov et al. 2000), RhoA (Tkachenko et al. 2011), and Cdc42 (Nalbant et al. 2004). With these and related approaches, active RhoA was localized mainly in lamellipodia and in membrane ruffles near the leading edge and tails of fibroblasts (Pertz et al. 2006; Kurokawa and Matsuda 2005), indicating a role for RhoA activation in the mechanical signaling events that occurs at these sites in migrating cells. Insights from these experiments have been very useful for understanding how Rho GTPases contribute to mechanotransduction since different Rho GTPase are activated at specific sites in the cell to regulate cytoskeletal organization in mechanically active events such as cell migration (Heasman and Ridley 2010).

5.9 Cell Migration

As discussed above, Rho GTPases play prominent roles in regulating actin assembly and organization, which affect a variety of cellular processes including single cell migration. Cell migration in turn intimately involves mechanosensing and cellular responses to forces (Schaefer and Hordijk 2015). Some of the critical mechanosensory functions that involve Rho GTPases include the formation of protrusions in response to local environmental determinants, determination of the fore-aft polarity of cells, the contractility of actomyosin, and the formation, maturation, and turnover of cell adhesions to the extracellular matrix (Fig. 5.4) or to other cells. Collectively, these processes contribute to the mode and efficiency with which cells migrate through connective tissues.

For cells on the move, Rho GTPases regulate the formation of several different types of protrusions, which include filopodia and lamellipodia (Fig. 5.3) (Jacquemet et al. 2015). These processes are thin extensions of membrane-bound cytoplasm that contain bundles of actin filaments arranged in parallel and which may coalesce following further maturational steps into lamellipodia. Filopodia enable mechanical sensing of the local environment and are partly under the control of Cdc42, which through the Mammalian Diaphanous-related formin mDia2, nucleates actin filament growth. Lamellipodia (Krause and Gautreau 2014) are sheet-like extensions of membrane-bound cytoplasm that often provide adhesion to the underlying matrix at

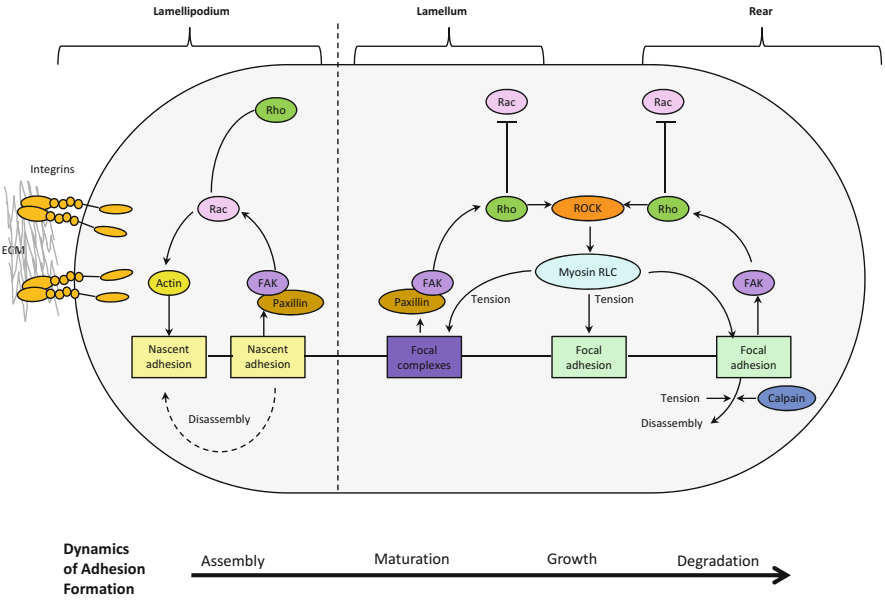

Fig. 5.4 A diagram showing maturation of adhesions in the lamellipodium, lamellum, and posterior aspects of the cell. Note the discrete GTPase and other signaling systems that regulate the maturation of different types of adhesions in various sites of the cell, and which are thought to be important for regulated cell migration. This figure was adapted from www.mechanobioinfo/modules/go-0041893

the leading cell edge and are regulated by active Rac1, Cdc42, RhoA, and RhoC. While Cdc42 can affect cell polarity and enhance the formation of protrusions by stabilizing microtubules (Cau and Hall 2005), Rac controls actin branching and cell steering through Arp 2/3 and WAVE (Dang et al. 2013; Derivery and Gautreau 2010). Actin branching can be further enhanced by cofilin, which is activated downstream of Rac (through PAK and LIM kinase) or through RhoC to ROCK and LIM kinase. Since cofilin can sever actin filaments at cell extensions and as a result generate more free barbed ends on existing actin filaments, this process facilitates Arp2/3-mediated extension of lamellipodia at these sites. As a result of PAK activation, Rac also promotes integrin-based adhesion to the extracellular matrix and mechanically stabilizes the advancing lamellipodia in the direction of cell migration. In certain types of cells, migration is mediated through Rho-dependent membrane blebbing (Kardash et al. 2010), which arises from a two-step process in which bleb-like membrane protrusions are formed (Fackler and Grosse 2008), followed by stabilization of the bleb by subcortical actin filaments. In cell migration associated with membrane blebbing, the phenomenon of blebbing seems to be dependent in part on the generation of intracellular hydrostatic pressure, which in turn is mediated by RhoA and actomyosin contraction.

The roles of Rho GTPases in single mesenchymal cell migration have been examined in some detail (Jean et al. 2014) and involve Cdc42- and Rac-dependent

cell protrusion at the leading edge of the cell, which is coupled to Rho-dependent contractility, a requirement for anterior translocation (Vega et al. 2011). Besides the formation of cell protrusions at the leading edge, Rho GTPases control cell contractility at the trailing edge of moving cells. RhoA activates ROCK, which engages myosin light chain kinase and myosin II for actomyosin contraction, preferentially towards the lateral and rear edges of the cell. RhoA appears to control tail retraction in migrating single cells and in collective cell migration, and possibly the mechanical coupling laterally of cadherin-based intercellular adhesions. More recent work by Ridley and colleagues (Vega et al. 2011) demonstrates specific functions of Rho isoforms in cell migration. While RhoA and RhoC exhibit similar amino acid sequences, they play unique roles in cell migration and the invasion of connective tissues by malignant cells. With the use of RNAi, they found that distinct migratory phenotypes were related to the expression of the Rho-regulated kinases ROCK1 and ROCK2. Notably, RhoA mediates polarity determination in migratory cells through ROCK2-mediated suppression of Rac1 activity in lamellipodia, while RhoC enables polarized migration as a result of the formin FMNL3, which restricts the width of the advancing lamellipodia. The same group showed that RhoB or depletion of the guanine nucleotide exchange factor GEF-H1 regulates cell migration by regulating the dynamics of focal adhesion formation (Vega et al. 2012). They considered that RhoB contributes to directional cell migration by affecting the cell surface expression levels and activity of β1 integrins, which in turn contributes to stabilization of lamellipodial protrusions.

Rho GAP has been suggested to play a central role in cell contractility and migration as a result of the ability of the Abl-related gene tyrosine kinase to affect p190RhoGAP and therefore its regulation of actomyosin contractility and focal adhesion dynamics (Peacock et al. 2007). Koleske and colleagues found that the Abl-related gene (Arg/Abl2) kinase slows the migration of fibroblasts by inhibiting actomyosin contractility and by affecting the formation of focal adhesions, processes which in turn are dependent upon distinct functional domains of Arg. Indeed, Arg uses its kinase activity and its ability to bind the cytoskeleton in order to affect the formation and transmission of force through focal adhesions. The Koleske paper provides a good example of how a GTPase activating protein can affect the mechanics of cell migration through restriction of actomyosin contractility and regulation of cell adhesion formation.

5.10 Collective Cell Migration

In contrast to single cell migration, cells that migrate as groups (i.e., collective cell migration) exhibit maintenance of intercellular junctions to ensure the cohesion of the migrating cell cluster. The cells at the leading edge are mechanically coupled and integrate the migration of the cells that follow behind them. In both two- and three-dimensional models of collective cell migration, Rho GTPases initiate and maintain leader and follower cell function, cell interactions with substrate, cell–cell cohesion,

and the overall coordination of cell movement. In cells that move individually, Rac and Cdc42 activity controls polarized actin polymerization, which is spatiotemporally separated from Rho-dependent actomyosin engagement and cell contraction. This separation ensures that each region of the cell is functioning in a coordinated fashion. In collective cell migration, subregions of active Rac and active Rho may exhibit reduced activity, or the reverse, which may reflect mutually interactive feedback and inhibitory systems for these two Rho GTPases. Indeed, Rho GTPase functions are subject to careful tuning in time and space to enable the complex regulation of cell adhesion and cytoskeletal organization that are needed for the mechanocoupling that is manifest in collective cell migration (Zegers and Friedl 2014). Recently, a novel mechanotransduction system has been demonstrated in collective migration of epithelial cells (Das et al. 2015). The tumor suppressor protein, merlin, may organize the collective migration of cell clusters ($n = 10–20$ cells), by acting as a mechanochemical transducer. In stationary epithelial monolayers and in human skin, merlin localizes to cortical intercellular junctions of epithelial cells but at the start of migration, some of the cortical merlin moves to the cytoplasm, a process that is apparently initiated by tensile forces from the leading cell and which is dependent on actomyosin-based cell contractility. These findings point to a central role for how merlin coordinates polarized Rac1 activation and lamellipodium formation over a length scale that involves multiple cells. Further, the data provide insights into a system that links intercellular forces to collective cell migration.

Other work in Drosophila has shown the importance of Rho GTPase in collective cell migration and its regulation by Crumbs for the control of tissue formation and cell layer involution in developing salivary glands (Xu et al. 2008). Notably, the use of a clever light-mediated activation protocol has highlighted an important function for Rac in collective cell movement (Wang et al. 2010). With photoactivatable analogues of Rac, activation or inactivation of Rac was achieved in border cells of Drosophila ovary. Manipulation of Rac activity in a single cell strongly affected responses in neighboring cells, indicating that cells may sense their direction of movement bases on relative levels of Rac activity. These data strongly indicate the central role that Rho GTPases play in regulating the mechanics of collective cell migration.

5.11 Rho GTPases and Exogenous Forces

The mechanical signaling processes described above involve cell-generated forces that typically are regulated by, and act upon, the same cell or groups of migrating cells. However, in vitro studies have shown that exogenously applied forces can also regulate Rho activity in different cell types, in some cases enhancing the ability of RhoA to increase cell contractility (Smith et al. 2003; Wojciak-Stothard and Ridley 2003). An early investigation of mechanotransduction in rat aortic vascular smooth muscle cells indicated a central role for Rho activation in response to mechanical stretch (Numaguchi et al. 1999). In non-stretched control cells, RhoA was mainly

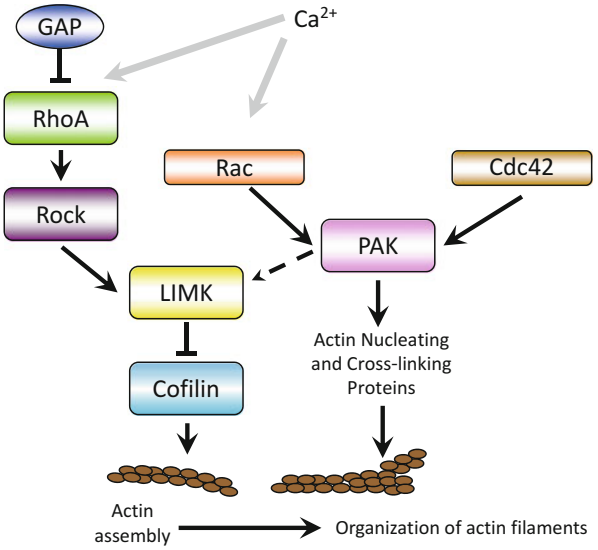

Fig. 5.5 Regulation of actin assembly through GTPases. GAPs inhibit GTPase activity, which may affect ROCK-associated enhancement of actin assembly through cofilin and LIM kinase. Rho GTPases including CDC42 can also affect actin filament architecture by promoting the formation of actin bundles through the enhanced activity of actin nucleating and bundling proteins. The activity of CDC42 is associated with the formation of filopodia, critical mechanosensory organelles involved in cell migration

found in the cytosolic fraction but translocated to the particulate fraction in response to mechanical stretch. Inactivation of Rho with Botulinum C3 exoenzyme and inhibition of Rho kinase reduced stretch-induced ERK activation. Collectively, this early work indicated that Rho is activated by stretch and that Rho kinase mediates stretch-induced ERK activation, directly implicating Rho GTPases in canonical signaling pathways with multiple downstream outputs. Further, the Rho-kinase pathway (downstream of Rho) can induce phosphorylation of LIM kinase and cofilin, which then enhances actin assembly and cell contractility. Cofilin is an abundant and widely expressed actin-binding protein (Lappalainen and Drubin 1997) that when phosphorylated (e.g., by LIM kinase) exhibits altered binding to actin filaments (Fig. 5.5). Accordingly, actin filaments are stabilized and the total actin filament content of cells is increased (Bamburg 1999). This pathway provides a good example of how exogenous forces can signal via Rho GTPases to regulate actin assembly and cell contractility through ROCK-induced phosphorylation of LIM kinase and phosphorylation of cofilin (Arber et al. 1998).

In serum-deprived fibroblasts, RhoA activity is selectively activated within 10 min after application of tensile forces through collagen-coated magnetite beads (Fig. 5.2), after which Rho activity is reduced to baseline levels (Zhao et al. 2007). By contrast, RhoA, Rac1, and Cdc42 are all activated during the early stages of endothelial actin cytoskeletal remodeling induced by shear stress (Wojciak-Stothard and Ridley 2003). Force-induced activation of RhoA is very transient and requires

integrins and intact actin filaments, suggesting a positive feedback loop involving an interaction of active RhoA and ROCK that leads to actin polymerization. Using an in situ rhotekin-binding assay, force-induced RhoA activation was localized to sites of force application (Zhao et al. 2007), consistent with the notion that Rho guanine exchange factors are recruited to adhesion sites (Guilluy et al. 2011).

In partial bladder outlet obstruction, remodeling of the bladder detrusor smooth muscle is associated with the regulation of contractile signals. In partial blockage of mouse urinary bladders, RhoA and ROCK are linked to calcium sensitization in muscle hypertrophy. In experiments involving mouse and human bladders, and in cell cultures subjected to equi-biaxial cell stretch, which models bladder wall smooth muscle hypertrophy, the expression of RhoA, ROCK, and the C-kinase-activated protein phosphatase I inhibitor were strongly enhanced. These data indicate that in vivo models of exogenous force exhibit increased expression of the signaling proteins in the Rho GTPase pathway that regulate bladder muscle tone (Boopathi et al. 2014).

Application of mechanical force can reorient endothelial cells in culture, a potentially useful model for determining how exogenous forces can affect cell organization through small GTPases. Cyclic stretch can induce the reorientation of vascular endothelial cells and their stress fibers in a direction perpendicular to the stretch axis. A screen of short hairpin RNAs targeting 63 Rho guanine nucleotide exchange factors (GEFs) showed that at least 11 Rho-GEFs target RhoA, Rac1, and/or Cdc42, and are involved in stretch-induced reorientation of endothelial cells (Abiko et al. 2015). One of these GEFs, named Solo, promoted RhoA activation and actin filament assembly at cell–cell and cell–matrix adhesions while Solo knockdown inhibited tensile-force-induced RhoA activation and perpendicular reorientation of endothelial cells. Thus Rho GEFs and Solo in particular in this model system are evidently important in transducing the exogenous force signals that enable stretch-induced endothelial cell reorientation.

5.12 Shear Stress Models

Early work employed in vitro models for shear stress and implicated GTPases in atheroma formation and a role for integrins in endothelial mechanosensing of shear stress (reviewed in Shyy and Chien 2002). It had been known for many years that the focal pattern of atherosclerotic lesions in arteries may indicate that local patterns of blood flow contribute to atherosclerosis. Many studies have shown that at branches and curved regions in arteries there is a higher probability of atheroma formation while laminar flow (in the straight part of arteries) protects against atheroma formation. With the use of cultured vascular endothelial cells in flow channels, evidence was presented to show that integrins and RhoA play critical roles in converting shear stress into downstream signals that affect the expression of genes that regulate apoptosis, cell cycle progression, and nitric oxide production, all of which can potentially dampen atheroma formation.

More recent work on shear stress in osteoblasts has implicated Rac1 and Cdc42 in β-catenin signaling leading to the expression of bone-related genes (Wan et al. 2013). The β-catenin-dependent T-cell factor/lymphocyte enhancing factor is sensitive to mechanical inputs and enhances bone formation, which is a mechanically sensitive process. In oscillatory shear stress experiments, osteoblastic cells were examined using fluorescence resonance energy transfer to assess signal transduction in living cells in real time. Oscillatory shear stress (10 dyn/cm^2) increased the activity of the T-cell factor/lymphocyte enhancing factor and also enhanced nuclear translocation of β-catenin, which was accompanied by increased activity of Rac1 and Cdc42. The ability of shear stress to activate T-cell factor/lymphocyte enhancing factor required Rac1 and Cdc42 activity since dominant negative or constitutively active constructs of these GTPases blocked or enhanced, respectively, enhancing factor activation. These data show that Rac1 and Cdc42 GTPases are important regulators of shear stress-dependent β-catenin signaling in osteoblasts.

5.13 Mechanoprotection and GTPase Activating Protein

Cells in mechanically active environments are subjected to high-amplitude exogenous forces that can lead to cell death. The actin cross-linking protein filamin A protects cells from mechanically induced death (D'Addario et al. 2002; Kainulainen et al. 2002) by mechanisms that are not wholly defined. In experiments using application of tensile forces applied through collagen-binding integrins, Rac mediated lamellae formation in filamin A-null cells but not filamin A-expressing cells (Shifrin et al. 2009). Force-induced lamella formation was suppressed by filamin A, and in particular this suppression was mediated by repeat 23 of filamin A, which binds FilGAP, a Rac GTPase-activating protein. FilGAP was targeted to sites of force transfer by filamin A and this force-induced redistribution of FilGAP was essential for the suppression of Rac activity and lamellae formation in cells treated with tensile forces. Depletion of FilGAP by small interfering RNA, inhibition of FilGAP activity by dominant-negative mutation, or deletion of its filamin A-binding domain, all resulted in a dramatic force-induced increase of apoptotic cells, indicating that FilGAP and filamin A synergize to protect cells against force-induced apoptosis. These data show that the control of GTPase activity, in this case by a GAP, plays an important role in preserving cell viability in mechanically challenged environments.

5.14 Hearing

A crucial developmental aspect of hearing-related structures involves the morphogenesis of sensory hair cells. The mechanotransduction organelles of these hair cells are stereociliary bundles; the proper development of these bundles in turn involves well-orchestrated remodeling of the actin cytoskeleton, which is required for proper

morphogenesis. Tissue-specific deletion of Rac1 in the otic epithelium of mice leads to severe defects in epithelial morphogenesis of the cochlea (Grimsley-Myers et al. 2009). The cochlea in mutant mice were shorter than wild type and exhibited reduced numbers of auditory hair cells and organization of the auditory sensory epithelium was abnormal. The hair cells showed disrupted planar cell polarity and inappropriate formation of the stereociliary bundle. There was fragmentation of bundles and inappropriate positioning or deletion of the kinocilium. The authors showed that an intact p21-activated kinase signaling pathway was needed for normal interactions between the kinocilium and stereocilia, which in turn was needed for cohesion of the stereociliary bundles. In the context of mechanobiology, these data show that Rac1 is a critical protein in morphogenesis of the auditory sensory epithelium and stereociliary bundles, which are essential for hearing.

5.15 Cancer

RhoC is a member of the Rho GTPase family that has been linked to cancer progression, possibly by enhancing the ability of cancer cells to invade tissues. A recent report indicates that RhoC regulates the process by which cancer cells interact with vascular endothelial cells (Reymond et al. 2015), which is an important process in metastases. Knockdown of RhoC in PC3 prostate cancer cells by short interfering RNA inhibited the adhesion of these cells to endothelial cells and reduced the ability of the cancer cells to migrate across sheets of endothelial cells in vitro. When two downstream effectors of RhoC, ROCK1, and ROCK2 were knocked down, cancer cell adhesion to endothelial cells was reduced. The paper also demonstrated that the formation of protrusions and the retention of PC3 cells in lungs and experimental metastasis formation were dependent on RhoC.

The potential involvement of GTPases in mechanical signaling in cancer has been advanced through a recent examination of the behavior of metastatic ovarian cancer cells plated on soft extracellular matrices and examined by traction force microscopy (McGrail et al. 2014). It is recognized clinically that metastatic ovarian cancer cells often adhere well to soft matrices and at these sites cancer cells exhibit enhanced migration, proliferation, and resistance to chemotherapy. In this study, human ovarian cancer cells showed increased traction forces and polarization on soft compared with relatively stiffer substrates. After culture on soft substrates, these cells exhibited some features of epithelial-to-mesenchymal transition. The observed mechanical tropism was dependent on signaling through Rho kinase, indicating that application of fundamental knowledge of mechanobiology relating to Rho GTPases may enable progress in the biology of metastasis. This supposition is consistent with an earlier report indicating that tumor-derived endothelial cells demonstrate altered Rho-mediated mechanosensing and perturbed angiogenesis in vitro (Ghosh et al. 2008). Conceivably, abnormal Rho-mediated sensing of the mechanical properties of the tumor microenvironment may contribute to the deviant behaviors of tumor cells that in turn affect the organization of the cancer microvasculature.

5.16 Conclusions

We have considered here the role of Rho GTPases in mechanotransduction, and in particular how Rho GTPases shape the mechanical signals that regulate cell migration and cellular responses to environmentally generated mechanical forces. The data reviewed here strongly indicate that the Rho subfamily of small GTPases are a critically important group of molecules that help to translate mechanical cues into appropriate cellular responses; these responses in turn are essential for maintaining the structure and function of normal cells, tissues, and organs. It is also apparent that high prevalence diseases of blood vessels as well as cancer involve disorders of mechanobiology. Newly emerging data are beginning to show the relationships between GTPase-dependent signaling and disruptions of mechanotransduction. These findings have the long-term potential of contributing to the discovery of new drug targets and therapeutics that focus on mechanobiology involving GTPases.

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Chapter 6
Illuminating Cell Adhesion: Modern Microscopy Approaches to Study Integrin-Based Focal Adhesions

Vinay Swaminathan and Clare M. Waterman

Abstract Integrin-based focal adhesions are plasma membrane-associated macromolecular structures that link the extracellular matrix to the cytoskeleton and play important roles in a variety of cellular processes. Focal adhesions dynamically change shape, size, and biochemical composition, all of which are carefully regulated by the cell to perform its function normally. Additionally, focal adhesions are also the primary conduits for relaying physical information between the cell and its environment. It is all these properties that make focal adhesions a unique research problem at the interface of biology and technology and have driven the development and application of many techniques to study them. In this article, we review how light and force microscopy-based approaches have expanded our understanding of integrin-based focal adhesions. We highlight specific questions about focal adhesions that each technique addresses, and the novel insight gained from studies using these approaches to understand how these complex adhesion organelles of more than 500 proteins are built and regulated, and the integration of the cell with its environment in mediating physiological functions.

Keywords TIRF • Super-resolution • FRET • Force spectroscopy • Correlation spectroscopy

6.1 Introduction

Integrin-based cell adhesions are the primary cellular organelles that mediate a wide variety of cell–extracellular matrix (ECM) and cell–cell interactions. These interactions are critical for a diverse set of physiological functions from gene expression

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and differentiation to cell migration during embryonic development, immune response, and wound healing. It is therefore of no surprise that a vast number of adhesion-associated proteins have been implicated in developmental disorders, immune diseases like inflammation, multiple sclerosis, Crohn's disease, and many different types of cancers. It is because of these important physiological roles that cellular adhesions have been the focus of biological research from almost 100 years.

As the name suggests, the primary cell–ECM adhesion receptors are the integrin family of proteins (Hynes 1992, 2002). These receptors mediate a range of mechanical, topological, and biochemical interactions and *integrate* the cell with its environment (Geiger and Bershadsky 2002; Geiger et al. 2009). Integrins bind to a variety of different ECM proteins like fibronectin and collagen and in immune cells can also bind to other cell surface proteins. In most mesenchymal cells, integrins connect the ECM to the cytoskeleton through the supramolecular complex of proteins called focal adhesions (FA). At the leading edge of a migrating cell, FAs are less than ~0.25 μm in radius and are called nascent adhesions (NA). During cell migration, these NAs increase in size (a process called "maturation") into focal complexes (FC) which are ~1 μm in diameter which will further mature into elongated FAs. The size of FAs depends on a wide variety of factors including cell type and ECM ligand density, but typically in fibroblasts migrating on fibronectin or collagen, FAs can be ~5–10 μm long. In an ECM rich environment, some of the FAs can mature into fibrillar adhesions which are more elongated and can remodel the ECM. While FAs strictly refer to integrin-based adhesions of a particular size and location in a cell, for the purpose of this review, we will refer to all integrin-based adhesion structures including NAs, FCs, FAs, and fibrillar adhesions as FAs unless otherwise specified.

Each morphological stage of a maturing FA is also accompanied by changes in its composition. Besides integrins, more than 500 different proteins including adapter and scaffolding proteins, kinases, phosphatases, molecular motors, ribosomes, and mRNA have been shown to associate with FAs and together all these proteins form a network called the integrin adhesome (Horton et al. 2016; Geiger and Zaidel-Bar 2012). Thus, by dynamically varying the morphology, composition, and biochemical and biophysical properties of individual components, FAs regulate cellular functions. For example, in the context of cell migration, FAs go from NAs to FCs and fully mature FAs before disassembling in minutes (Webb et al. 2002), while at the same time different proteins in FAs get dynamically recruited, phosphorylated, change interactions with other proteins, or undergo changes in their conformation. All these changes are critical for proper directed cell migration in response to cues such as diffusible growth factors (chemotaxis), ECM-associated cues (haptotaxis), and mechanical cues (durotaxis) (Pelham and Wang 1998; Huttenlocher et al. 1995; Lauffenburger and Horwitz 1996; Palecek et al. 1997; Ridley et al. 2003).

Some of the earliest evidence for FAs in live cells was obtained in the 1960s using an imaging technique called interference reflection microscopy (IRM) (Ambrose 1961; Curtis 1964). This technique relies on reflections of the incident beam as it travels through different refractive index media. As light passes from the

coverslip to the aqueous medium and from the aqueous medium to the cell membrane, the waves reflected of each surface interfere constructively and destructively with each other resulting in contrast enhancement with areas of close contact appearing dark and rest of the cell appearing light. This makes IRM a very useful technique to image FAs and cell motility. First seen by EM as areas of close contact with substrates at the termination of actin filaments, IRM confirmed the existence of FAs in live cells and was used in a number of subsequent studies to classify FAs on the ventral cell surface (Izzard and Lochner 1976; Heath and Dunn 1978; Abercrombie and Dunn 1975). Vinculin, isolated from a cytoskeletal fraction of chicken gizzard, was identified as the first FA protein after it was found to localize to FA sites using IRM in combination with fluorescence microscopy (Geiger 1979). These early studies directly led to a number of subsequent works identifying and characterizing different FA proteins in cells and revolutionized the field of adhesion biology and cell migration (Burridge et al. 1988).

Thus, from the 1960s, adhesions have been at the forefront of biological systems which have driven the development and advancements of microscopy techniques. The complex architecture, composition, dynamics, and mechanical and biochemical regulation of these organelles present unique challenges in light microscopy, many of which have been overcome in the past 35 years. In this review, we will highlight these techniques with emphasis on how questions specific to FAs have driven these advancements and how these technological advancements have taught us something new about the architecture, regulation, and function of FAs.

6.2 Total Internal Reflection Microscopy (TIRF): Studying FA Dynamics During Cell Migration

The proximity of FAs to the substrate makes these structures optimal for total internal reflection fluorescence microscopy (TIRFM) techniques which allows for selective excitation of fluorophores in a region within <100 nm adjacent to the coverslip (Fig. 6.1a). Similar to IRM, TIRFM relies on the refraction and reflection of fluorescent light when it travels from a high index of refraction medium to a low index of refraction medium (for example, glass to water) resulting in the incident light to be completely reflected back at a specific angle of incidence. This total internal reflection results in a thin electromagnetic field in the low refractive medium called the evanescent field with low penetration depth and rapid decay. This affords high contrast fluorescence imaging with no background from out-of-focus fluorescence deeper in the specimen. Although development of fluorescent protein fusions to FA proteins allowed imaging of the dynamics of FA growth and turnover during cell migration (Laukaitis et al. 2001), FA proteins possess a large cytoplasmic pool, with only a small fraction associated with FAs. This poses problems when FAs are imaged by conventional widefield fluorescence techniques because both cytoplasmic and FA-associated molecules are excited, and their fluorescence is collected independent of whether they are in focus. By selectively exciting fluorophores close to the cell surface, TIRFM

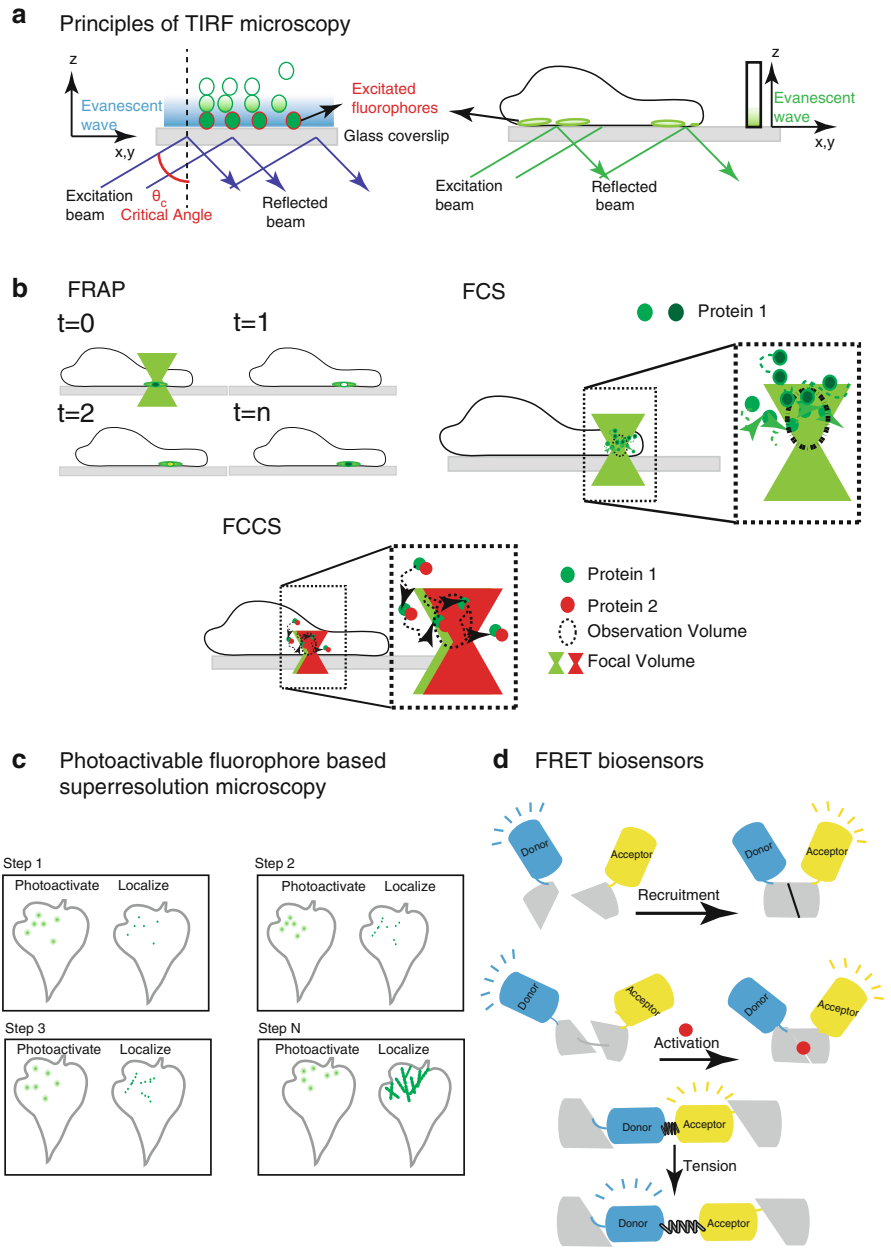

Fig. 6.1 Optical microscopy-based approaches for studying FAs. (**a**) (*Left*) Principle of TIRF microscopy showing selective excitation of fluorophores (*green filled circle*) close to the substrate as excitation light is incident at an angle greater than the critical angle (θ_c). (*Right*) Illustration of selective excitation of focal adhesions (*green*) in a cell by TIRF microscopy. (**b**) (*Left*) Principle of FRAP where a small region of fluorescently labeled molecules in an FA is irreversibly bleached and the subsequent movement of the surrounding non-bleached molecules into the bleached region is monitored. (*Right*) FCS microscopy where the fluctuations in intensity within a specific image region is monitored as fluorescent molecules (*green circles*) move in and out of the region (focal volume shown here) over time. (*Bottom*) FCCS microscopy, where fluorescence fluctuations of two

results in better signal-to-noise ratio (SNR) for the proteins specifically localized in FAs and lower levels of photobleaching and photodamage, both of which have made TIRFM the go-to method for studying FA dynamics in living cells.

One of the earliest applications of TIRF to study the dynamics of fluorescent paxillin in live cells led to the discovery of NAs and the characterization of their dynamics during cell migration (Choi et al. 2008). The loss of out-of-focus fluorescence in TIRFM made it possible to resolve the earliest stages of NA formation and assembly because the small numbers of molecules forming NA clusters were obscured by large cytoplasmic fluorescence in widefield imaging techniques. Through this and other subsequent work using TIRFM, we now know that NAs form de novo in the lamellipodia in a myosin II independent mechanism, and that NAs are precursors of FAs (Choi et al. 2008). Studies on their dynamics reveal that once NAs form, a vast majority of them rapidly turnover and only a small fraction stabilize and mature into FAs (Choi et al. 2008; Thievessen et al. 2013; Oakes et al. 2012). What drives formation of NA, determines fraction that mature, and how NAs are mechanically coupled to the lamellipodia actin are currently still not clear and are active areas of research.

Live cell imaging by TIRFM has improved our knowledge in many aspects of FA biology. By imaging FA and other cytoskeletal proteins, TIRFM has shown that not only actin, but even microtubules target to FA proximal areas (Krylyshkina et al. 2003), and can induce their disassembly by a FAK-dynamin pathway (Ezratty et al. 2005). TIRFM was also used to highlight the role of the FA protein talin in providing mechanical linkage between ECM and actin cytoskeleton after initial cell spreading (Zhang et al. 2008). Studies using TIRFM have also shown the role of vinculin in linking the ECM and the actin cytoskeleton for promoting traction forces, NA formation and turnover, as well as vinculin's role in reorganizing FAs during establishment of cell polarity (Thievessen et al. 2013; Carisey et al. 2013). TIRFM has also been used to image integrin dynamics in FAs, showing the potential role for clathrin-mediated endocytosis of integrins in FA disassembly (Ezratty et al. 2009), the critical role for ligand spacing in integrin clustering (Cavalcanti-Adam et al. 2007), and the role of locally generated lipid kinase PIP_2 in coupling integrins to the ECM and the cytoskeleton (Legate et al. 2011).

Because of its ability to image rapid dynamics close to the membrane–coverslip interface, TIRFM has also been used to investigate the mechanism of coupling between FA and leading edge dynamics during processes like migration. Some of

Fig. 6.1 (continued) fluorophores (*red* and *green circle*) in a sampling volume is monitored and correlated to measure co-localization, binding, and concentrations. (**c**) Principle of PALM-based SRM techniques showing selective photo-activation (*green diffused spot*) and localization (*green dot*) over time of a dense structure in cell. (**d**) FRET-based biosensors. (*top*) A protein recruitment-based biosensor where FRET only occurs when two proteins get in close proximity (less than 10 nm) or bind to each other. (*middle*) Conformation biosensor where change in the conformation due to activation results in change in distance between the fluorophores and a change in FRET. (*bottom*) Tension sensor where fluorophores are linked by an elastic spring-like molecule and inserted into protein of interest. Change in tension across the protein results in extension or compression of the spring resulting in change in FRET

the key studies have combined TIRFM with other imaging modalities like high resolution differential interference contrast (DIC) microscopy (Giannone et al. 2007) which is a brightfield imaging technique in which the reference beam is sheared along a specific direction resulting in a gradient of optical paths which gives the image a shadow-cast appearance. Simultaneous imaging of leading edge dynamics with DIC and FA dynamics using TIRFM showed that periodic polymerization of actin in the lamellipodia and initiation of FA sites are coordinated via a mechanical link during cell motility (Giannone et al. 2007).

TIRFM has also been combined with fluorescent speckle microscopy (FSM) (Hu et al. 2007) which relies on fluorescent labeling of a small number of molecules which co-assemble along with a larger fraction of unlabeled molecules to randomly incorporate into a macromolecular structure. The distribution of fluorophores in the structure results in a non-uniform intensity pattern which appears as a distinct puncta or *speckle* and can be analyzed for motion and turnover of the macromolecular assembly. FSM imaging of different FA proteins together with actin by TIRFM showed a dissipation of motion between retrograde flow of the actin and FA proteins which enables force transmission and protrusion of the leading edge (Hu et al. 2007; Guo and Wang 2007).

Thus, TIRFM has shown that there is strong mechanical and biochemical coupling between the FA system and the actin cytoskeleton with proper coordination required for proper cell migration and other processes (Gardel et al. 2010).

6.3 Measuring FA Protein Dynamics Using FRAP and Fluctuation-Based Microscopy Techniques

FAs are highly dynamic structures and consist of more than 500 different proteins and even greater number of protein–protein interactions. Because FAs are not membrane bound, FA-associated proteins can freely exchange subunits with the cytosol. Thus, understanding the mechanisms and dynamics of molecular transport, protein association-disassociation rates and concentration in FAs and the cytosol are critical to understand how FAs are assembled and regulated during different cellular processes. Fluorescence recovery after photobleaching (FRAP), Fluorescent correlation spectroscopy (FCS), and Image correlation spectroscopy are three microscopy techniques that can be exploited for these measurements.

In FRAP, a small region of fluorescently labeled molecules within the cell is irreversibly bleached and the subsequent movement of the surrounding non-bleached molecules into the bleached region, i.e., the "fluorescence recovery," is monitored and quantified (Fig. 6.1b Left). Both the diffusion constant and the mobile fraction (fraction of fluorescent proteins that can dissociate from the bleached region) can be assessed by fitting typical FRAP data to exponential functions. FRAP analysis has been used to measure the dynamics of a wide variety of FA proteins including integrins (Ballestrem et al. 2001; Cluzel et al. 2005), vinculin (Cohen et al. 2006; Lele

et al. 2006; Humphries et al. 2007), FAK (Hamadi et al. 2005; Pasapera et al. 2010), and zyxin (Lele et al. 2008). All these studies have highlighted the complex and heterogeneous dynamics of FA proteins in a cell. For example, zyxin, paxillin, and vinculin show force-dependent mobility in FAs with reduction in cellular contractility resulting in higher FRAP rates and larger mobile fractions (Lele et al. 2006; Pasapera et al. 2010; Wolfenson et al. 2009a, b). Proteins also have different mobilities based on the morphological state of FAs (growing, stable, disassembling) or region within an FA (towards cell periphery or in the cell center) (Wolfenson et al. 2011, 2013). Thus, FRAP has not only led to quantification of mobility parameters of different FA proteins but also has highlighted the heterogeneity in FA structures and the wide array of regulations that different FA proteins undergo.

Similar to FRAP, FCS and ICS can be used to measure transport and association-disassociation rates but can also provide concentrations of molecules. Both FCS and ICS techniques rely on monitoring the fluctuations in intensity within a specific image region as fluorescent molecules move in and out of the region over time. While FCS measurements are made in a single focal volume (Elson 2001) (Fig. 6.1b Right), ICS measurements are made on a pixel by pixel basis in a series of confocal images (Petersen et al. 1998). By calculating an autocorrelation function from fluctuations of intensity, the diffusion rate and concentration of molecules can be calculated. The advantage of ICS-based techniques is that it allows for scanning of a bigger cellular region than traditional FCS which is restricted to sampling in a single focal volume though FCS has higher sensitivity compared to ICS-based techniques. FCS has found limited application in studying adhesion dynamics in cells because of the relative ease of FRAP to study mobility. However, ICS-based approaches have been used to show that paxillin enters FAs as a monomer and dissociates in a large complex (Wiseman et al. 2000; Brown et al. 2008; Digman et al. 2008).

Extensions of FCS and ICS on the other hand have found a wider use in the study of protein interactions in cells and in FAs. As the densities of proteins in FAs are below the diffraction limit, simple co-localization analysis does not measure true molecular interactions occurring in an FA or in the cytosol. Thus, cross-correlation-based extensions of FCS (FCCS) and ICS (Cross-correlation number and brightness, ccN&B) have been developed to not only look at protein–protein interactions but also to measure the dynamics, stoichiometry, and mobility of protein assemblies in a cell. Cross-correlation-based techniques rely on fluorescence fluctuations of two fluorophores in a sampling volume (single focal volume for FCCS, pixels of an image in ICS) and looks for correlations in the fluctuation of the two colors (Fig. 6.1b Bottom). If two molecules interact with each other or are bound to each other, they diffuse through the sampling volume in a synchronous way resulting in positive cross-correlation readout. Both FCCS and ICCS have been used to look at interactions of proteins both in FAs and in NAs (Digman et al. 2009; Choi et al. 2011; Bachir et al. 2014; Hoffmann et al. 2014). ICS-based cross-correlation microscopy (ccN&B) recently has revealed a number of key interactions between proteins in NAs and the role of those interactions in NA dynamics.

One study showed that paxillin and FAK interact with each other in NAs in a 1:1 stoichiometry. The size of this complex was dependent on phosphorylation of paxillin and the complex increases in size with phosphorylation. Interestingly, this study also showed that paxillin interacts with FAK in the cytosol though in this case, only phosphorylated paxillin can associate with FAK (Choi et al. 2011). More recently, this technique has elucidated other interactions between FA proteins in newly formed NAs as well as in the cytosol prior to NA formation. Integrin and kindlin appear as a complex very early during NA formation while integrin and talin appear together much later during FA maturation. Interestingly, talin and vinculin form a complex prior to formation of the integrin–talin complex, suggesting a hierarchy of interactions between these proteins (Bachir et al. 2014). The evidence for preformed assembly of FA proteins prior to FA formation was further strengthened by a study utilizing FCCS and FRAP. In this study, 15 different interactions between FA proteins were found in the cytosol (Hoffmann et al. 2014). This included interaction between paxillin and FAK (as previously seen using ICS) but also included interaction between paxillin and vinculin. Some other interactions found were between VASP, zyxin, alpha-actinin, and vinculin and a complex between zyxin, VASP, and CAS. These mutually exclusive complexes suggest that some FA proteins get recruited to FAs from different pools bound to other different proteins (Hoffmann et al. 2014). This study also found that the preformed FA complexes enter and exit FAs symmetrically and this may provide an explanation on how FAs so rapidly assemble or disassemble in a cell.

Thus FCS/FCCS/ICS have extended the ability of light microscopy to study protein interactions dynamically and with high spatial resolution. Evidence from studies highlighted and others suggests that FA as a macromolecular assembly is built not by recruitment of single proteins but by recruitment of assemblies of proteins which can ensure an error-free, rapid, and well-regulated system of assembly and disassembly. The exact nature of these complexes (phosphorylation state, conformation), regulation, and localization mechanism are currently unknown. This "modular building block" phenomenon can have implications for how other macromolecular assemblies form and that remains to be investigated.

6.4 Single Molecule Tracking (SMT) Reveals Heterogeneous Dynamics of FA Proteins

For most typical microscopy systems which are diffraction limited, the resolution is about 250 nm in the lateral/XY plane and 600–800 nm in the axial/Z plane in the visible spectral range. Thus, FRAP and correlation-based techniques (FCS/ICS) report on the average behavior of an ensemble and understanding how the dynamics of single proteins contribute to the ensemble requires SMT-based techniques.

SMT or single particle tracking (SPT) was a very important development in optical microscopy (Moerner and Orrit 1999) which has now been used in a number of

different biological contexts, both in vitro and in cells (Kusumi et al. 2014; Joo et al. 2008). SMT relies on the principle of single molecule imaging and localization, i.e., if it is known a priori that a diffraction-limited image is from a single fluorescent molecule then one can estimate with a precision higher than the diffraction limit the location of that molecule. One frequently used method to achieve this localization is to fit the point spread function (PSF) of the image of the single molecule to a 2D Gaussian distribution with the peak of the Gaussian serving as a proxy for the position of the molecule (Schmidt et al. 1996). Mapping the trajectory in time of single molecules using this technique has been facilitated in recent years due to application of TIRF (to improve SNR), improvement in camera technology (use of CCDs and EMCCDs), and development of brighter and more photostable fluorophores. In fact, these advances in SMT techniques have directly led to most pointillist super-resolution microscopy (SRM) techniques like PALM and STORM highlighted below. SPT allows for direct measurement of the position of molecules in cells as they bind, associate and disassociate, engage ligand, and interact with their surroundings, and has been used extensively in cells to study the physical properties of the membrane and membrane receptors (Kusumi et al. 2014).

FAs are ideal structures for SPT as they are built on the plasma membrane. Indeed, tracking of non-FA membrane proteins like the transferrin receptor and Thy1 at ~4 ms time scale found that they enter, diffuse, and rapidly exit the FA zone (Shibata et al. 2012). Tracking of β3 integrins, on the other hand, revealed completely different transport dynamics, with integrins immobilizing in the FAs before diffusing away. These observations were consistent with measurement of integrin dynamics made by combining SPT with PALM (Manley et al. 2008). Not only was it observed that integrins exhibited diffusion and immobilization in the FAs, but in fact the dynamics were not the same for different integrin types, suggesting a regulation mechanism dictating this phenomena (Rossier et al. 2012). These observations have led to a physical model for FAs where the majority of the FA region consists of a fluid plasma membrane, and integrins form clusters which dot the FA region like islands with free diffusion in the space between the islands (Kusumi et al. 2014; Shibata et al. 2013).

Recently, SMT-based approach was also used to look at protein conformational changes in FAs in a live cell. Here, the authors tagged the N and C terminus of talin with fluorophores of different color and using single molecule localization techniques found that individual talin molecules can stretch almost 5–8 times its length due to myosin contractility. This stretching facilitates the binding of vinculin to its otherwise hidden domains and provides evidence for force-mediated FA strengthening via conformation changes and recruitment of FA proteins (Margadant et al. 2011).

Thus, SMT techniques enable measurements of protein mobility revealing the nature of the FA environment and can also be used to image changes in protein conformations. These parameters are critical to understand how FAs are physically and biochemically regulated at different time scales.

6.5 Super-Resolution Microscopy: Nanoscale Architecture of FAs

Because of resolution limits, conventional light microscopy has revealed very little about the ultrastructure of FAs. While recent work using cryo-electron tomography has shown the basic structural scaffold of FA architecture (Patla et al. 2010), EM-based approaches cannot dissect the organization of the wide variety of proteins within it. These obstacles have been overcome by recent advances in super-resolution microscopy (SRM) which have begun to highlight the complex architecture and organization of FAs.

One strategy to overcome diffraction limit in fluorescence microscopy is to reduce the overall size of the focal spot used for imaging which results in a smaller PSF of the microscope. Stimulated emission depletion (STED) microscopy is one such technique which uses a second laser to suppress fluorescence emission from fluorophores around the center of excitation (Klar et al. 2001; Hell and Wichman 1994; Wildanger et al. 2009). The strength of STED over other SRM techniques is its imaging speed which is why it has been combined with FCS to study protein dynamics at time scales not achievable by many other techniques (Kastrup et al. 2005). In FA biology, STED microscopy has been used to show that small GTPases, RhoA, RhoD, and Rac1 promote a homogenous distribution of nanoscale FA particles on the ventral surface of cells (Gad et al. 2012). STED has also been use to show that β3 integrins are twofold higher in abundance than β1 integrins within a single FA (Rossier et al. 2012).

Another technique for improving the x-y resolution based on changing excitation light pattern is structured illumination microscopy (SIM). Here, a patterned illumination field is applied to the sample and multiple images are collected by changing the phase and orientation of the illumination pattern. The mixing of spatial frequencies of the pattern and the sample results in shifting of high frequency information to lower frequencies which are then collected by the objective lens (Gustafsson 2000, 2005). SIM has been used to study the high resolution localization of vinculin in macrophage podosomes (Walde et al. 2014) and has more recently revealed that a single FA consists of an aligned array of linear structures consisting of β1 integrins, FAK, paxillin, vinculin, and zyxin (Hu et al. 2015). Interestingly, each linear subunit is attached to a single actin cable, and growth of the subunit is linked mechanically to the growth of the actin cable and actomyosin contractility (Hu et al. 2015). This could be the first direct evidence for the "islands" of FA proteins predicted from SPT measurements described earlier.

Another strategy to overcome the diffraction barrier in the x-y dimension relies on the principle of SMT detailed earlier and the use of photo-activable or photo-switchable fluorescent molecules to localize single molecules within dense structures. The individual molecules are photo-activated, imaged, and then bleached, which allows for temporal separation of molecules which are spatially inseparable (Fig. 6.1c). By mapping the location of individual molecules and then merging all the locations, a super-resolution image of the structure can be obtained. Photoactivated

localization microscopy (PALM) (Betzig et al. 2006), fluorescence photoactivated localization microscopy (FPALM) (Hess et al. 2006), and stochastic optical reconstruction microscopy (Rust et al. 2006) are examples of techniques which use this approach. PALM and STORM-based approaches have been used in various studies on FAs with PALM being used in both live cell imaging and in two color mode (Shroff et al. 2008a, b). Dual color STORM imaging has recently revealed the nanoscale organization of different proteins in podosomes, which are FA structures functionally distinct from FAs but are composed of some of the same proteins (Van den Dries et al. 2013).

Most structures in a cell are however not two-dimensional but are three-dimensional in nature which has necessitated the extension of SRM techniques to the axial dimension. While a number of approaches have been used to achieve higher axial resolution (Gustafsson et al. 1999, 2008; Huang et al. 2008a, b), interferometry-based approaches have found the most application in studies of FAs.

The use of interferometry in PALM systems has led to one of the highest axial resolutions (~10 nm) in a technique called iPALM that has been applied to map the location of proteins along the z-dimension of FAs (Shtengel et al. 2009). Interferometry is a technique that relies on light interfering with itself after taking two different position-dependent paths. In iPALM, two objectives are used and fluorescence emission from each fluorescent molecule is collected through both objectives and combined using a specialized beam splitter. The difference in path length taken by a photon between the two objective lenses results in a phase difference that is proportional to the axial coordinate of that photon source. iPALM revealed for the first time that FAs are a stratified, ~100 nm high macromolecular complex with different FA proteins localized within different layers of the FA. Interestingly, talin and vinculin that link integrins to the actin cytoskeleton either directly (talin) or indirectly (vinculin) were the only FA proteins imaged that were found to span multiple layers of the FA (Kanchanawong et al. 2010; Case et al. 2015).

Another interferometry technique which has led to increased axial resolution is scanning angle interference microscopy (SAIM) (Paszek et al. 2012). Though not a true super-resolution microscopy technique, the ease of implementation makes SAIM an excellent complimentary technique to other microscopy modalities. SAIM has been used to show that microtubules require CLASP2 to target to FA (Stehbens et al. 2014), that extrinsic and intrinsic forces are required for vinculin activation (Rubashkin et al. 2014), and more recently has been used to show that the glycocalyx drives integrin clustering and activation (Paszek et al. 2014). SAIM has also been used in complement with iPALM to show that talin spans the height of the FA and is polarized at very precise angles w.r.t. the FA plane (Liu et al. 2015). Interestingly, this study also showed that the length of talin determines directly the height of the FA in cells and thus is a molecular ruler in FAs (Liu et al. 2015).

Another technique developed for axial super-resolution and used to image FAs is a confocal-based technique where a z stack is obtained at step sizes smaller than the PSF of the system (Chiu and Gratton 2013). A phasor approach is then used to determine the axial center of mass to get axial information of the molecule

(Chiu and Gratton 2013). This approach has also confirmed the layered architecture of FAs as reported using iPALM.

All these techniques have revealed a well-organized hierarchical structure for FAs with different proteins occupying different layers. How this structure is built and how it is organized when cells are in a 3D environment are currently not known. Additionally, within this anisotropic structures how the individual molecules are oriented and what implications that has for the molecule and for the cell is also unknown and needs to be investigated.

6.6 Forster Resonance Energy Transfer (FRET) Sensors for FA Protein Conformations, Activity, and Measuring Molecular Forces

The molecular basis of FA-mediated cellular functions like those highlighted above have resulted in the need for techniques that can measure small changes in protein conformation, measure forces exerted on specific molecules within FAs, and visualize protein–protein interactions in living cells. FRET is one such technique which facilitates these requirements by its ability to measure distances in the length scale of 1–10 nm. FRET is a phenomenon of energy transfer from an excited fluorophore (donor) to another fluorophore (acceptor) which only occurs when the two are in close proximity with each other. The efficiency of energy transfer is highly dependent on the distance ($\sim 1/R^6$) between the fluorophores making FRET an extremely sensitive molecular ruler. It is this property of FRET that has made it a versatile "fluorescent biosensor" tool in cell biology in which changes in protein conformation or interactions detected by FRET are induced by the activity of enzymes or physical perturbations. This principle has been applied to read out kinase and phosphatase activities or measure the physical tension that acts on FA proteins.

FRET-based biosensors for FA proteins including for FAK (Papusheva et al. 2009; Cai et al. 2008), vinculin (Chen et al. 2005), and integrins (Askari et al. 2010; Kim et al. 2003) have been used to measure distinct conformational changes and activity in these proteins in FAs. A recent study using a vinculin activation FRET sensor in combination with iPALM revealed that vinculin localizes to different layers of the FA dependent on its activity (Case et al. 2015). FRET studies on integrins in FAs have shown that leg separation between the α and the β subunit of integrins was required for integrins to go to a higher affinity state for its ligand (Kim et al. 2003). FRET was also used to measure distance between the integrin and the cell membrane and it was found that in FAs, integrins are mostly in an extended conformation (Askari et al. 2010).

More recently, FRET-based biosensors have been used to measure tension across individual FA proteins like vinculin (Grashoff et al. 2010) and α-actinin (Verma et al. 2012). In these biosensors, the FRET module consists of the fluorophore pair separated by an elastic linker with known mechanical properties. By inserting this module into vinculin, tension across vinculin molecules within FAs was measured for the

first time in a cell (Grashoff et al. 2010) (Fig. 6.1d). This showed that forces across vinculin were highly dynamic and varied depending on FA location (front versus back) or state (growing versus disassembling). Modified versions of this tension sensor module have resulted in better FRET efficiency (by changing fluorophores with organics dyes (Morimatsu et al. 2013)) as well as higher sensitivity to measure molecular tension (by replacing the linker to calibrated double-stranded DNA (Wang and Ha 2013) or PEG polymers). These probes can now be genetically expressed or be anchored to the substrate to measure force between a receptor and an immobilized ligand. All these advances have led to remarkable sensitivity to measure forces of the order of a few piconewtons and measurements of forces exerted by single integrin molecules on its ligand in a cell (Morimatsu et al. 2013; Zhang et al. 2014).

6.7 Force Microscopy: Understanding the Role of Forces and Mechanics in Cell Biology

The first clear demonstration that cells exert forces on the substrate was based on observations that cells induce wrinkles on a flexible substrate (Harris et al. 1980). Using IRM, it was further demonstrated that FAs were the sites where these "traction" forces were exerted. The subsequent 30 years have focused on developing quantitative assays and using them to understand the role of forces in cellular functions and dissecting the mechanism by which cells exert and sense forces (Fig. 6.2).

The first accurate quantitative measurement of cell traction utilized micromachined arrays of cantilever beams showed that cells exert asymmetric forces, with maximum force exerted at the front of the cell (Galbraith and Sheetz 1997). However, the need to measure traction with high spatial resolution led to development of traction force microscopy or TFM. TFM methods can be classified under two categories. In substrate-based TFM, fluorescent beads are embedded in an elastic gel and the movement of beads in presence of cells is tracked to calculate traction force magnitudes and dynamics (Oliver et al. 1995; Dembo and Wang 1999; Pelham and Wang 1999; Balaban et al. 2001) (Fig. 6.2e). The second approach uses a bed of micropillars of silicone elastomer which get bent when cells exert traction on them (Tan et al. 2003; Du Roure et al. 2005; Schoen et al. 2010) (Fig. 6.2f). Substrate-based TFM, while easier to implement, requires complex continuum modeling to deconvolve forces from bead displacement fields. On the other hand, micropillars are harder to make and require microfabrication tools but calculation of traction forces is easier as individual pillars under small deformations can be approximated as linear springs. Both these techniques have been used extensively to measure traction forces in many cell types and have highlighted the complexities in traction forces exerted by a cell on a 2D substrate. Seminal work using flexible polyacrylamide substrates for TFM has shown that NAs exert the highest forces during migration, while bigger FAs apply relatively weaker forces (Beningo et al. 2001). Additionally, as FAs mature, traction forces decrease (Beningo et al. 2001) and

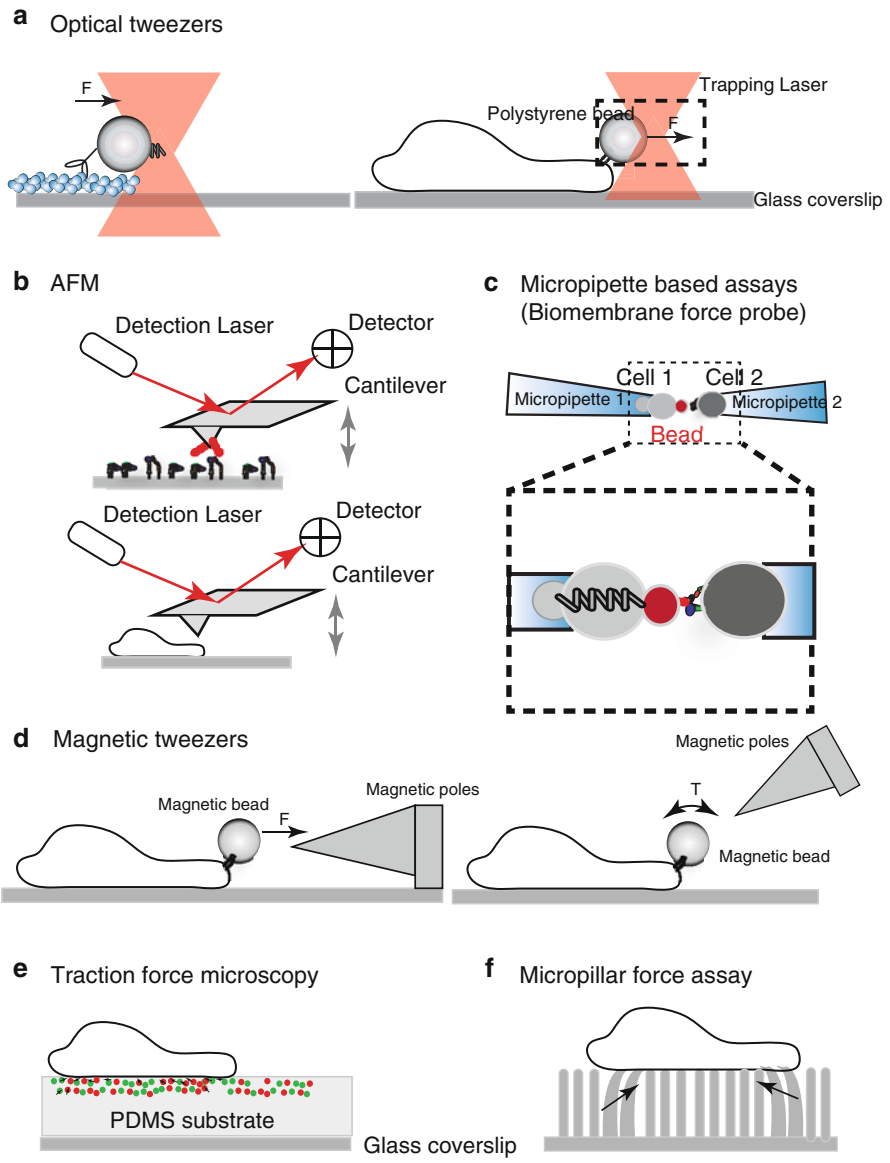

Fig. 6.2 Force microscopy-based approaches to study FAs. (**a**) (*Left*) Optical tweezers uses a laser trap (depicted as a focal spot and a spring) to apply restoring forces on a glass bead attached to molecule of interest. (*Right*) For studying FAs, beads coated with ECM proteins are attached to cells and force is then applied by the trap. (**b**) Atomic force microscopy (AFM) uses a cantilever (*grey*) with a sharp tip mounted on a piezo stage to drive its interaction with a specimen (cells or purified proteins in vitro as depicted here). Deflection of the cantilever is measured using a laser and a photodiode-based detector. (**c**) Illustration of a biomembrane force probe. A red blood cell (*red circle*) with ligand of interest is aspirated on one micropipette and another micropipette with a cell expressing receptor of interest is brought into contact with it. (**d**) Magnetic tweezers to apply forces on cells. Magnetic bead coated with ECM protein of interest is allowed to attach to cells and an external magnetic field is used to apply linear forces (*left*) or twisting forces (*right*). Recruitment of proteins can be imaged or stiffness of cell-bead attachment can be calculated by calibrating for

these forces correlate biphasically with retrograde actin flow speeds (Gardel et al. 2008). Recently, substrate-based TFM have been extended to the third dimension and have shown that cells exert forces via FAs in all three dimensions with the overall effect being a rotation moment about FAs (Legant et al. 2012). Additionally, increase in resolution of TFM has revealed new insights into the dynamics of traction exerted within individual FAs where cells exert and use tugging forces on the substrate to probe its environment (Ghassemi et al. 2012; Plotnikov et al. 2012). One of molecular pathways that mediates this tugging involves the FAK-paxillin pathway and is required for cells to migrate along a stiffness gradient (Plotnikov et al. 2012).

While TFM and micropillar assays measure the forces applied by cells, active techniques apply forces on individual cells or protein complexes to dissect mechanisms of mechanosensing. A number of techniques have been developed to mechanically probe biological materials, each with their advantages, disadvantages, and applications.

Optical tweezers, also called optical trap or laser tweezers, are one of the most versatile and widely used assays for in vitro and cellular force spectroscopy. A laser trap is created by focusing a laser to a diffraction limited spot with a high NA objective (Fig. 6.2a). Any dielectric particle near the laser spot experiences a restoring force produced by the momentum of light, which can be calculated by approximating the trap as a linear spring. Optical tweezers have been used to manipulate biological systems over a wide range of length scales (cells to RNA polymerase advancing a single base pair) and force scales (disrupting covalent bonds to nucleic acid folding kinetics). One of the early observations of how cells respond to changes in ECM mechanics used optical tweezers to change the local ECM stiffness (Choquet et al. 1997) and identified the ECM–integrin linkage as site of force-mediated strengthening of the cell. Since then, optical tweezers have been used to study the mechanism of many of these processes, including to show that FA proteins like vinculin get recruited to sites of force application (Galbraith et al. 2002), and that talin is required for engagement of integrin to the actin cytoskeleton when forces are exerted to FAs (Jiang et al. 2003). Combining FRET-based biosensors with optical tweezers has also shown that forces on FAs can activate Src kinase (Wang et al. 2005).

Magnetic tweezers, like optical tweezers, is a bead-based technique that uses the force due to an external magnetic field gradient to apply calibrated forces on the material attached to the bead (Matthews et al. 2004; Trepat et al. 2003; Fisher et al. 2005) (Fig. 6.2d). The advantage of magnetic tweezers over optical tweezer lies in the fact that at any given time, force can be applied to a number of beads instead of a single bead as is the case in most conventional optical tweezers system.

Fig. 6.2 (continued) forces and measuring bead displacement. (**e**) Substrate-based TFM showing a cell on an elastic substrate embedded with beads. For high resolution TFM, two color beads (as shown here) can be used and bead displacements are captured as cells exert traction on the substrate. (**f**) Micropillar-based TFM utilizes pillars fabricated from an elastic material. Force applied by cells on the pillar results in pillar deflections that can be measured to estimate traction forces

Additionally, magnetic tweezers can apply forces from a few piconewtons to nanonewtons whereas single optical tweezers apply forces up to about 100 pN. Magnetic tweezers have been used to show that the ability of FAs to withstand high forces is mediated by clustering of α5β1 and that αvβ3 integrin and talin are required for reinforcement of FAs (Roca-Cusachs et al. 2009). Magnetic tweezers have also been used to show that FA protein vinculin is required for maintaining cell stiffness (Mierke et al. 2008) and this mechanical function of vinculin is through its binding to talin (Yao et al. 2014). Recently, it was shown that application of forces on FAs with magnetic tweezers resulted in activation of RhoA through the recruitment of Rho-GEFs, GEF-H1, and LARG (Guilluy et al. 2011). Use of magnetic tweezers in vitro on the FA protein talin showed that force induced a change in its conformation resulting in increased vinculin binding (Del Rio et al. 2009). The result from this study was one of the earliest evidences suggesting a molecular basis for mechanosensing via FA proteins (Del Rio et al. 2009).

Atomic force microscope (AFM) and micropipette-based force techniques are nonoptical-based techniques that have also been used to study force response of FAs, both in vitro and in vivo. The AFM which uses a cantilever with a sharp tip mounted on a piezo stage to drive its interaction with a specimen has also been used in a variety of studies both on cells and on protein systems in vitro (Fig. 6.2b). Since AFM was developed to measure sub-nanometer changes in surface topography, it is an extremely sensitive and rapid technique for single molecule assays to study rupture of molecular bonds and conformational changes to proteins (Schwaiger et al. 2004; Oesterhelt et al. 2000; Rief et al. 1997). In fact the catch bond-like behavior of integrins was first demonstrated in vitro using an AFM (Kong et al. 2009). AFM has been used to measure bulk mechanical properties of a cell (Costa et al. 2006; Cross et al. 2007) and the ECM, and has also been used as force spectroscopy tool to study integrin–ECM linkage in cells (Zhang et al. 2004; Taubenberger et al. 2007; Sun et al. 2005; Lehenkari and Horton 1999).

Micropipette-based probing in cells showed that force induces FA growth (Riveline et al. 2001) and in vitro has been used to measure receptor-ligand binding kinetics (Chesla et al. 1998). A modified micropipette assay to measure protein–protein interaction is the biomembrane force probe (BFP) where a red blood cell (RBC) expressing ligand of interest is aspirated on one micropipette and another micropipette with a cell expressing receptor of interest is brought into contact with it (Evans et al. 2004) (Fig. 6.2c). This approach has been used to study integrin–ECM bind dynamics with great detail and has shown that unlike regular bonds where force reduces bond lifetimes, the integrin–ECM bond is a catch bond, with force-dependent increase in bond lifetime (Chen et al. 2012; Kong et al. 2013).

Overall these studies have indicated that mechanosensing in cells is regulated by changes across many length, time, and force scales. Piconewton level forces can induce changes in protein conformation or receptor ligand bonds which can induce changes in FA morphology, cytoskeletal architecture, and result in changes in force exerted by cells during migration or other cellular functions.

6.8 Concluding Remarks

Integrin-based focal adhesions are the primary physical and biochemical link between the cell and its environment. It is therefore of no surprise that these organelles have evolved such functional, compositional, and morphological complexities as different cells are exposed to different physical and biochemical environments. As microscopy advances over the past 50–60 years have deciphered many of these complexities, they have also led to new questions that require more technological developments. Further advances in super-resolution microscopy (for live cell imaging), force microscopy in 3D environments, structural level imaging in cells, and in vivo rapid imaging with high resolution will provide us with the next level of information on how single proteins are activated and organized in a cell or how adhesions are organized and regulated in vivo.

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Part III
Epigenetic and Genetic Regulations in the Nucleus

Chapter 7
Perspectives of FRET Imaging to Study Epigenetics and Mechanobiology in the Nucleus

Qin Peng, Binbin Cheng, Shaoying Lu, Shu Chien, and Yingxiao Wang

Abstract Epigenetic modulations are crucial for the regulation of chromatin structures and genomic organizations, which are key factors determining gene expressions and cellular functions. Mechanical cues have also been shown to play important roles in modulating gene expressions and cellular functions. While there have been significant advances in our understanding of mechanotransduction in the nucleus, there is a lack of knowledge on the molecular details by which mechanical cues affect epigenetic and chromatin regulations to determine genetic outcomes. In this chapter we first introduce the current understanding on epigenetic regulations, particularly on histone modifications and DNA methylations. This is followed by the epigenetic regulations related to mechanobiology in the nucleus. We then introduce the development of genetically encoded molecular biosensors and the principles based on fluorescence proteins (FPs) and fluorescence resonance energy transfer (FRET) for the visualization of dynamic epigenetic regulations in single cells. Lastly, we present examples of the application of biosensors to visualize mechanotransduction events occurring in the nucleus in live cells. The single cell imaging of nuclear mechanotransduction can shed new lights on the molecular mechanisms regulating physiological and pathophysiological processes in living cells under different mechanical environments.

Keywords Live cell imaging • Fluorescence proteins (FPs) • Fluorescence resonance energy transfer (FRET) • Mechanobiology • Mechanotransduction

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7.1 Introduction

Epigenetics refers to molecular variations in cells that change gene expression without altering DNA sequence, typically through changes in DNA modifications and chromatin proteins, e.g., DNA methylations and histone posttranslational modifications that alter DNA accessibility for the activation or silencing of transcription. It has been well documented that epigenetics and its modulation can govern genomic regulation and ultimately cell fates (Li et al. 2012). In particular, epigenetics has been shown to mediate the cellular functions in response to the changing mechanical cues in the cell microenvironment (Downing et al. 2013; Tan et al. 2014). However, it remains largely unclear how epigenetics is regulated in space and time in relation to the genomics upon receiving the mechanical cues. Most current studies utilize the DNA sequencing, Chip-on-Chip, and Chip-Seq technologies to investigate epigenetic regulation (Li et al. 2012). However, these assays in general require the signals to be averaged from a large number of lysed cells obtained at a given time. As such, the dynamic nature of epigenetic signals crucial for cellular functions in single cells, such as cell cycle control, can be masked by the noise engendered from the cell–cell heterogeneity, particularly in non-synchronized cells with relatively varied cellular processes. Therefore, there is an urgent need for the development of new imaging technologies and detection methods in order to elucidate the spatiotemporal landscape and epigenetic regulation.

7.2 Epigenetic Regulation and Mechanobiology in the Nucleus

7.2.1 Chromatin

Chromatins are highly ordered nuclear structures that contain DNAs, histones, and other chromosomal proteins. A fundamental subunit of chromatin in eukaryotes is the nucleosome, which consists of approximately 147 base pairs (bp) of DNA associated with a complex of eight core histones (two copies each of histone H2A, H2B, H3, and H4). The wrapping of genomic DNA sequence in 1.6 turns around the octamer results in a five- to tenfold compaction of DNA (Kornberg 1974; Kornberg and Thomas 1974; Black et al. 2012). The compact DNA is only partially accessible to regulatory proteins, but it can become more available if there is a conformational alteration of the nucleosome, or if the DNA is partly unwound from the histones. The histone tails protrude from the core complex and are readily accessible. Enzymes can chemically modify these tails to create docking motifs and recruit various chromatin modulators for the regulation of nucleosome remodeling and DNA unwinding, which can have profound effects on the chromatin complex and genomic regulation (Felsenfeld and Groudine 2003). Since chromatin has a very compact organization and most DNA sequences are structurally inaccessible at rest,

it is in general unfavorable for transcription. It has been shown that enzymes recognizing DNA sequences have easier access to nucleosomes associated with active genes (Weintraub and Groudine 1976), suggesting a selective unfolding of the compact structure to activate gene transcription (Felsenfeld and Groudine 2003).

Chromatins and nucleosomes are subject to modifications to potentially alter their structures and regulate gene activity. There are three general ways in which chromatin structure can be altered by nucleosome modification. First, utilizing ATP, nucleosome remodeling can be directly induced by chromatin remodeling complexes designed specifically for the task (Becker and Horz 2002). Second, covalent modifications of histones can occur in the histone tails to recruit chromatin remodeling proteins (Zhang and Reinberg 2001). Third, histone variants may replace one or more of the core histones (Ahmad and Henikoff 2002; Redon et al. 2002; Smith 2002; Felsenfeld and Groudine 2003).

7.2.2 Chromatin and Histone Posttranslational Modifications (PTM)

Since the pioneering studies by Allfrey in the early 1960s, posttranslational modifications on histones have been well documented (Allfrey et al. 1964). A large number of different histone posttranslational modifications (PTMs) have been reported and categorized (Table 7.1 and Fig. 7.1) (Luger et al. 1997; Cota et al. 2013). Among all the PTMs, histone acetylation, methylation, and phosphorylation are the most prevalent forms. In fact, all histones can be modified at different sites by acetylation, methylation, and phosphorylation. The determination of the high-resolution X-ray structure of the nucleosome in 1997 led to the insights into how these histone PTMs could affect chromatin structure (Luger et al. 1997). The resolved structure indicates that the highly basic histone amino (N)-terminal tails can extend from their

Table 7.1 Different classes of modifications identified on histones (Kouzarides 2007)

Chromatin modifications	Residues modified	Functions regulated
Acetylation	**K**-ac	Transcription, repair, replication, condensation
Methylation (Iysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, repair, condensation
Ubiquitylation	**K**-ub	Transcription, repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

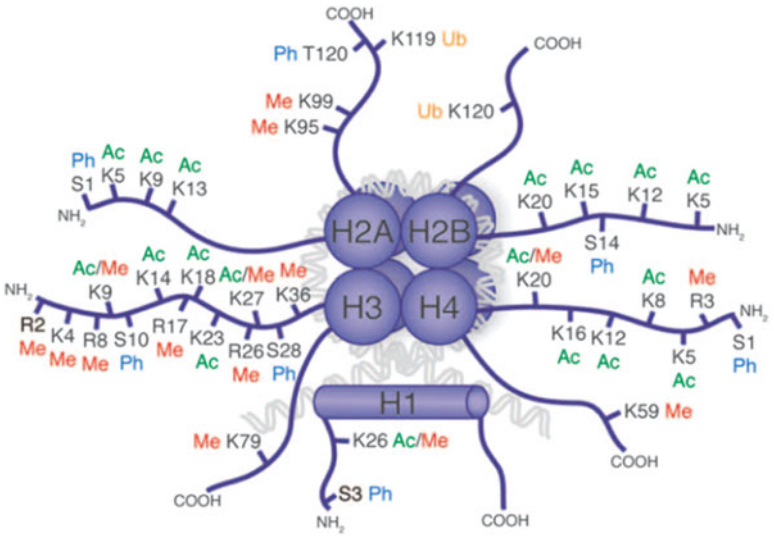

Fig. 7.1 Nucleosome with histone posttranslational modifications (Cota et al. 2013)

own nucleosome and make contact with adjacent nucleosomes. It is likely that the modification of these tails can affect inter-nucleosomal interactions and thus affect the structure of the whole chromatin (Bannister and Kouzarides 2011).

Histone acetylation was first reported in 1964 (Allfrey et al. 1964). The acetylation on lysine sites is highly dynamic and regulated by the opposing action of two families of enzymes, histone acetyltransferases (HATs) and histone deacetylase (HDACs) (Fig. 7.2) (Xhemalce et al. 2011). The HATs utilize acetyl CoA as a cofactor and catalyze the transfer of an acetyl group to the lysine side chains. This action neutralizes the lysine's positive charge, and has the potential to weaken the interactions between histones and DNA for chromatin remodeling (Bannister and Kouzarides 2011).

Histone phosphorylation takes place on residues including serine, threonine, and tyrosine, predominantly in the N-terminal histone tails (Xhemalce et al. 2011). Like histone acetylation, the phosphorylation of histones is also highly dynamic. The phosphorylation level is controlled by kinases and phosphatases that add and remove the phosphate group, respectively (Oki et al. 2007). Histone kinases transfer a phosphate group from ATP to the hydroxyl group of the target amino acid side chain, adding negative charge to the histone to affect the chromatin structure. However, it is unclear how the kinases are accurately recruited to the phosphorylation sites on histones at chromatins. Even less information is available regarding the roles of histone phosphatases, although it is clear that a high level of phosphatase activity exists in the nucleus to direct a rapid turnover of histone dephosphorylations. For example, the proteins phosphatase 1 (PP1) can rapidly neutralize the action of Aurora B kinase, which causes genome-wide phosphorylation events at H3S10 during mitosis (Fig. 7.3) (Goto et al. 2002; Sugiyama et al. 2002; Bannister and Kouzarides 2011).

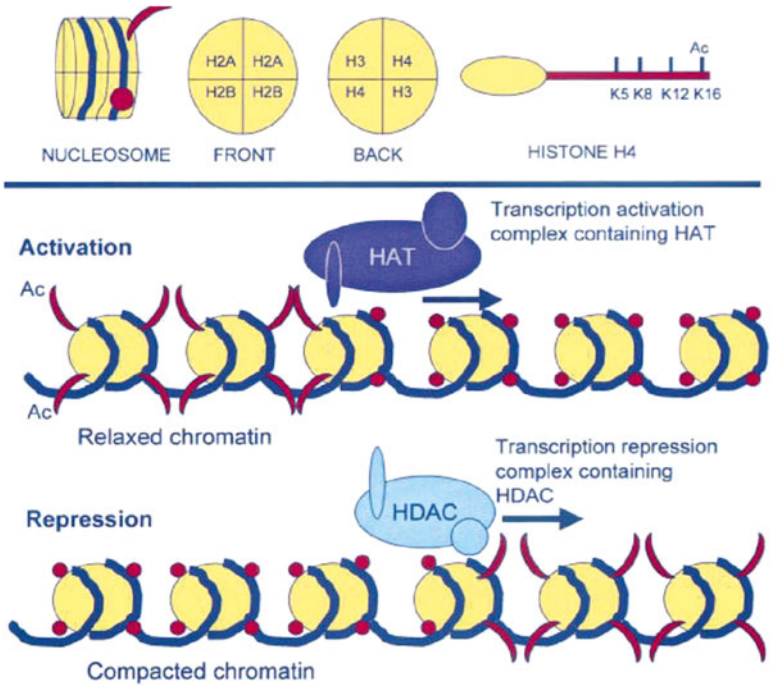

Fig. 7.2 The diagram of histone acetylation regulation (Cota et al. 2013)

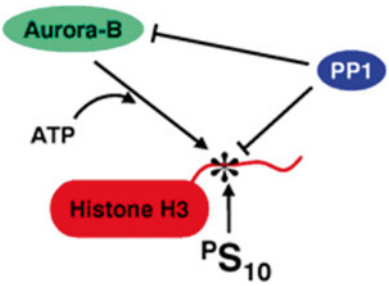

Fig. 7.3 The phosphorylation and dephosphorylation of H3S10 by Aurora kinase and PP1, respectively (Oki et al. 2007)

Unlike acetylation and phosphorylation, histone methylation does not alter the charge of histone proteins. Lysines and arginines are the main targets for histone methylation, with lysines being mono-, di-, or tri-methylated (Fig. 7.4) whereas arginines being mono-, or symmetrically or asymmetrically di-methylated (Bannister and Kouzarides 2005; Bedford and Clarke 2009; Lan and Shi 2009; Ng et al. 2009; Bannister and Kouzarides 2011; Xhemalce et al. 2011). The methylation level is controlled by methyl-transferase and demethylase that can add and remove the methyl group, respectively.

Among the different methyl-transferases, SUV39H1 is the first identified histone lysine methyl-transferase (HKMT), which targets H3K9 for tri-methylation

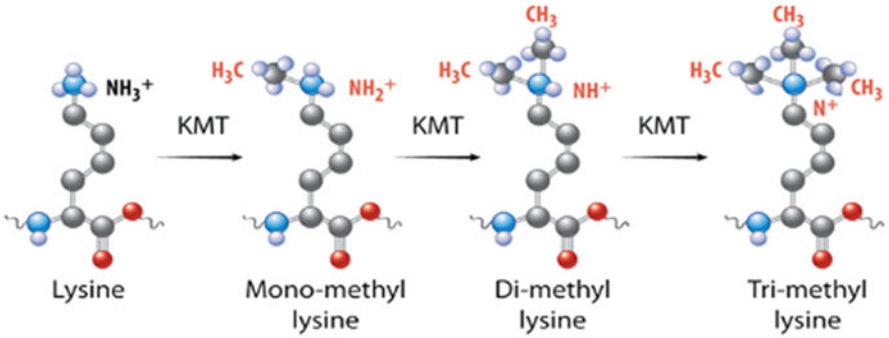

Fig. 7.4 The diagram of different lysine methylations (Rea et al. 2000)

(Bannister and Kouzarides 2005). Numerous HKMTs have since been identified, with the vast majority of them methylating lysines at the N-terminal tails of histone proteins. Except the Dot1 enzyme that methylates H3K79 in the histone globular core, HKMTs that methylate N-terminal lysines contain a so-called SET domain harboring the enzymatic activity, which can specifically catalyze the transfer of a methyl group from *S*-adenosylmethionine (SAM) to a lysine's ε-amino group. The resulted outcome can be either activation or repression of corresponding gene transcriptions (Martinez-Balbas et al. 1995; Bannister and Kouzarides 2011).

Current information suggests that some lysine methylation sites on histones are associated with transcriptional activation, e.g., H3K4, H3K36, and H3K79, with H3K4me and H3K36me implicated in transcriptional elongation. Other lysine methylation sites are connected to the repression of transcription, e.g., H3K9, H3K27, and H4K20. Methylation at H3K9 is implicated in the silencing of euchromatic genes, as well as forming silent heterochromatin such as pericentric heterochromatin surrounding centromeres. Gene repression related to the H3K9 methylation may involve the recruitment of methyl-transferase and heterochromatin protein 1 (HP1) to the promoter region of repressed genes. It is interesting that H3K9me3 and HP1γ are enriched in the coding region of active genes (Martinez-Balbas et al. 1995). It is possible that H3K9 methylation represents active signals within the coding regions, while promoting gene repression at the promoter sites (Kouzarides 2007).

Via histone posttranslational modifications, chromatin can undergo dynamic structural alterations to control the activation or repression of specific genes, e.g., during the cell cycle processes or in response to mechanical environmental cues. Indeed, the distinct H3 and H4 tail modifications may act sequentially or in combination to effect unique biological outcomes, constituting the so-called histone code (Strahl and Allis 2000). For example, H3K14 acetylation is required in addition to H3S10 phosphorylation to repel HP1 during G2/M transition in a cell cycle (Mateescu et al. 2004), with a minor influence of H3S10 phosphorylation observed on the HP1 binding to the tri-methylated H3K9 (Vermeulen et al. 2010). H3S10 phosphorylation can also lead to the recruitment of histone deacetylase HST2 for

the deacetylation of H4K16 and its interaction with H2A for chromatin condensation (Wilkins et al. 2014). However, it remains unclear on how this hierarchy of multiple modifications extends (depicted as "higher-order combinations"), or how distinct combinatorial sets are established or maintained in the localized regions of the chromatin structure.

7.2.3 Cell Cycle and Histone Modifications

In eukaryotic cells, chromosomes occupy relatively large and intertwined regions in the interphase nucleus. As such, the packing and unpacking of chromosomes into discrete units are key tasks during cell division. The mechanics of chromatin organization is also important during cell division. For example, centromeres, the distinct chromatin regions of chromosome on which kinetochores form during mitosis, allow kinetochore formation and attachment to the mitotic spindle, which mechanically moves chromosomes into daughter cells. It has been shown that the large displacement of transcription factors from chromatin and general transcription are suppressed during mitosis (Gottesfeld and Forbes 1997; Egli et al. 2008). Since the defining features of a specific cell lineage can be maintained epigenetically during cell proliferation, mechanisms must also exist to ensure a "memory" of transcriptional program is inherited through mitosis (Egli et al. 2008; Moazed 2011; Sarkies and Sale 2012), which usually involves chromatin remodeling (Wang and Higgins 2013).

In order to replicate the DNA sequence and pass it on in the genome, chromatin undergoes dynamic cycles of compaction and unpacking during cell cycle procession. Previous studies on replication timing have demonstrated that distinct chromatin replications happen at different times during S phases (Kennedy et al. 2000; Dimitrova and Berezney 2002). Euchromatic regions in general replicate early in the interior of the nucleus, followed by the replication of heterochromatin regions associated with the nuclear lamina. During this process, the local changes of chromatin microenvironment could dictate cell cycle and proliferation rate by regulating genes directly. The requirement of KMT4/Dot1 for the efficient entry into S phase is an example of transcriptional control of cell cycle genes through H3K79 di-methylation (Schulze et al. 2009). The chromatin microenvironment can also affect cell cycle indirectly by changing the replication timing. For example, KMT6/EZH2, by regulating epigenetic H3K27me3 levels, can target transcriptional regulations of cell cycle proteins Cyclin D1, E1, and A2 (Bracken et al. 2003; Black and Whetstine 2011). The overexpression of the H3K9me3 demethylase KDM4A results in a faster progression through S phase, possibly due to the better chromatin accessibility, increase in replication forks, and altered replication timing at heterochromatin regions (Black et al. 2012). Similarly, the loss of KDM4A in MDA-MB-231 breast cancer cells leads to a G1/S arrest and decreased proliferation rates (Black et al. 2010). Consistent with these results, H3K9me3 levels are reduced in S phase, accompanied by an increase in H3K9me1/2 levels (Fig. 7.5) (O'Sullivan et al. 2010). Although the complete molecular mechanisms remain to be elucidated, these results suggest that H3K9me3 and

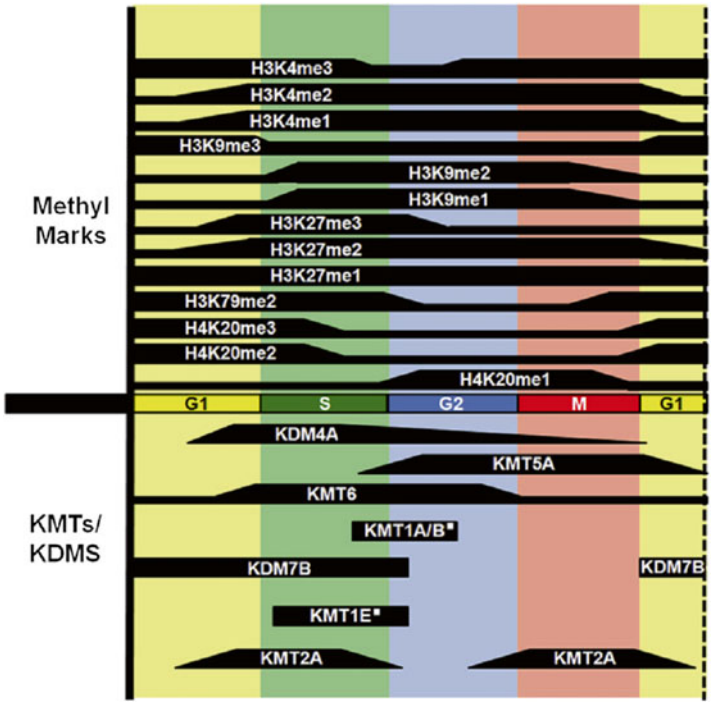

Fig. 7.5 Histone methylations, as well as KMTs and KDMs, are dynamically regulated during the cell cycle (Black et al. 2012)

other histone epigenetic marks may serve as conserved determinants of replication timing to regulate cell cycle processes. Therefore, the modulation of cell cycle genes can be a key determinant in cell cycle, which can be regulated by a dynamic balance between histone methyl-transferases and demethylases.

7.2.4 The Role of H3K9me3 and Chromatin Condensation in Cell Mitosis

In the interphase nucleus, chromatin is highly ordered for the storage of the genetic material (Campos and Reinberg 2009). Chromosome condensation occurs during mitosis to facilitate the separation of sister chromatids and the maintenance of genomic stability. It was demonstrated that histone modification within the nucleosome can trigger the structural changes for chromosome condensation (Trojer and Reinberg 2007; Duan et al. 2008). In fact, H3K9me3 was identified as a marker of heterochromatin and epigenetic silencing regions, which typically are located close to the centromeres (Schotta et al. 2004; Stewart et al. 2005). Accordingly, H3K9me3 is dynamically regulated in a cell cycle-dependent manner (McManus et al. 2006;

Duan et al. 2008; Park et al. 2011). H3K9me3 increased rapidly in G2 to reach a maximum, followed by a quick decline (McManus et al. 2006). Loss of H3K9me3 is correlated with mitotic defects such as failure of chromosomes alignment, as shown in mouse embryonic fibroblasts (MEFs) following the elimination of histone-lysine-*N*-methyl transferases (SUV39h1/h2−/− cells) (Peters et al. 2001; McManus et al. 2006). Both SUV39h1 and SUV39h2 are primary enzymes catalyzing the tri-methylation of H3K9 (Rea et al. 2000; Heit et al. 2009). In these SUV39h1/2 knock-out cells, the mitotic checkpoint is active and kinetochore proteins localize properly. However, pericentric chromatin in these cells is less condensed. Mis-segregation of chromosomes is also observed in cells treated with the methylation inhibitor adenosine dialdehyde (AdOx), which affects the tri-methylation of both H3K9 and H4K20. The reduced integrity of pericentric heterochromatin might be responsible for the loss of tension at the centromere and activation of the spindle assembly checkpoint in AdOx-treated cells, which leads to the mitotic defects. Therefore, an increase in H3K9me3 at late G2 phase and early mitosis may be needed to stabilize the pericentric heterochromatin so that the centromeres and kinetochores have a rigid structure needed for proper tension transmission to the inner centromere (Heit et al. 2009). However, this result based on immunostaining is in contrast to the more recent results of genome-wide dynamic change of H3K9me3 in the overall cell cycle (Fig. 7.5). A recent study further revealed an association between chromatin and a lysine demethylase KDM4C during mitosis, which is accompanied by a decrease in the mitotic levels of H3K9me3 (Kupershmit et al. 2014).

Epigenetic modifications including histone methylation at different residues are early events that can guide gene regulation networks and expressions. The histone methylations and their combinations can serve as codes to determine the overall gene expressions and phenotypic outcomes. For example, mounting evidence suggests a direct role of histone H3K9 methylation as a histone marker positively correlating with DNA methylation and participating in repressive heterochromatin formation in tumorigenesis. In the cell cycle, H3K9me3 is important for HP1γ recruitment to regulate gene expression, chromatin packaging, and heterochromatin formation. However, the dynamics of H3K9me3 remains controversial, mainly due to the lack of the appropriate tool for H3K9me3 detection in living cells.

7.2.5 Epigenetics in Mechanobiology

Increasing evidence suggests that epigenetic modulations in DNA methylation and histone modification play a crucial role in regulating gene expression and cellular functions under different mechanical environments. Early studies have demonstrated that shear stress can cause the chromatin remodeling on both histones H3 and H4 to result in the eNOS gene expression modulation at the transcriptional level (Illi et al. 2003; Fish et al. 2005). Recently, hemodynamic force-induced histone modifications in cardiovascular systems have been extensively studied (Chen et al. 2013), mainly on histone acetylation/deacetylation (HAT/HDAC).

Three main groups of HDACs sensitive to hemodynamic force were characterized: class I (HDAC-1/2/3 and HDAC-8), class II (HDAC-4/5/6/7 and HDAC-9/10), and class III sirtuins (SIRT). Laminar flow was shown to increase the activity of class I HDACs and induce the association between HDAC1 and p53, leading to the deacetylation of p53 at Lys-320 and Lys-373 in ECs for the cell cycle arrest (Zeng et al. 2003). Oscillatory flow was also shown to modulate cell proliferation by increasing the activity of class I HDAC 1/2/3, but not class II HDAC4/7 (Lee et al. 2012). Both Class II and III HDACs have been demonstrated to play a critical role in the shear stress-induced eNOS expression (Illi et al. 2008; Chen et al. 2010), with Class III HDACs also playing a protective role in atherosclerosis (Stein and Matter 2011). Besides the modification of histones, DNA hypermethylation was shown to result in atheroprone gene expression in human umbilical vein endothelial cells (HUVECs) and rat carotid arteries as well as hematopoietic cell development (Lund et al. 2004; Zhou et al. 2014). Kim et al. further demonstrated that both the global DNA methylation profiles in atherosclerotic tissues and the DNA methylation patterns of estrogen receptor-β are correlated to the atherosclerotic development (Post et al. 1999).

Mechanical signals are also important regulators of epigenetics in guiding stem cell behavior and cell/tissue differentiation. Recent study has demonstrated that cues from the mechanical microenvironment can alter the DNA methylation in promoter regions of osteogenic genes in bone marrow mesenchymal stem cells (bMSCs) to control the osteogenic cell fate (Arnsdorf et al. 2010). Biophysical cues such as microgroove topography were also shown to increase the histone H3 acetylation in somatic fibroblasts to promote cell reprogramming (Downing et al. 2013). Mechanical matrix stiffness was further shown to affect structural protein lamin-A and guide cell differentiations (Swift et al. 2013). In fact, stem cell differentiation into fat cells on soft matrices and into bone cells on stiff matrices can be enhanced by the low and high lamin-A levels, respectively (Swift et al. 2013). These observations are consistent with the subcellular location and function of lamin-A proteins, lying inside the nuclear envelope and interacting with both chromatins and the cytoskeleton to transmit mechanical signals into the nucleus for the regulation of epigenetics and gene expression. Recent observation revealed that a high level of H3K9 tri-methylation is correlated with the peripheral localization of chromosomes proximal to Lamins, with the inhibition of H3K9 tri-methylation leading to the relocation of chromosomes (Bian et al. 2013). Consistently, Lamin-A/C-deficient ($Lmna^{-/-}$) and $Lmna^{N195K/N195K}$ mutant cells have impaired nuclear morphology and downstream signaling of the mechanosensitive transcription factor MKL1 (Ho et al. 2013). Moreover, a single point mutation (G608G) in lamin-A gene (*LMNA*) caused aging-associated nuclear defects including disorganization of nuclear lamina and loss of heterochromatin (Scaffidi and Misteli 2005; Scaffidi and Misteli 2006; Dechat et al. 2008; Pegoraro et al. 2009). This critical role of lamin A in regulating epigenetics and gene expression can also be seen in Hutchinson–Gilford progeria syndrome (HGPS) fibroblasts, which showed abnormal nuclear morphology and lamina, loss of heterochromatin markers H3K9me3, HP1α, and HDAC1 (Liu et al. 2011). Therefore, lamin A, by mechanically connecting cytoskeleton outside and

chromatin inside the nucleus, may serve as a mechanotransducer to relay mechanical signals into nucleus to regulate epigenetic modifications and gene transcriptions for the determination of cell fates.

7.3 FRET Imaging of Epigenetic Regulations in Single Cells

7.3.1 FRET Imaging

Recent advances in genetic technologies have allowed the sequencing of the whole human genome, providing invaluable information toward our molecular understanding of human pathophysiological regulations. However, genomic sequences lack spatial and temporal information of the target molecules, as well as information on post-genomic modifications, which directly link to biological regulations in health and disease. Therefore, imaging technologies are crucial for the direct determinations of the spatiotemporal characteristics of different functional molecules in cells and organisms (Wang et al. 2008; Wang and Wang 2009). Parallel progresses in the developments of molecular imaging probes and microscopic technologies have allowed the dynamic monitoring of molecular localization, activities, and interactions in real time in live cells. In this aspect, the fluorescence proteins (FPs) and fluorescence resonance energy transfer (FRET) technology have become timely developments. FPs such as the green-colored GFP have allowed a variety of revolutionary discoveries, but the limitation of the GFP-tagging approach is that only the location of the molecules can be monitored. In contrast, FRET technology, which utilizes FPs to provide ratiometric readout on molecular activities and interactions, has been increasingly powerful in live cell imaging (Wang et al. 2008; Wang and Wang 2009).

The concept of FRET is based on the interactions of a pair of FPs. When two FPs are very close in distance, with the emission spectrum of the donor FP overlapping the excitation spectrum of the acceptor FP, energy transfer occurs if these two FPs are in favorable orientations (Clegg 1996; Clegg 2005; Wang and Wang 2009). The FRET efficiency between the donor and acceptor FPs of a FRET pair is mainly dependent on three factors: The first is the overlapping area between the emission spectrum of the donor and the excitation spectrum of the acceptor (Fig. 7.6a), with a larger area providing a higher efficiency. The second factor is the distance between the donor and acceptor. The FRET efficiency is inversely proportional to the 6th power of the distance between donor and acceptor. Hence, a slight modification of this distance can markedly affect the FRET signals. Typically, sufficient FRET between FPs occurs only when this distance is within 10 nm (Fig. 7.6b). The second factor is the relative orientation between donor and acceptor. It has been reported that FRET can change dramatically by changing the orientation while maintaining the distance between the two FPs (Nagai et al. 2004; Giepmans et al. 2006). Because FRET can provide such a high sensitivity in detecting a change in the distance or orientation between the donor and acceptor FPs, it has been widely applied to

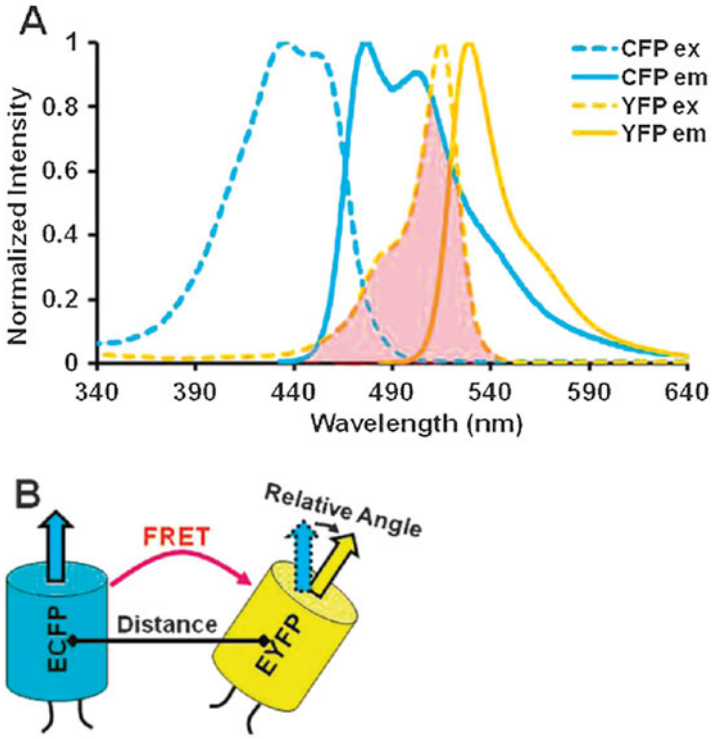

Fig. 7.6 (**a**) The excitation and emission spectra of a typical FRET pair, with cyan fluorescent protein (CFP) as the donor and yellow fluorescent protein (YFP) as the acceptor. The *broken lines* represent the excitation spectra and the *solid lines* the emission spectra of CFP and YFP. The spectra curves of CFP and YFP are color-coded with *cyan* and *yellow*, respectively. The *shaded red area* represents the overlap between the CFP emission and the YFP excitation. (**b**) The cartoon shows that the FRET efficiency between a typical FRET pair, enhanced CFP (ECFP) as the donor and enhanced YFP (EYFP) as the acceptor, is mainly dependent on the distance and the relative orientation between the donor and acceptor (Wang and Wang 2009)

designing biosensors for the measurement of molecular signals with high precision (Piston and Kremers 2007; Wang et al. 2008; Wang and Wang 2009).

7.3.2 FRET Biosensors

Based on the principle of FRET, different kinds of biosensors have been developed for biological applications. For example, hybridized sensors consisting of polypeptides and protein domains recognizing active target molecules have been applied to detect the small GTPases RhoA and Cdc42 (Nalbant et al. 2004; Pertz and Hahn 2004; Hodgson et al. 2008). However, genetically encoded FRET biosensors are particularly appealing for live cell imaging, because these biosensors can be conveniently introduced into cells for targeting subcellular compartments to continuously

monitor local molecular signals (Wang et al. 2008; Wang and Wang 2009). For the development of genetically encoded FRET biosensors, researchers have originally chosen the enhanced and blue-colored EBFP and green-colored EGFP as the FRET donor and acceptor pair (Romoser et al. 1997; Mahajan et al. 1998). Although the acceptor EGFP is quite bright, the donor EBFP is dim and poor in photo-stability, hence hampering further usage of the EBFP/EGFP FRET pair. Later, it was discovered that the enhanced cyan-colored ECFP and yellow-colored EYFP, including the yellow-colored variants of EYFP such as Venus and Citrine, provide excellent FRET pairs (Miyawaki et al. 1997; Griesbeck et al. 2001; Itoh et al. 2002; Nagai et al. 2002; Yoshizaki et al. 2003). Therefore, these FRET pairs have become increasingly popular for the biosensor development. In fact, ECFP and a YFP variant, YPet, have been shown to serve as a high-efficiency FRET pair for a variety of biosensors (Ouyang et al. 2008). At this stage, it seems that the brightness of ECFP is still a limiting factor for cell imaging. Recently, new variants for EBFP and ECFP have been developed, including Azurite (Mena et al. 2006), EBFP2 (Pedelacq et al. 2006), Cerulean (Rizzo et al. 2004), and mTFP1 (Ai et al. 2006). All these newly developed FP variants display significantly enhanced fluorescent properties, such as brightness and photo-stability, and they provide promising candidates for further improvement of FRET biosensors (Wang and Wang 2009).

A single-molecule FRET biosensor typically consists of a FRET pair of FPs and two intramolecular domains capable of interacting with each other. For example, a FRET-based biosensor capable of detecting Src kinase activation has been developed consisting of an N-terminal ECFP, a SH2 domain derived from Src kinase, a flexible linker, a substrate peptide derived from p130cas and specifically sensitive to Src phosphorylation, and a C-terminal Citrine (EYFP) (Wang et al. 2005; Wang et al. 2008; Wang and Wang 2009). This Src FRET biosensor has been applied to monitor Src activity continuously in live cells (Wang et al. 2005; Wang et al. 2008; Wang and Wang 2009). Alternatively, the donor and acceptor FPs can also be fused with two different target molecules, with the distance between donor and acceptor representing the interaction or separation of two target molecules (Zaccolo et al. 2000; Ai et al. 2006; Schulze et al. 2009; Wang and Wang 2009). Comparing these design approaches, the single-molecule FRET biosensors have two advantages: (1) the intramolecular interaction between the biosensor domains is robust and resistant to the interference caused by the interactions between the endogenous target molecules and the biosensor domains; (2) the acceptor/donor ratio will not be influenced by the relative expression levels of donors and acceptors in a single cell. Therefore, the single-molecule FRET biosensors are quite popular for monitoring intracellular signals (Wang and Wang 2009).

7.3.3 FRET-Based Epigenetic Biosensor

FRET-based biosensors have been recently developed to study the dynamics of histone epigenetics at single living cell level, and to investigate the mechanism of epigenetic regulations on cell fates. The Ting group first developed FRET-based histone methylation reporters to visualize H3K9me3 and H3K27me3 in vitro and in single

living cells (Lin et al. 2004). To visualize the spatial patterns of aurora B kinase in anaphase, Fuller et al. developed a strategy using FRET-based sensors to report quantitative changes in substrate phosphorylation in living cells (Fuller et al. 2008). Sasaki et al. developed a FRET-based histone acetylation biosensor, which monitored the dynamic fluctuation of histone H4 acetylation levels during mitosis (Sasaki et al. 2009). Later, Chu et al. developed another FRET-based and centromere-targeted H3K9me3 biosensor to visualize the methylation dynamics during chromosome segregation (Chu et al. 2012). However, the usage of current FRET-based epigenetic biosensors are relatively limited, possibly due to: (1) the substrates of those epigenetic biosensors are in general short histone peptides (N-terminal tail), which cannot be incorporated correctly into target nucleosomes. Therefore, the specificity of the resulting biosensors is not high; (2) the sensitivity of most current epigenetic biosensors is still awaiting further improvement (Sasaki et al. 2009) (Tan et al. 2014). As such, some physiologically important epigenetic signals with moderate magnitude may not be readily detectable. These limitations have led to the scarce application of epigenetic FRET biosensors in mechanobiology. An H3K9me3 FRET biosensor was applied to reveal a low level of H3K9me3 in tumor-repopulating cells (TRCs), which is unresponsive to matrix stiffness or applied forces (Tan et al. 2014). It is apparent that more FRET biosensors will be available to monitor epigenetic modulations, and this may promote the study of epigenetic regulations in mechanobiology.

7.4 Conclusions and Perspectives

Given the crucial role of epigenetics in genomic regulations and cellular fate determinations, there is a great need to monitor the epigenetic regulation in space and time. FRET biosensors should provide powerful tools in elucidating these spatiotemporal landscapes of epigenetic regulations, particularly in single live cells. The results should advance our precise understanding on the dynamic coordination of epigenetics and genomic regulations at different chromatin locations inside the nucleus, which should exceed the capability of traditional bulky assays based on the averaged extraction of a large number of cells.

It is expected that, with the rapid development in computational molecular modeling and high-throughput screening of large mutant libraries, highly sensitive and specific FRET biosensors will be developed in a systematic and rapid fashion. These biosensors, together with DNA binding domains and genome targeting motifs, should allow the precise monitoring of spatiotemporal epigenetic regulations at specific locus-sites. Similar approaches should also lead to the development of novel FRET biosensors capable of monitoring the spatiotemporal landscapes of DNA methylation evolution during different cellular processes including mechanotransduction, i.e., how cells perceive the mechanical environmental cues and transmit them into the regulation signals of epigenome and genome. In summary, the single cell study of epigenetic regulations in mechanobiology is at its infant stage. FRET

imaging integrated with rapidly progressing sequencing technologies should allow the revelation and construction of the spatiotemporal landscape of epigenetic regulations in response to mechanical cues in the near future.

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Chapter 8
Mechanotransduction to Epigenetic Remodeling

Douglas Kelkhoff, Timothy Downing, and Song Li

Abstract Biophysical factors such as mechanical cues and micro/nano features of extracellular matrix regulate a variety of cell functions, including signal transduction from the cell surface to nucleus. Recent studies indicate that biophysical factors not only modulate immediate mechanotransduction and signaling, but also exert long-term effects on phenotypic changes, including stem cell differentiation and cell reprogramming. Both cell differentiation and reprogramming involve epigenetic state changes such as DNA methylation and histone methylation and acetylation, each of which affects gene expression independent of DNA sequence. There is emerging evidence that biophysical factors, in addition to transcriptional factors and biochemical factors, can regulate the epigenetic state of the cells and thus its phenotypic changes. This chapter will highlight the potential mechanisms through which mechanotransduction may lead to epigenetic modifications.

Keywords Mechanotransduction • Nucleus • Chromatin • Epigenetic modification • Cell reprogramming

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8.1 Introduction

It has largely been accepted that biophysical cues can regulate a variety of cell functions, including signal transduction from the cell membrane through the cytoplasm to the nucleus. The regulation of signaling molecules by biophysical factors represents the immediate responses of cells, which can lead to the activation of transcriptional factors resulting in differential gene expression and cell functions. On the other hand, recent studies have also demonstrated that biophysical factors have a long-term effect on phenotypic changes, modulating stem cell differentiation and cell reprogramming. The change of cell phenotype stems from the modulation of its epigenetic state, the "memory" of a cell. This state can be regulated by DNA methylation as well as histone methylation and acetylation, each of which affects gene expression independent of DNA sequence. There is emerging evidence that biophysical cues, in addition to transcriptional factors and biochemical factors, can regulate the epigenetic state of the cells and thus its phenotypic changes. This chapter will highlight the potential mechanisms through which mechanotransduction may lead to epigenetic modifications.

8.2 Mechanotransduction from Cell Surface to Nuclear Matrix Through the Cytoskeleton

It is well understood that cells utilize focal adhesion complexes to adhere to and sense their environment. The activation of an integrin receptor by ligand binding or mechanical perturbation can lead to a series of intracellular signaling events such as the activation of focal adhesion kinase and downstream signaling (Li et al. 2005). Concurrently, actin fibers cross-link with α-actinin and myosin II forming a stress fiber which tethers the adhesion site to the cellular cytoskeleton. It is well known that the cytoskeleton is a predominant structural component of the cell. In addition, recent findings suggest that the cytoskeleton serves a role for mechanosensing and transduction, allowing the transmission of mechanical signals to the nucleus, ultimately affecting gene expression and epigenetic modifications. The primary cytoskeletal component, actin filaments, is believed to be the principle signal transducer. Where small molecule diffusion or active transport of protein to the nucleus takes seconds, stress wave propagation can occur on the millisecond timescale, making the actin cytoskeleton the preferred transmitter for a rapid response to mechanical cues (Wang et al. 2009). Although it is now believed that there is a linkage between mechanical input and nuclear behavior, it remains unclear how this signal propagates to the nucleus.

There is a definitive mechanical coupling between the cell surface and the nucleus through a cytoskeletal linkage. By allowing cell attachment to adhesive microbeads and subsequently pulling on those beads using a micropipette, it has been shown that there was a distinct morphological correlation between cell and

nuclear shape. Furthermore, even after soluble cytoplasmic components were removed using a detergent, leaving only the cytoskeleton, the mechanical coupling persists. This finding suggests a tethering of the cytoskeleton to the nuclear lamina is responsible for the shape of the nucleus (Maniotis et al. 1997).

In the past decade, the NUANCE protein (now known as Nesprin-2) was discovered. Nesprin-2 was found to contain a transmembrane domain, localizing cytoskeletal protein to the outer nuclear membrane (Zhen et al. 2002). Furthermore, this protein contains a binding site for α-actinin, one of the principal components of actin stress fibers. Colocalization of the nesprin-2 and cytoskeletal proteins suggested a linkage between the cytoskeletal microfilaments and the nucleus-bound protein. For the first time, this work suggested a protein linkage between the cytoskeleton and the physical structure of the nucleus.

Now it is known that the cytoskeleton is linked to the structural component of the nucleus, the nuclear lamina, through the linker of nucleoskeleton and cytoskeleton (LINC) complex. This protein complex is composed of a number of inner and outer nuclear membrane proteins that are linked through transmembrane domains. This linkage relies on KASH domain proteins that contain transmembrane domains on the cytoplasmic leaflet. The KASH containing proteins bind SUN 1/2 in the perinuclear space and span the inner nuclear leaflet. Inside the nucleus, the SUN 1/2 proteins contain binding domains for lamin A, tethering the complex to the nuclear lamina. Nesprin-2 is one such KASH domain protein. LINC complexes serve as the primary mechanical linkage of cellular mechanical structures, tethering the nuclear lamina to stress fibers, intermediate filaments, and microtubules. In this way, nuclear mechanics are directly linked to the extracellular matrix and cell junctions through physical, covalent linkages.

With this knowledge, it is clear that the cytoskeleton and nucleus are mechanically continuous. However, it remains unclear how this results in altered gene expression. On the cellular scale, there is evidence that the overall shape of the nucleus can have a profound impact on histone modification and cellular phenotype (Thakar et al. 2003; Li et al. 2011). One direct coupling of cellular shape to nuclear deformation is through the perinuclear actin cap (Kim et al. 2012). This actin cap is a structure that appears in two-dimensional culture of cells characterized by a collection of stress fibers spanning the apical surface of the nucleus and terminating in highly elongated actin cap associated focal adhesions. The tension of the stress fibers causes a distortion of the nucleus, causing it to adopt a disk-like morphology. It is now clear that the actin cap is necessary for effective distortion of the nucleus when the cytoskeleton is in tension. Since cytoskeletal tension is largely dependent on substrate rigidity, this work suggests that the perinuclear actin cap is an important downstream regulator of nuclear shape in response to environmental mechanical cues.

Similarly, a recent work shows a clear correlation between cell elongation and nuclear shape (Versaevel et al. 2012). By seeding cells on micropatterned adhesion ligand substrates to confine cells to shapes with predefined anisotropy, it was shown that nuclear shape as well as chromatin condensation shows a strong reliance on cell morphology. Highly aligned cells showed smaller, more elongated nuclei with considerably higher levels of chromatin condensation. Disruption of the cytoskeletal

structure within elongated cells using blebbistatin, an inhibitor of myosin II, results in chromatin unpacking comparable to isotropic cells.

Although it is becoming increasingly clear that mechanical signals are relayed to the nucleus, the exact mechanism remains unidentified. Proposed pathways include (1) mechanically coupled nuclear pores, (2) mechanically coupled ion channels, (3) strain-induced chromatin unfolding, or (4) strain-induced DNA unraveling (Li et al. 2005). The opening of nuclear pores or ion channels along the nuclear membrane alters local nucleus-targeted protein or ion concentration within the nucleus. Targeted proteins may serve as transcription factors altering gene expression where an influx of ions may offset histone interactions resulting in chromatin remodeling. Likewise, transduction of mechanical signals to the nuclear lamina may result in spatial separation of DNA, either resulting in strain-induced DNA denaturation or the mechanical disruption of histone binding. Any of these mechanisms, therefore, would affect gene expression or result in a direct epigenetic modification.

8.3 Nuclear Matrix Lamin as a Mechanotransducer and Modulator

The mechanosensitive nature of the nucleus is thought to rely largely on the physical linkage that exists between the cytoskeleton and nuclear membrane (or envelope). Given this connection, it becomes sensible to speculate that physical stresses and strains imposed on cells from the extracellular space might propagate through the cytoplasm and on to affect the intranuclear happenings of the cell. Over the past several years, a growing body of literature has confirmed this speculation and demonstrated the functional implications of direct nuclear mechanosensing. More recently, researchers have even begun to identify several proteins that are critical to the nucleus' capacity for mechanosensing. Lamin proteins in the nuclear matrix have been identified as critical players in nuclear mechanotransduction and interact heavily with other proteins of the LINC complex. Lamins, a type V intermediate filament and major component of the nuclear lamina, contribute greatly to the structure and mechanical integrity of the nucleus. With the help of lamin networks, cells are able to precisely regulate the shape, rigidity, and permeability of their nucleus. In addition, through its ability to form networks with DNA, lamin plays an important role in chromatin packing and gene accessibility. While much of lamin's function is associated with structural support of the nucleus and proper force transmission/response, lamins have been shown to play a pivotal role in gene regulation, stem cell fate commitment, and disease pathology.

Lamins are categorized as either A-type or B-type. Two of the most abundant A-type isoforms of lamins are lamin A and lamin C, which are both produced from a single gene, LMNA. A-type lamins are present in nearly all somatic cell types but prove absent in early embryonic cells. Lamin B1, lamin B2, and lamin B3 (B-type lamins, encoded by genes LMNB1 or LMNB2) are expressed in somatic cells types and have been shown to be involved in the early development specification of *Xenopus*

oocytes, for example (Dechat et al. 2008). While B-type lamins are important in organogenesis, their presence does not appear to be necessary for embryonic stem cell differentiation. Still, the coordinated expression of lamin proteins across critical developmental stages and in various cell types suggests that lamins might contribute in some way to cell state transitions or even stem cell differentiation.

In agreement with this, previous work has shown that lamin A mutation or overexpression within muscle progenitor (mouse C2C12) cells interferes and/or slows their differentiation into immature muscle fibers (myotubes) (Favreau et al. 2004). In the same regard, others have also shown that the overexpression of both wild-type and mutant lamin A inhibits lipid accumulation, triglyceride synthesis, and expression of adipogenic markers during the differentiation of 3T3-L1 pre-adipocytes into adipocytes (Boguslavsky et al. 2006). Interestingly, lamins have also been shown to play a role in stem cell fate determination. Specifically, knockdown of lamins A and C in human mesenchymal stem cells (MSCs) inhibited differentiation into osteoblasts and curbed MSCs toward an adipocyte-like fate (Akter et al. 2009). These results corroborate with the previous study and suggest that the presence of A-type lamins may slow or inhibit adipocyte formation.

Previous studies have also shown that adult stem cells exhibit altered differentiation propensities when cultured on cell-adhesive substrates of different stiffness. Recently, Discher and colleagues investigated the relationship between nuclear lamin levels and tissue stiffness, while shedding light on the role that nuclear lamins have in directed differentiation. Specifically, this work demonstrates that protein levels of nuclear lamin A (relative to lamin B) correlate with matrix elasticity and collagen content in adult tissues. Furthermore, lamin A knockdown enhanced MSC differentiation on soft matrices towards a low-stress, fat phenotype while overexpression enhanced differentiation on stiff matrix toward a high-stress, bone phenotype. It's also worth noting that high levels of lamin A coregulate key factors such as serum response factor (SRF), which promoted expression of stress fiber-associated proteins involved in differentiation, and the Hippo pathway factor YAP1 (Swift et al. 2013).

While it remains unclear exactly how lamin A is involved in stem cell regulation, there is growing evidence that lamin mutations can influence key signaling pathways that are known to influence stem cell maintenance and differentiation. For example, when the progerin/LAΔ50 protein (a mutant form of lamin A, originating from truncated prelamin A mRNA) is introduced into MSCs, major downstream effectors of the Notch signaling pathway become activated. This activation appears to induce major changes in MSC molecular identity and differentiation potential. In addition, Wnt signaling was shown to be involved in regulating hair follicle stem cells in mouse models of progeria. Specifically, Zmpste24$^{-/-}$ mice, which lack production of proteins involved in the processing of lamin A and, as a result, display age-related nuclear lamina defects and progeroid-like symptoms, have significantly higher numbers of resident epidermal stem cells within the hair bulge with decreased proliferative potential. Losses in active β-catenin and MITF protein levels is thought to be the cause of this stem cell misregulation since both proteins have roles in the Wnt signaling pathway. Beyond these two pathways, there is evidence that lamin A also influences TGF-β/Smad and Rb/MyoD pathways (Meshorer and Gruenbaum 2008).

A number of human diseases have been connected to aberrations in lamin expression or function. A majority of these laminopathies arise from mutation in the LMNA gene specifically, giving rise to aberrant lamin A and C proteins. These diseases include Emery–Dreifuss muscular dystrophy, dilated cardiomyopathy, limb-girdle muscular dystrophy, and Hutchinson–Gilford progeria syndrome (Burke and Stewart 2002; De Sandre-Giovannoli et al. 2003). In addition, lamins appear to be involved or modulated in a number of cancers (Esteller 2007). Given the broad influence of lamins in cell function, researchers speculate that these disease-related lamin mutations are the result of, for example, impaired nucleoskeletal force transmission, reduced nuclear stability, altered mechanotransduction signaling, misregulation of gene transcription, or impaired stem cell function. Still, however, much of the mechanism relating to lamin-induced disease development remains unknown. As research continues to deconvolute the intricacies of lamin mechanotransduction, it is likely that new opportunities for novel therapeutics will emerge to help combat the progression of lamin-related disease.

8.4 Biophysical Effects on Chromatin

Gene expression relies on a number of regulatory components including the presence (or absence) of specific transcription factors, epigenetic marks on DNA or histone proteins, as well as small RNAs. While the global levels of each of these components can be informative to the identity or behavior of a particular cell, the spatial positioning of these regulatory components within the subnuclear space continues to gain major interest. The architecture of the nucleus is hierarchical in nature, composed of chromatin territories where chromosomes are ordered in discrete regions within the nucleus based on chromosome size and gene richness, for example. Heterochromatin tends to lie at the periphery of the nucleus, where it closely associates with the nuclear lamina. These regions of DNA are often transcriptionally inactive and highly compacted. In contrast, regions of euchromatin—which tends to be transcriptionally active with a less compact structure—typically localize to the center of the nucleus.

In recognizing the physical link that exists between the cytoskeleton and the nucleus in addition to the association of nuclear membrane bound proteins to chromatin (for example, via Lamin Associated Domains or LADs), researchers speculate that deformations imparted onto cells from extracellular forces might alter chromatin structure, conformation, or location. Such changes could position chromatin segments away from or into transcriptionally repressive regions, thereby activating or repressing gene expressing. With the help of new technologies in sequencing and the ability to capture the three-dimensional chromatin architecture, researchers are able to further advance our understanding of biophysical regulation of chromatin structure and the subsequent cell response.

Chromatin compaction is necessary for many cell processes. During cell division, for example, cells organize their DNA into tightly coiled chromatin structures during the prophase of mitosis. As migratory cells move throughout the body, they

necessarily undergo drastic deformation in cell shape in order to squeeze through narrow openings between cell–cell junctions or dense regions of extracellular matrix. Along with these changes in cell shape, cells are likely to experience changes in the shape and size of their nucleus, necessitating a rearrangement of the cell's DNA. Moreover, several studies have shown that changes in cell cytoskeletal shape leads to a subsequent change in the shape of the nucleus (Dahl et al. 2008).

In mammalian cells, histone proteins closely associate with DNA and play a major role in chromatin compaction. Modification of these histone proteins, collectively referred to as an epigenetic signature, also seems to correlate with certain chromatin states. While much is still unknown regarding the appropriate classification of all histone modifications, the methylation or acetylation of histone residues strongly correlates with the local chromatin compaction. Recent work has revealed that the modification of histones can be directly impacted by a cell's shape. When cells are patterned into various geometries using micro-island patterning, the cell's histone modification profile is altered and gene expression is changed (Jain et al. 2013). In addition, stem cells show dynamic changes in chromatin compaction, which might be critical to enabling the sampling of various chromatin states by transcription factor-rich domains within the nucleus during differentiation (Bernstein et al. 2006). Together, these insights highlight the idea that cell geometry, regulated through manipulation of the extracellular environment, plays a significant role in chromatin compaction and that biophysically induced changes in chromatin states could be critical in establishing adult or stem cell identity.

Emerging adaptations of fluorescence in situ hybridization (FISH) has brought to light the non-random three-dimensional organization of chromosomes within the nucleus during interphase. In addition, the development of chromatin immunoprecipitation (ChIP) assays in conjunction with the advancement of sequencing technologies has allowed researchers to map the location of specific proteins or histone modifications across the genome at high resolution. These methods have brought new ways to explore and understand the impact of chromatin positioning (and repositioning) on changes in gene expression and the overall cell state.

Chromosome conformation capture (3C), for example, allows for the high-throughput assessment of chromosome organization with respect to other chromosome regions and proteins using various molecular biology and sequencing techniques. Over the years, this technology has advanced from 3C to 4C, 5C, and Hi-C, with each progression broadening the number of detectable chromosome interactions. 4C and Hi-C techniques, for example, have been able to demonstrate that for a given chromosome, multiple active gene-rich segments within the genome have a tendency to cluster together in the nuclear space. Interestingly, this clustering was not seen within the gene-poor domains of these chromosomes. In addition to the clustering of active gene regions, these same techniques also revealed the preferential clustering of inactive gene regions within the nucleus. These data strongly support the hypothesis that chromatin position plays a critical role in transcriptional regulation.

Furthermore, a recent study by Solovei et al. reported on the organization of heterochromatin in several different tissues and across multiple species. This work finds that heterochromatin positioning is coordinated throughout development and cellular

differentiation through Lamin B receptor and Lamin A/C proteins. With knockdown of these two proteins, heterochromatin is lost from the nuclear periphery and can lead to gene deregulation in myogenic differentiation (Solovei et al. 2013).

8.5 Biophysical Regulation of Epigenetic State and Cell Reprogramming

Cell reprogramming is a major advancement of biology in the past decade. Stem cells can spontaneously be guided to differentiate into a variety of cell types; in addition, cells can be reprogrammed and converted into distinct cell types by using transcriptional factors and biochemical compounds. All of these cell type changes involve epigenetic modifications (Fig. 8.1), but it is not yet well understood how biophysical cues modulate epigenetic state and cell reprogramming, and there is still much to learn before such techniques can be utilized for epigenetic cellular engineering. Largely, the study of biophysical regulation is still in its infancy, with only a rudimentary understanding of how mechanical signals are conveyed to the cell to result in differential gene expression. By exploring how biophysical regulation can result in varying expression of genes through modification of a cell's epigenetic state, we can uncover important regulatory mechanisms for development and prevention of disease. Though recent work has been driven forward by a select few mechanotransductive

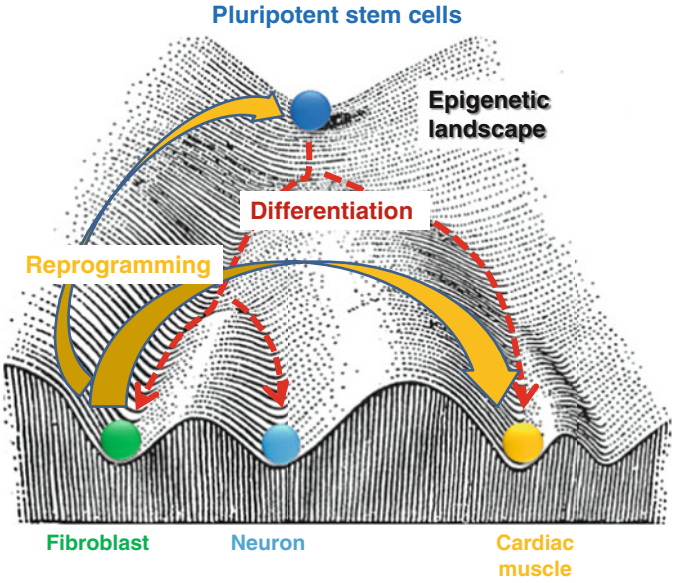

Fig. 8.1 Schematic representations of cell differentiation (*red arrows*) and reprogramming (*orange arrows*) on the epigenetic landscape. How biophysical cues will modulate the epigenetic state and help cells overcome the barrier of phenotype changes remain to be investigated

pathways in a small subset of model systems, it is clear that biophysical regulation is an essential regulatory mechanism in many, if not all cells.

Recently, the mechanical stimulation of cells has been shown to impact histone modifications and consequently the expression of genes. For example, various histone deacetylases are regulated by cyclic mechanical loading in a vascular smooth muscle system, resulting in a repressed migratory phenotype (Chen et al. 2013). This work links mechanical biophysical cues to phenotypic changes in a cell through epigenetic modifications. Interestingly, seeding of MSCs on nano-patterned substrates resulted in decreased activity of histone deacetylase (HDAC) corresponding to an increase in histone acetylation (Li et al. 2011). Furthermore, when these substrates were stretched orthogonal to the direction of the grooves, a further decrease in HDAC activity was observed. Lamin A/C knockdown was used to determine whether these proteins play a role in the transduction of mechanical signals to the nucleus. Since complete knockdown of lamin results in severely retarded cell viability, partial knockdown of lamin A/C to only half the endogenous amount was characterized. This partial knockdown inhibited any mechanotransduction of loading conditions to nuclear epigenetic changes, suggesting that lamin proteins play a critical role in the mechanosensing of MSCs.

In a subsequent study, fibroblasts derived from adult mouse ears were transduced with a virus encoding factors known to induce reprogramming to pluripotency. The cells were seeded onto a nano-patterned, grooved surface and a fourfold increase in reprogramming was observed on 10-μm wide grooves (Downing et al. 2013). This system, like the MSC system discussed previously, highlights how topology can affect a cell's epigenetic state. To contrast the previously mentioned work, this study broadens the earlier finding to show that somatic cell histone modification can be altered through the topology of a substrate. To further explore the mechanism through which this mechanosensing was taking place, reprogramming was compared to known chemical systems of induced pluripotency. It was observed that this increased efficiency showed the same effect as valproic acid (VPA) and tranylcypromine hydrochloride (TCP), which were previously known to increase reprogramming by inhibiting histone deacetylase and lysine-specific demethylase, respectively. When these chemicals were added in conjunction with biophysical regulation, no significant change in reprogramming was observed compared to biophysical regulation alone, suggesting a shared mechanism. This work further supports a linkage of biophysical cues to the activity of specific histone modification enzymes. As this work draws a connection between pluripotency and biophysical regulators, it draws into question what effect biophysical regulation may have on cell differentiation and development. Additionally, as this work ties mechanical regulation to pluripotency it connects the importance of the cell microenvironment to tumorgenesis, reinforcing the linkage of substrate mechanics to tumor development and metastasis (Nelson and Bissell 2006; Paszek and Weaver 2004).

In addition to application of forces through the deformation of a substrate, stresses caused by flow of the fluid surrounding a cell imposes a biophysical cue which can be transduced to influence cell function. Recently, a growing body of work has been tying physiological fluid flow patterns to phenotypic changes in cells

through epigenetic modifications. Specifically, the circulatory system serves as one target system which is thought to be largely maintained by the mechanical signals conveyed through fluid flow conditions. By exposing vascular endothelial cells to turbulent or laminar flow patterns, recent work has shown an epigenetic dependence on flow conditions (Lee et al. 2012). By uncovering a dependence of two classes of HDACs on the fluid-mediated shear stresses, it was determined that multiple shear-responsive genes were epigenetically regulated in response to the applied stress (Xia et al. 2010). This regulation correlates to the presentation of oxidative, inflammatory, or proliferative phenotypes observed in endothelial cells suggesting a mechanically transduced dependence of cell fate and a potential mechanism for vascular disorders such as atherosclerosis.

8.6 Future Directions

Though there is still much unknown about how biophysical cues can impact nuclear signaling, there is an evolving foundation of knowledge that advances our understanding of epigenetic regulation through mechanotransduction. This linkage allows for a mechanical dependence of histone modifications and differential, biophysically regulated gene expression. There are several important steps of mechanotransduction from the cell surface to the chromatin structure that require further investigation. A number of questions remain unaddressed or require further exploration: How can mechanical cues modulate the linkage and interactions between the nuclear matrix and chromatin? How do mechanical factors regulate histone and DNA modifications? How does the regulation by global and local histone modification differ? How do reversible and irreversible epigenetic changes compare—among a multitude of further transduction-centric research. To answer these questions, a multidisciplinary approach using a variety of tools and technologies is paramount to dissecting these mechanisms, combining the knowledge of biology, physics, and engineering.

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Chapter 9
The Nuclear Lamina: From Mechanosensing in Differentiation to Cancer Cell Migration

Jerome Irianto, Irena L. Ivanovska, Joe Swift, and Dennis E. Discher

Abstract How cells respond to physical cues in order to meet and withstand the physical demands of their immediate surroundings has been of great interest for many years, with current efforts focused on mechanisms that transduce signals into gene expression. Pathways that mechano-regulate entry of transcription factors into the cell nucleus are emerging, and our most recent studies suggest that mechanical properties of the nucleus itself are actively controlled in response to matrix elasticity in mature, injured, and developing tissue. Here, we discuss the mechano-responsive properties of nuclei as determined by intermediate filament lamin proteins that line the inside of the nuclear envelope and that also impact transcription factor entry and broader epigenetic mechanisms. We summarize signaling pathways that regulate lamin levels and decisions of cell fate in response to matrix mechanics combined with molecular cues. We also discuss recent work that highlights the importance of nuclear mechanics in niche anchorage and cell motility in development, hematopoietic differentiation, and cancer invasion whilst also emphasizing a role in protecting chromatin from stress-induced damage.

Keywords Cell mechanics • Mechanotransduction • Extracellular matrix • Nucleus • Nucleoskeleton • Proteostasis • Lamina • Differentiation • Cancer

9.1 Introduction

Mature tissues need to be particularly resistant to the mechanical demands of an active life. Our bones, cartilage, skeletal muscle, and heart tissues are stiff, making them robust to routine physical exertion such as walking or running when they are subjected to high-frequency shocks, stresses, and strains. With every heartbeat, the

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left ventricular wall experiences a 20 % radial strain (Aletras et al. 1999), and local strains of ~20 % also occur in the cartilage of knee-joints with every step (Guilak et al. 1995). Tissue-level deformations might even be amplified within cells and their nuclei (Henderson et al. 2013). Our softer tissues have less need for robustness because their function does not require them to bear load. Furthermore, some of our softest tissues, such as brain and marrow, are protected from an otherwise hard world by our bones. Nonetheless, when soft tissues are subjected to impact, such as a collision of heads in American football or rugby, occurrences of rapid straining can cause lasting damage (Viano et al. 2005).

We have recently sought to characterize the composition of cells and extracellular matrix (ECM) in tissues of increasing stiffness, and by implication, in tissues that are subjected to the greatest stress (Swift et al. 2013a, b). A close correlation between the concentration of ECM components and bulk tissue elasticity was discovered. More surprisingly, we also discovered a systematic scaling between tissue elasticity and concentration of lamins in the nucleoskeleton that was partially recapitulated in cultured cell systems. Corresponding changes in the mechanical properties of the nuclei suggest that this response may act to protect the precious chromatin cargo of the nucleus from shocks that are transmitted through the surroundings, across the cytoskeleton, and into the nucleus. An active regulation of cell or matrix composition in response to the environment implies feedback into pathways of protein turnover and remodeling, or control of the rate of new protein production. Responsive matching of mechanical properties to physical demands has classically been described as a "mechanostat" in the context of bone regulation (Frost 1987), but a recent explosion in mechanobiological studies has uncovered a host of other mechanically sensitive cellular phenomena, including contraction (Discher et al. 2005), migration (Hadjipanayi et al. 2009a, b; Winer et al. 2009), proliferation (Lo et al. 2000; Hadjipanayi et al. 2009a, b; Klein et al. 2009), differentiation (Engler et al. 2004, 2006), and apoptosis (Wang et al. 2000). Despite the recent progress, questions of how mechanical signals are transduced into specific transcriptional or regulatory pathways continue to challenge the field.

The lamina is a network structure formed from intermediate filament (IF) lamin proteins that lies inside the nuclear envelope and interacts with both chromatin and the cytoskeleton (Fig. 9.1a). In the somatic cells of humans, mice, and most vertebrates, the major forms of lamin protein are expressed from three genes: lamins -A and -C are alternative splicing products of the *LMNA* gene (collectively "A-type" lamins); lamins -B1 and -B2 are encoded by *LMNB1* and *LMNB2* genes, respectively ("B-type" lamins). The lamins share structural features, and indeed have some commonality in amino acid sequence, but differ in their posttranslational modification, with B-type lamins permanently appended by a farnesyl group that is cleaved from mature lamin-A (reviewed by Dechat et al. 2010). Like other IFs, such as keratin and vimentin, the lamins form coiled-coil parallel dimers that assemble into higher-order filamentous structures which fulfill important structural roles (Herrmann et al. 2009).

Here we aim to summarize recent efforts to characterize the proteins that vary systematically with tissue stiffness. The effects of the composition of lamina on nuclear mechanical properties will be elaborated in detail, and we will consider the

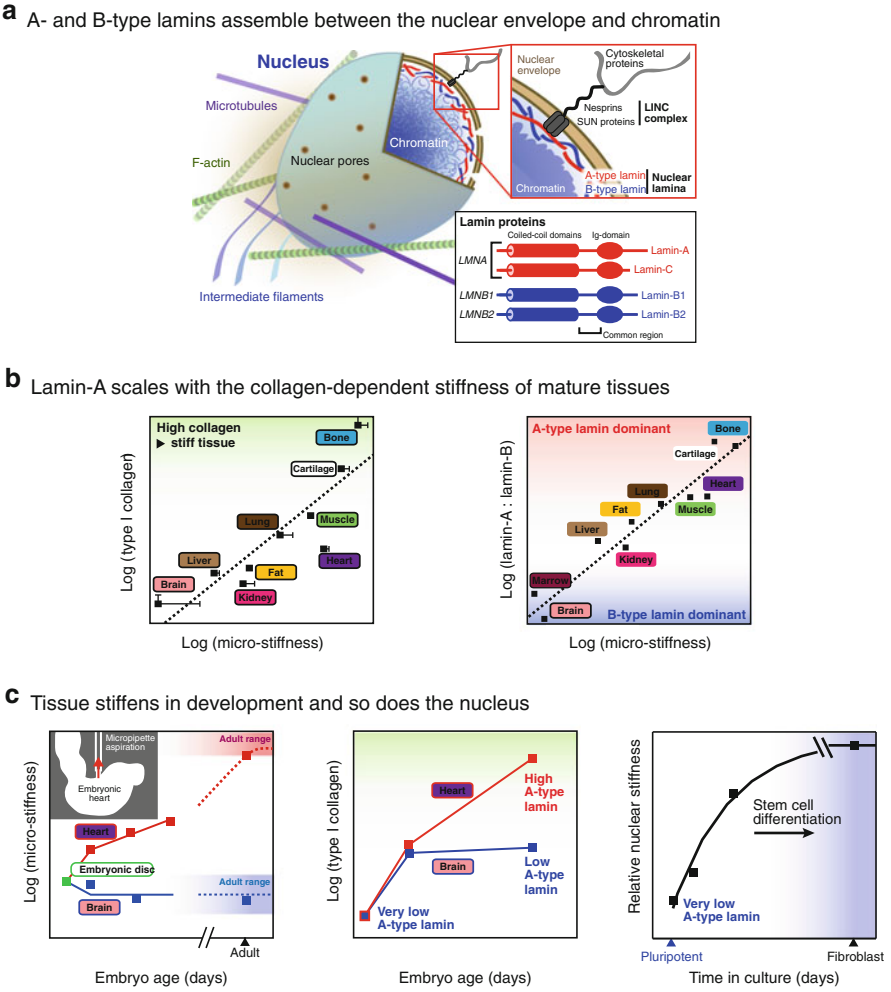

Fig. 9.1 Scaling of matrix and lamin in mature tissue and during development. (**a**) A-type and B-type lamins form juxtaposed networks on the inside of the nuclear envelope; they are effectively located at an interface between chromatin and the cytoskeleton, to which the lamina is attached through the "LINC" (linker of nucleo- and cytoskeleton) complex. "A-type lamins," lamins -A and -C are alternative spliceoform products of the *LMNA* gene; "B-type lamins," lamins -B1 and -B2 are protein products of *LMNB1* and *LMNB2*, respectively (adapted from ©Buxboim et al. 2010, originally published in The Journal of Cell Science). ((**b**)—**left**) The quantity of collagen-1 present in tissues scales with tissue micro-elasticity (Swift et al. 2013a, b). As collagen is one of the most prevalent proteins in the body, it is perhaps expected that it defines mechanical properties. ((**b**)—**right**) The composition of the nuclear lamina scales with tissue microelasticity. A-type lamins dominated the lamina in stiff tissue, whereas B-type lamins are prevalent in soft tissue (Swift et al. 2013a, b). ((**c**)—**left**) Observations made in adult tissue were also reflected in developing chick: the embryonic disc was initially soft, but divergent tissues either remained soft, such as brain, or became increasingly stiff, such as heart. *Inset*: developing chick hearts were probed by micropipette aspiration to determine micro-elasticity. ((**c**)—**center**) Tissue stiffening during development is accompanied by increased levels of collagen and A-type lamins (Lehner et al. 1987; Majkut et al. 2013). ((**c**)—**right**) Embryonic stem cells initially have negligible quantities of A-type lamins, but these levels increase as the nucleus stiffens during lineage commitment (Pajerowski et al. 2007)

functions of the lamina in transducing mechanical signals from matrix and surroundings into cellular response, both in terms of an active regulation of the lamina itself and its broader role as a linkage in mechanotransduction pathways. Although we focus on a primarily protective purpose of lamin in the nucleus, there are additional regulatory consequences of such a stiff and bulky organelle, and we will summarize recent evidence that such properties limit the freedom of cells to move through tissue. The proximity of the lamina to heterochromatin within the nucleus has led it to be widely associated with epigenetic regulation (e.g., Kim et al. 2011; Meuleman et al. 2013). This review will seek to highlight the pervasive influence of the mechanical role of the lamina and hence proposes that lamin acts as both guardian and gatekeeper for chromatin.

9.2 Scaling of ECM and Lamina Components in Mature and Developing Tissue

Collagens and other protein constituents of the ECM are the most prevalent proteins in our bodies, largely determining the mechanical properties of tissue. Collagens are found at higher levels in stiff, mature tissues where, consistent with an expectation for proteins to behave as "biological polymers" (Gardel et al. 2004), their increased concentration is the basis of tissue elasticity (Fig. 9.1b—left). By using quantitative label-free mass spectrometry (MS) for proteomic profiling (Swift et al. 2013a, b), we have shown that collagens and other ECM-associated proteins scale with tissue elasticity (Swift et al. 2013a, b). MS was also used to quantify roughly 100 of the most abundant proteins in the cytoskeleton and nucleus, and we found the strongest correlation with bulk tissue elasticity in the composition of the nuclear lamina (Fig. 9.1b—right). Although primarily characterized by the ratio between the two main families of lamins, A-type and B-type, the compositional scaling is dominated by a 30-fold increase in the concentration of lamin-A, C from brain to bone. Although our recent observations are broadly in agreement with an extensive literature in lamin quantification (e.g., Krohne et al. 1981; Rober et al. 1990; Cance et al. 1992; Broers et al. 1997), they provide a new perspective on systematic variations across many tissues.

The relationship between tissue stiffness, ECM, and lamina during development was also determined; micropipette aspiration of embryonic chick tissue showed that the homogeneous embryonic disc is initially very soft, with proteomic profiles indicating correspondingly low levels of collagen (Fig. 9.1c—left and center). However, the properties of different tissues diverge during development with the brain remaining soft, whereas the heart stiffens as ECM proteins are deposited (Majkut et al. 2013). Cells in stiffening tissues, such as heart, are also likely to have respectively higher levels of lamin-A,C (Lehner et al. 1987). Nuclei in embryonic stem cells have indeed been shown to be very soft and to have low levels of lamin-A,C (Pajerowski et al. 2007; Eckersley-Maslin et al. 2013). As these cells commit to a

lineage-specific fate, the levels of lamin-A,C increase and the nucleus becomes correspondingly stiffer (Fig. 9.1c—right).

Importantly, despite an apparent role in amplifying decisions of animal cell fate in conjunction with matrix elasticity, lamin-A,C is not essential to development as knockout mice still form all tissues (Sullivan et al. 1999). Likewise, lamin-B knockout mice survive embryogenesis (Kim et al. 2011). The most critical role of lamin may therefore be to tune the properties and regulation of maturing tissues in higher organisms, and its absence can perhaps be compensated for during development. However, the distinction here may be blurred: there is still a need to understand nuclear structure during some stages of development, such as during cell migration; and processes of trafficking and differentiation continue throughout an organism's life span. Nonetheless, there appears to be consistency with the current notion that lamins are not expressed in yeast and plants (Dittmer and Misteli 2011), despite the latter possessing genomes that are larger and more complex than those in animals. It seems very likely that the hard cell walls of these organisms protect the chromatin in ways that are not possible for animal cells with soft cell membranes. Cell biologists could thus benefit from thinking about such physical properties that of course fit within a structure-function paradigm.

9.3 The Influence of Lamina Composition on the Mechanical Properties of the Nucleus

Micropipette aspiration experiments have enabled the detailed study of nuclear mechanical properties by measuring the rate of deformation under pressure (Fig. 9.2a, b; Dahl et al. 2005; Pajerowski et al. 2007). By examining nuclei with different lamina compositions, it is thus possible to approximate the characteristic contributions to nuclear mechanical properties from A-type and B-type lamins (Fig. 9.2c; Shin et al. 2013; Swift et al. 2013a, b; Harada et al. 2014). The nuclear response in deformation is a combination of elastic (spring-like) and viscous (liquid-like, flowing) properties, with lamin-B's contributing primarily to the elastic response and lamin-A,C contributing viscosity. Thus the difference between a nucleus stoichiometrically dominated by A-type vs. B-type lamins might be akin to comparing a balloon filled with honey to one filled with water. The importance of A-type lamin in maintaining nuclear structural integrity and cell viability has been appreciated for many years (e.g., Broers et al. 2004; Lammerding et al. 2006), and its influence on nuclear viscosity has been more recently demonstrated in studies where nuclei are deformed during migration through microfluidic circuits (Rowat et al. 2013) or transwell pores (discussed later, Shin et al. 2013; Harada et al. 2014).

"Laminopathies" are a family of diseases that are caused by mutations in lamin-A,C (reviewed, for example, by Butin-Israeli et al. 2012; Worman 2012). These disorders include muscular dystrophies (Bonne et al. 1999), cardiomyopathies (Fatkin et al. 1999), lipodystrophies (Hegele et al. 2000; Shackleton et al. 2000;

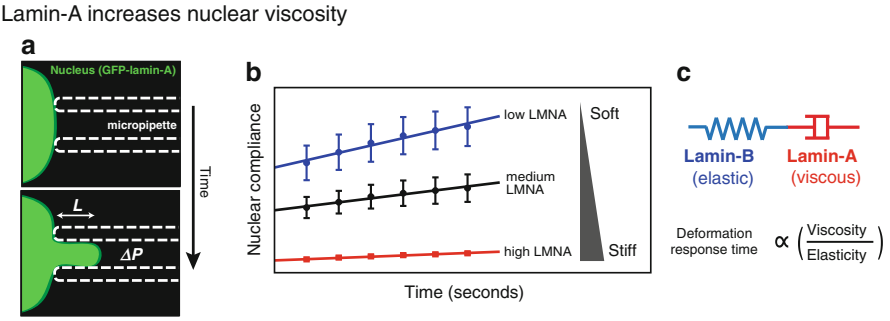

Fig. 9.2 The mechanical role of lamin in the nucleus. (**a**) Deformations applied by micropipette aspiration were used to quantify nuclear compliance (effectively a measure of "softness"; the inverse of stiffness) in a range of nuclei with altered lamina compositions (for example, by overexpressing a GFP-lamin-A fusion construct). Compliance can be calculated over a range of deformation timescales as a function of the micropipette diameter, the extent of deformation (L), and the applied pressure (ΔP). (**b**) When a constant deforming pressure was delivered by micropipette over timescales on the order of seconds, nuclei with low LMNA were found to be more compliant than those with high LMNA. (**c**) The mechanical properties of the lamina can be considered as a combination of elastic (spring-like) and viscous (flowing) properties, which together define the "deformation response time," the timescale over which the nuclear shape deforms under force. Nuclei with greater quantities of A-type lamins relative to B-type lamins were found to deform more slowly under stress (Swift et al. 2013a, b)

Speckman et al. 2000), and premature aging ("progeria," Merideth et al. 2008). Indeed, one of the confounding aspects of lamin-related disease is how such a widely expressed protein can cause tissue-specific symptoms. Whilst much work remains to be done to resolve this question, it is broadly consistent that laminopathies cause defects in tissues where lamin-A,C is the dominant lamin in the nucleus, i.e., bone, heart, muscle, and fat (although there are exceptions: Charcot-Marie-Tooth disorder affects the nervous system, De Sandre-Giovannoli et al. 2002). Mouse models of lamin-A,C knockout have defects in muscle and connective tissue and typically die several weeks after birth from heart failure (Sullivan et al. 1999; Kubben et al. 2011; Jahn et al. 2012). Despite the apparently constitutive expression of B-type lamins in tissue, mouse models with lamin-B1 and -B2 ablation progress through embryogenesis with eventual death due to defects in brain development (Coffinier et al. 2011; Kim et al. 2011).

9.4 Mechanisms of Lamin Regulation

Earlier discussion has posited that lamins are closely regulated to match the mechanical properties of the nucleus with the physical demands of tissue. In addition to being set by the epigenetic programming required to make a given tissue or organ, it is also important that protein levels vary in response to feedback from their surroundings. Even within bulk tissues, mechanical loading can cause inhomogeneous

straining (for example, in human articular cartilage, meniscus, and ligaments, Chan and Neu 2012), making it beneficial to have mechanisms of lamin regulation at the local, individual cell level.

The many mechanisms by which the level of lamin-A,C can be regulated are summarized in Fig. 9.3a. We showed that the transcript and protein levels of lamin-A,C are highly correlated in tissue (Swift et al. 2013a, b), suggestive of a tight regulatory feedback. A recent study of the proteome and transcriptome in mouse fibroblasts suggested that there are around ten million copies of lamin-A,C protein per cell—accounting for about 0.7 % of cellular protein mass—and around 200 copies of the *LMNA* transcript (Schwanhausser et al. 2011), which seems similar to single cell measurements (Dingal et al. 2015). Half-life in the cell on rigid plastic dishes was found to be around 4 days for the protein and about 20 h for the mRNA, both slightly higher than the cellular average for all proteins and genes (Schwanhausser et al. 2011). Measurements made on proteins in a human lung cancer cell line showed the half-life of lamin-A,C to be around 12 h, roughly in the middle of the span of protein-half lives recorded in the study (Eden et al. 2011). DNA methylation is known to be an epigenetic mechanism by which gene activity can be regulated, but was discounted as the foremost means of controlling *LMNA* levels: no consistent changes were observed in the methylation of the *LMNA* promoter in a range of cell lines known to express different levels of lamin-A,C protein (Swift et al. 2013 b), or in tissues from patients with laminopathic disorders (Cortese et al. 2007). *LMNA* transcription has been reported to be controlled by transcription factors of the retinoic acid (RA) receptor (RAR and RXR family proteins, Olins et al. 2001; Okumura et al. 2004a, b; Shin et al. 2013; Swift et al. 2013a, b), with the resulting mRNA alternatively spliced to give the lamin-A and truncated -C forms. Soft tissue generally favors the lamin-C spliceoform (Swift et al. 2013a, b), and in brain the micro interfering-RNA *MIR-9* specifically targets and deactivates the mRNA of the lamin-A spliceoform (Jung et al. 2012, 2013).

9.5 Stress-Responsive Regulation of Lamin: "Use It or Lose It"

To better understand how lamin proteins are actively regulated in response to stress, mesenchymal stem cells (MSCs) were cultured on collagen-1 coated polyacrylamide hydrogels with stiffnesses that set to mimic the ECM of either brain (0.3 kPa) or pre-calcified bone (40 kPa) (Buxboim et al. 2010; Swift et al. 2013b). Images of the cultured MSCs showed that the nuclear envelopes of cells on soft matrix are wrinkled and relaxed, whereas, on stiff matrix, the nuclei are flattened by stress fibers and appear taut and smooth (Fig. 9.3b—left). Accompanying proteomic analyses revealed that, on stiff matrix, the conformation of lamin-A,C protein is maintained, the total quantity is upregulated, and the extent of phosphorylation at four sites is decreased. Phosphorylation is recognized as a key mechanism for modulating the solubility, conformation, and organization of IF proteins (Omary et al. 2006),

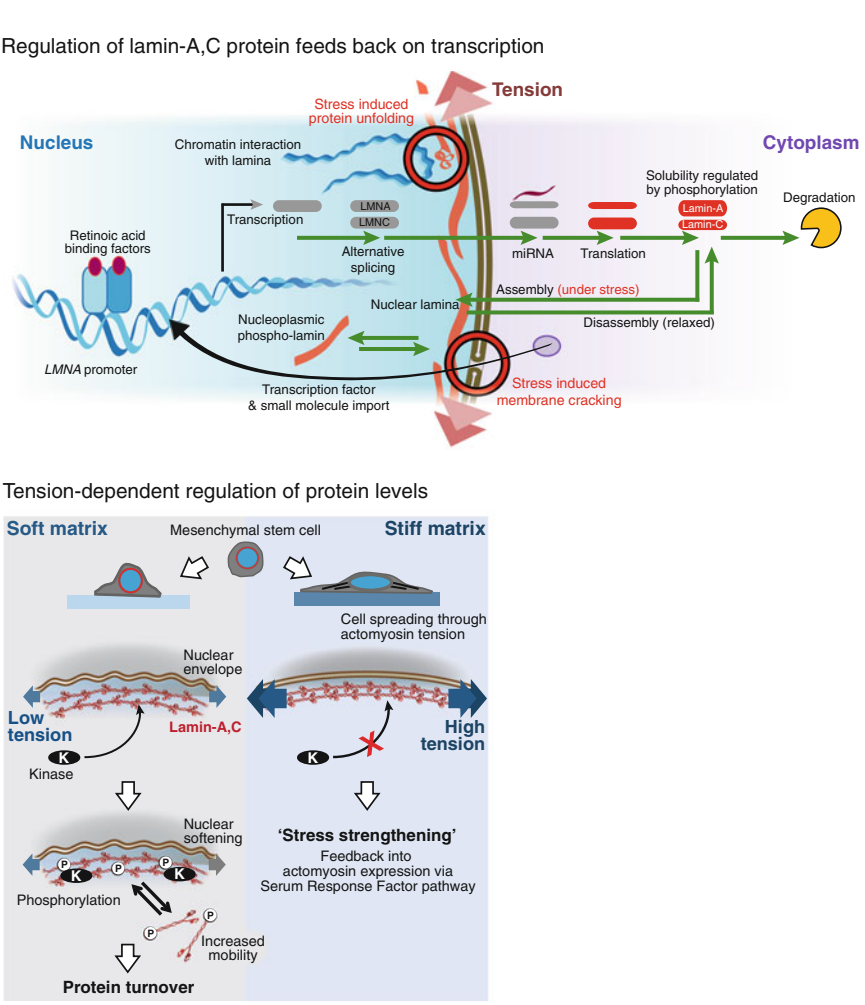

Fig. 9.3 Protein regulation as a function of stress. (**a**) Schematic showing the factors that can regulate the levels of lamin-A,C in the cell: *LMNA* transcription is promoted by retinoic acid binding factors (Olins et al. 2001; Okumura et al. 2004a, b). The transcript is alternatively spliced to give rise to lamin-A and -C forms. In some tissues such as brain the -A form is suppressed through micro interfering RNA (Jung et al. 2012). Mature lamin-A (following posttranslational processing) and lamin-C assemble into the nuclear lamina, although some protein remains mobile in the nucleoplasm (Shimi et al. 2008). Phosphorylation leads to increased solubility, and may precede enzymatic protein turnover. Further stress-dependent pathways have been reported: stress on the nucleus causes unfolding of the Ig-domain of lamin-A,C and phosphorylation is suppressed under tension (Swift et al. 2013a, b). Laminopathic nuclei have been shown to have transient membrane defects that allow ingress of transcription factors (De Vos et al. 2011). (**b**) The nuclei of MSCs cultured on soft substrate were wrinkled, whereas those in cells on stiff substrate had a smooth, stretched appearance suggestive of greater tension. We have shown that lamin-A,C is less phosphorylated under tension (Swift et al. 2013a, b). By concentrating on one of the matrix-stiffness-regulated phosphorylation sites, we confirmed that lamin-A,C is rapidly phosphorylated with reduced cytoskeleton tension and phosphorylation leads to nuclear softening and lamin-A,C turnover (Buxboim et al. 2014)

and indeed lamins are highly phosphorylated during normal mitosis, driving disassembly of the lamina in preparation for chromosomal separation (Gerace and Blobel 1980; Heald and McKeon 1990). Thus the response we observe from matrix-induced stress is the converse of this process, with decreased phosphorylation acting to decrease lamin-A,C solubility and thereby strengthening the lamina. On soft matrix, lamin-A,C is more extensively phosphorylated, more mobile, and so, more susceptible to turnover (Buxboim et al. 2014). These observations hence point to a "use it or lose it" dynamic, whereby inessential lamin-A,C is eventually degraded. Lamin-A,C level has been reported to drive the translocation of the lamin-promoting transcription factor retinoic acid receptor gamma (RARG) to the nucleus, pointing to a feedback mechanism by which lamin protein levels promote their own transcription (see gene circuit in Swift et al. 2013a, b).

9.6 Mechanotransduction to the Nucleus: Downstream of Matrix and Lamin

Lamin is a key component in a system of protein linkages that allow the transmission of signals from a cell's surroundings into the transcriptional machinery of its nucleus (Fig. 9.1a and discussed in recent reviews, e.g., Simon and Wilson 2011; Gundersen and Worman 2013; Rothballer and Kutay 2013; Sosa et al. 2013). Cell–cell interactions link to the cytoskeleton through tight and adherens junctions that tether to actin, and desmosome complexes that interact with cytoplasmic IFs such as keratin (Jamora and Fuchs 2002). Cell–matrix interactions are mediated by integrins and focal adhesion complexes that bind to cytoplasmic actin (Puklin-Faucher and Sheetz 2009; Watt and Huck 2013). The appropriately named LINC complex ("linker of nucleo- and cytoskeleton") acts as an intermediary between cytoplasmic and nuclear structural proteins: F-actin binds to the nuclear envelope components nesprins -1 and -2, and IFs bind to the desmosome protein plectin, which in turn binds nesprin-3. Nesprins can also interact with kinesin and dynein complexes to tether to the microtubule network; Nesprins bind the SUN domain-containing family of inner nuclear membrane proteins and these in turn bind to the lamina on the inside of the nuclear envelope. Current problems for progress on understanding the roles of Nesprins are that there are few if any good antibodies to Nesprins and there are many spliceforms of Nesprins.

Lamin interactions within the nucleus are highly promiscuous (Wilson and Berk 2010; Wilson and Foisner 2010); as emphasized by Wilson and Berk in their review: "almost all characterized [inner nuclear membrane] proteins bind to A- or B-type lamins (or both) directly." These interactions include binding to structural proteins, like actin (Simon et al. 2010), and a range of proteins that bind to the nuclear membrane, including emerin, barrier-to-autointegration factor (BAF, de Oca et al. 2009), lamina-associated polypeptide 2 (LAP2), and lamin-B receptor (LBR, Solovei et al. 2013). Of these, emerin has attracted considerable recent interest for its roles in mediating changes in the stiffness of isolated nuclei in response to tension applied

to nesprin-1 (Guilluy et al. 2014), and in mechanosensing by affecting the translocation of transcription factor MKL1 (Ho et al. 2013). Furthermore, some transcription factors such as Oct-1 interact with the lamina directly (Malhas et al. 2009). Many lamin-binding proteins also interact with chromatin, particularly in its silenced heterochromatin form (Wagner and Krohne 2007), and indeed the lamins have been shown to bind DNA directly (Shoeman and Traub 1990; Luderus et al. 1992; Stierle et al. 2003). This chain of interactions thus completes a continuous physical linkage through which deformations can be transmitted from the cell exterior to chromatin (Maniotis et al. 1997). What is missing from this picture, however, is how blunt inputs—forces and perturbations acting without microscopic coherence—can be converted from mechanical to biochemical signals to activate individual genes at precise spatial locations within the nucleus. Perhaps specificity can be delivered through changes in binding, local concentration, conformation, and modification of cofactors or transcription factors.

As described above, mechanical cues from outside the cell alter protein conformations, protein modifications, and protein levels—all of which can broadly affect cell morphology and function. It is therefore of particular interest to understand the multiplicity of mechanisms that likely underlie how external factors induce stem cell lineage, with far-reaching implications for therapeutics and regenerative medicine. Populations of MSCs can be expanded in culture in a relatively naïve undifferentiated state, but they can certainly differentiate into multiple mesenchymal lineages, including fat, cartilage, muscle, and bone, dependent on external cues, such as the presence of nutrients, growth factors and cytokines, cell density, spatial constraints and mechanical forces (Pittenger et al. 1999). Cell shape influences cell fate through modulation of the activity of the small GTPase RhoA, with round cells favoring adipogenesis and well-spread cells favoring osteogenic lineage (McBeath et al. 2004). RhoA drives commitment to lineage in conjunction with its effector Rho-associated protein kinase (ROCK) through its regulation of nonmuscle myosin-II that controls cytoskeletal tension.

Although the focus of this review on the nucleus limits a deeper discussion of nonmuscle myosin-II, at least two points should be made. Knockout mice that completely lack nonmuscle myosin-IIA (MYH9) die at such an early embryonic stage that they exhibit little to no differentiation: no heart and no vasculature (Conti et al. 2004). This myosin-II isoform tends to be the dominant and early form of nonmuscle myosin-II isoforms in many tissues. Nonetheless, heterozygous mutations in human MYH9 are common and exhibit weak dominant negative effects on the wild-type protein from the normal allele (Spinler et al. 2015), which strongly suggests that even less than half of nonmuscle myosin-IIA is sufficient for near-normal differentiation in most tissues.

Cell fate can be influenced by matrix stiffness (Engler et al. 2006), and we have recently shown that this effect can be modulated by the nuclear lamina (Fig. 9.4a; Swift et al. 2013a, b). MSCs cultured on soft hydrogel substrates favor adipogenesis, but the extent of adipogenesis, as determined by oil red staining of lipid droplets, is double or more when combined with lamin-A,C knockdown. Likewise, stiff matrix induction of osteogenesis is greatly increased by lamin-A overexpression. Stiff

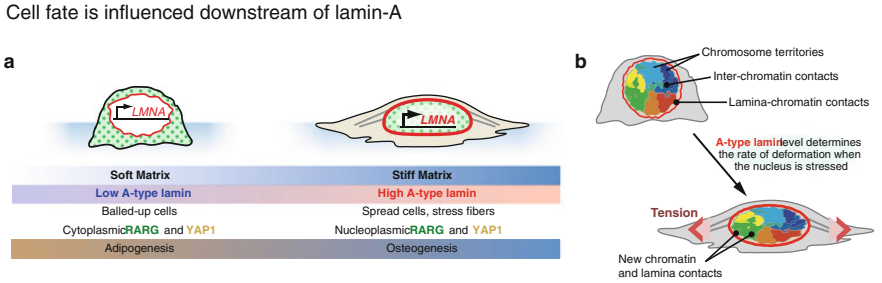

Fig. 9.4 Decisions of cell fate downstream of lamin-A,C regulation. (**a**) MSCs cultured on soft and stiff substrates take on differing phenotypes and favor alternate cell fates (Engler et al. 2006). On soft substrate, MSCs exhibit small nuclear and cellular spread areas and the nuclear lamina is thinned by a stress-sensitive phosphorylation feedback mechanism (Fig. 9.3b—left; Swift et al. 2013a, b). The transcription factors RARG and YAP1 (YAP1, Dupont et al. 2011) remain in the cytoplasm, and adipogenic cell fate is preferred. On stiff substrate, cells spread extensively with nuclei that are pinned down by well developed stress fibers. Lamin-A,C is less phosphorylated under strain, thus strengthening the lamina; RARG also translocates to the nucleus, increasing *LMNA* transcription. Activity of the transcription factor SRF (downstream of lamin-A,C) increases expression of cytoskeletal components (Ho et al. 2013; Swift et al. 2013b). Under these conditions, YAP1 translocates to the nucleus and cells favor osteogenesis. On both soft and stiff substrates, the effects of matrix elasticity and lamin level cooperate to enhance differentiation: lamin-A,C knockdown on soft matrix leads to more adipogenesis; lamin-A overexpression on stiff matrix leads to more osteogenesis. (**b**) Transcriptional activity is believed to be regulated by conserved interchromatin contacts that give rise to the spatial ordering of chromosomes (chromosome territories shown here in different colors, Cremer and Cremer 2001). Lamin-A,C can interact with DNA directly ("lamina-chromatin contacts") or through protein intermediaries (Simon and Wilson 2011), but could have additional regulatory roles by mechanically determining the extent and rate that the nucleus deforms under tension, a process that could lead to the formation of altered interchromatin and lamina-chromatin contacts

matrix has been found to drive the nuclear translocation of the transcription factors RARG (Fig. 9.4a; Swift et al. 2013a, b) and yes-associated protein 1 (YAP1) (Dupont et al. 2011). RARG directly regulates lamin-A,C as part of differentiation and regulation of the serum response factor (SRF) pathway that amplifies levels of cytoskeletal components such as nonmuscle myosin-IIA (Fig. 9.4a; Swift et al. 2013a, b). Findings with MSCs on stiff matrix indeed are consistent with greater SRF activity in epithelial cells (Connelly et al. 2010; Ho et al. 2013). NKX2.5 is yet another transcription factor that is matrix elasticity sensitive, but it accumulates in the nucleus of MSCs on soft matrix and represses expression of at least one tension stabilizing protein, smooth muscle actin (SMA) (Dingal et al. 2015). Nuclear translocation of transcriptional regulators in response to matrix mechanics is thus an emerging theme in mechanosensing. It has also been shown to occur upon transfer of ions and changes in osmotic pressure (Finan et al. 2009; Irianto et al. 2013; Kalinowski et al. 2013). Such translocation could be driven by a change in concentration of protein-binding sites (e.g., on lamin-A,C or emerin, Ho et al. 2013; Swift et al. 2013a, b) or conceivably by protein modifications (e.g., YAP1 nuclear localization can be mediated by phosphorylation, Murphy et al. 2014). Besides

conventional transport through nuclear pores via nuclear localization sequences (NLS) (Dingal et al. 2015), transient breakdown of the nuclear envelope in cells with defects in the lamina, perhaps as a consequence of a reduced robustness to mechanical stress, has been shown to affect nuclear localization of transcription factors such as RelA (De Vos et al. 2011).

The ability of lamins and/or its binding partners to tether to DNA has lead to interest in its role in chromatin organization and regulation (Guelen et al. 2008; Kim et al. 2011; Zullo et al. 2012; Kind et al. 2013; Lund et al. 2013; Meuleman et al. 2013). Lamina-associated domains (LADs) located at the nuclear periphery have been thought to associate with low gene expression levels whereas actively transcribed euchromatin is usually found in the nuclear interior. This might contribute to "chromosome territories" (Cremer and Cremer 2001; Iyer et al. 2012) and to transcriptional hotspots within specific locations (Fraser and Bickmore 2007). However, it has been recently determined that nuclear lamins are not required for LAD organization in embryonic stem cells (Amendola and van Steensel 2015). Chromatin and DNA are also generally considered to make negligible contributions to overall nuclear mechanics (Pajerowski et al. 2007; Guilluy et al. 2014) unless the nucleus is condensed (Pajerowski et al. 2007), although particular cases are emerging—for example, in ESCs passing though a metastable transitional state before differentiation—where the condensation state of chromatin can become mechanically significant (Pagliara et al. 2014). It is not yet fully understood which proteins could give rise to mechanically responsive, locally defined structures and organization within the nucleus, nor how a protein as ubiquitously expressed as lamin could play a part in such specificity. Knowledge in this field will continue to improve as new experiments and models emerge to study protein-mediated changes in chromatin organization in response to perturbation (Shivashankar 2011; Talwar et al. 2013). However, based on current work, we have hypothesized that the effect of lamins on nuclear mechanics could determine the sensitivity and timescale of nuclear reorganization in response to stress (Fig. 9.4b; Swift et al. 2013a, b).

9.7 Cell Migration Is Slowed by the Nuclear Stiffness Needed to Protect Chromatin

As the nucleus is generally the largest and stiffest organelle, it can be a limiting factor in the migration of a cell through the 3D matrix. This means that the mechanical properties of the nucleus can have regulatory roles in processes, such as development, wound healing, hematopoiesis, cancer metastasis, and others (Fig. 9.5a—left). Studies of migration through narrow pores that mimic those in tumor tissue and require the deformation of the nucleus demonstrated a dependence on lamina composition; migration was limited when lamin-A was overexpressed, but promoted by a ~50 % knockdown of the protein (Harada et al. 2014). However, a deeper knockdown to <10 % was found to cause apoptosis in migration through small pores,

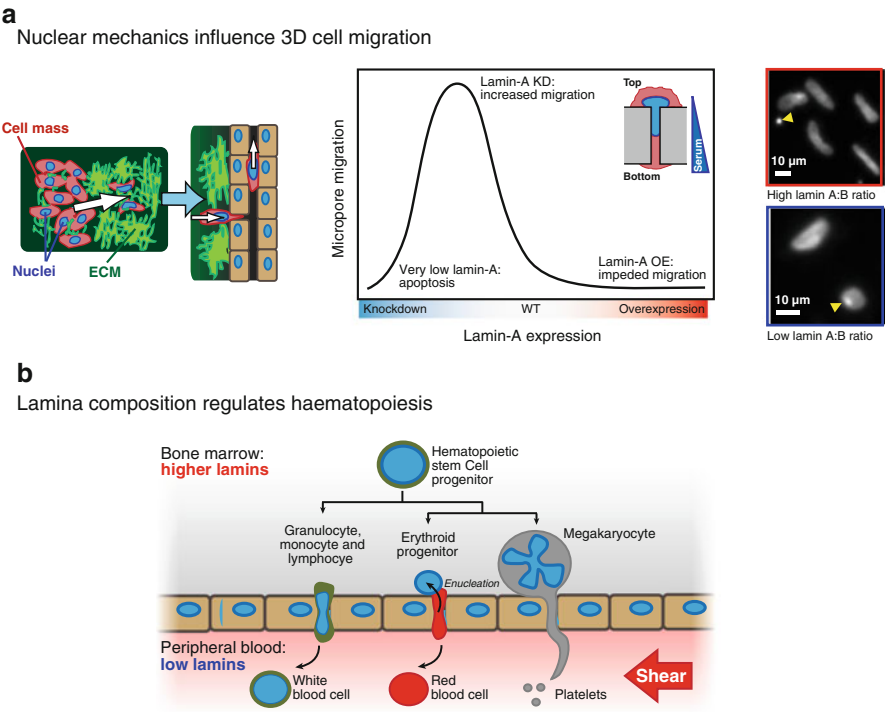

Fig. 9.5 The influence of the mechanical properties of the nucleus on cell migration. ((**a**)—**left**) As the largest and stiffest organelle in the cell, the nucleus can act as an "anchor" and prevents cell movement through the matrix or into surrounding vasculature. ((**a**)—**center**) As a model of migration through matrix, cells are induced to pass through 3 μm pores, a diameter sufficiently small to require deformation of the nucleus (*inset*). Lamin-A overexpression inhibits migration, whereas knockdown increases migration, up to a point at which significant apoptosis is observed. Thus extremely low or high lamin-A,C levels are unfavorable for cell migration, an observation with potential impact on understanding of processes such as cell migration during development and cancer metastasis. ((**a**)—**right**) Lamin-A rich nuclei (top image) showed persistence of a sausage-like morphology upon emergence from the pores (*yellow arrow*), while lamin-B rich nuclei (bottom image) rapidly recovered their shape (Fig. 9.5a adapted from ©Harada et al. 2014. Originally published in The Journal of Cell Biology. doi: 10.1083/jcb.201308029). (**b**) Effect of lamina composition on nuclear deformability during hematopoiesis. Stem cells that are retained in the marrow niche have higher lamin levels than differentiated blood lineages (Shin et al. 2013). A downregulation of nuclear cytoskeletal components in granulocytes, for example, ostensibly makes the cells better suited for passage through narrow blood vessels, but the lack of nuclear stability may contribute to their relatively short circulation times (Olins et al. 2009)

underscoring the importance of lamin in providing physical protection to the nucleus (Fig. 9.5a—center). Consistent with earlier observations that lamins -A and -B, respectively, contribute primarily viscous and elastic mechanical properties to nuclei (Fig. 9.2), nuclei in which high lamin-A,C levels dominated the mechanical characteristics were observed to recover slowly following deformation, maintaining an elongated morphology after emerging from the pores (Fig. 9.5a—top right

image). In contrast, nuclei with dominant levels of elastic B-type lamins rapidly returned to their more spheroid pre-migratory shapes following deformation (Fig. 9.5a—bottom right image).

Cell migration is an important part of the development process and it is possible that the elasticity imparted by lamin-B is needed to allow nuclei to recover from the deformation (typically elongation) that occurs during migration, perhaps explaining why the brain fails to develop in lamin-B knockout mice (Coffinier et al. 2011; Kim et al. 2011; Jung et al. 2013). Neutrophilic cells also have very low levels of nucleoskeletal proteins to allow their deformation as they squeeze into confined spaces (Olins et al. 2009; Rowat et al. 2013), and indeed the composition of the nuclear lamina is continuously regulated during hematopoiesis (Fig. 9.5b; Shin et al. 2013). We hypothesize that by downregulating components of the lamina, white blood cells compromise their robustness in favor of mobility, and that this contributes to the short lifetimes of many of these cells in circulation. Cancer metastasis is an equally complex process that depends on factors including matrix remodeling and nuclear deformability (Wolf et al. 2013; Harada et al. 2014). Other work has shown that myosin-II's ability to deform the nucleus can be a decisive factor in limiting glioma migration into brain tissue (Beadle et al. 2008; Ivkovic et al. 2012), but cancer cells in general show no universal lamina phenotype (reviewed in Foster et al. 2010). Although low levels of lamin-A,C have been correlated with increased reoccurrence of colon cancers (Belt et al. 2011), lamin-A,C was found to be upregulated in certain skin and ovarian cancers (Tilli et al. 2003; Hudson et al. 2007) and higher lamin-A,C expression was associated with better clinical outcomes in breast cancer (Wazir et al. 2013). Our own studies of tumor expansion in mouse flank have associated moderately lower levels of lamin-A,C with an increased invasiveness into the surrounding tissue, but a more complex dependence of the level of lamin-A,C with clinical prognosis might be explained by the tenuous balance between the effect of the lamina on nuclear deformability compared with that on cell survival.

9.8 Lamins in Cancer

Many studies have shown that lamin levels change in cancer of many organ types when compared to normal tissue (Table 9.1). Direct mechanistic links between lamins and cancer progression remain mysterious nonetheless. In cancer progression, a proliferation-competent cell acquires a cancer phenotype by either epigenetic changes (DNA methylation and histone modifications, Berdasco and Esteller 2010) or direct genomic changes (mutational, Salk et al. 2010) that lead to activation of oncogenes or inactivation of tumor suppressor genes (for review Hanahan and Weinberg 2011). Many in vitro studies have suggested a role for lamin-A in DNA damage response (Musich and Zou 2009; Mahen et al. 2013; Singh et al. 2013), but mice and humans with lamin-A deficiencies and defects are not reported to have an increased risk of cancer. Nonetheless, deep knockdown of lamin-A increases

Table 9.1 Lamins in cancer

Type of cancer	Lamin-A,C	Lamin-B
Lung cancer	↓	
Breast cancer	↓	↑
Colon cancer	↓	↓
Colorectal cancer	↑	↑
Colonic and gastric adenocarcinomas	↓	
Primary gastric carcinoma	↓	
Basal cell skin carcinoma	↓	
Skin cancer	↑	
Leukemia	↓	
Ovarian serous cancer	↑	
Ovarian cancer	↓	↑
Prostate cancer	↓	↑
Liver cancer		↑
Pancreatic cancer		↑

apoptosis after constrained migration through small matrix-like pores, consistent with increased DNA damage (Harada et al. 2014). The same study also showed migration—induced damage of the nuclear lamina, and nuclear ruptures have been observed in cancer cells (Vargas et al. 2012). These findings collectively suggest a protective role of lamins as an "armor" for guarding the genome. Further work is required to drill into the mechanistic link between lamins and cancer, which may lead to new treatments or at least a clearer basis for lamins as bio-marker in cancer progression.

9.9 Conclusions and Prospects

We have sought to outline the importance of nuclear mechanics in the context of tissue function, considering how it reflects the protective properties of the lamina, influences cell fate, and also regulates cell migration. In understanding that one of the key functions of the lamins is to ensure that the mechanical properties of the cell meets the demands of a tissue—either directly or by driving broader changes with regard to cell fate—lamins stress response factors. The response to cellular stress is classically thought in terms of how cells mitigate "heat-shock" that otherwise result in high levels of unfolded proteins (Hartl et al. 2011), but mechanical stress might also cause chromatin unfolding. Nonetheless, we are still a long way from understanding cellular protection mechanisms and how stress response pathways affect the regulation of structural features within the cell—motivating more work in nuclear biophysics.

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Chapter 10
Role of Cell Geometry on Nuclear Mechanics, Chromosome Reorganization, and Gene Expression

Yejun Wang, Ekta Makhija, Karthik Damodaran, and G.V. Shivashankar

Abstract In this book chapter, we summarize the current findings for the physical and chemical connections between the extracellular matrix (ECM) and 3D chromosome organization, which ultimately lead to modular gene regulation. An overview is first provided to delineate the linkage between the nucleoskeleton and cytoskeleton through LINC complexes and on how this linkage regulates nuclear mechanotransduction. This involves alterations in nuclear morphology and dynamics, by reorganization of cytoskeletal network and actomyosin contractility in response to different ECM constraints. These external mechanical signals, once transduced to the nucleus, facilitate remodeling of chromatin dynamics, epigenetic landscape, and 3D chromosome organization. Finally, we present the role of cell geometric constraints on 3D chromosome organization for modulating gene expression. Extreme alterations in matrix signals could lead to a number of diseases, including fibrosis and cancer. In this context, analysis of nuclear mechanotransduction and genome regulation could provide a better understanding of tissue homeostasis.

Keywords Cell geometry • Nuclear mechanotransduction • Epigenetics • 3D chromosome organization • Genome regulation

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Abbreviations

Chr	Chromosome
ECM	Extracellular matrix
FISH	Fluorescence in situ hybridization
FRAP	Fluorescent recovery after photobleaching
H3K27me3	Tri-methylation on lysine 27 of histone H3
H3K9ac	Acetylated histone H3 on lysine 9
H3K9me3	Tri-methylation on lysine 9 of histone H3
HDAC	Histone deacetylase
HP1α	Heterochromatin protein 1α
IAD	Interchromosomal activity distance
INM	Inner nuclear membrane
IPD	Interchromosomal physical distance
KASH	Klarischt/ANC-1/Syne domain
LINC	Link the nucleoskeleton with the cytoskeleton
MRTF-A	Myocardin-related transcription factor
NRD	Normalized radial distance
ONM	Outer nuclear membrane
PDMS	Polydimethylsiloxane
SUN	Sad1p/UNC-84 domain
TF	Transcription factor
UTP	Uridine-50-triphosphate

10.1 Introduction to Nuclear Mechanotransduction and Genome Regulation

Signals from the extracellular matrix (ECM) are important for controlling cell behaviors such as differentiation and developmental programs (McBeath et al. 2004; Kilian et al. 2010; Engler et al. 2006; Chen et al. 1997; Farge 2003). Mechanotransduction from ECM to the nucleus for genome regulation is carried out through the physical connections between plasma membrane and nuclear envelope, via an intricate cytoskeletal network (Wang et al. 2009).

Evidence has highlighted the role of SUN–KASH proteins which link the nucleoskeleton with the cytoskeleton (LINC) via interactions across the nuclear membrane (Crisp et al. 2006; Starr and Fridolfsson 2010; Tapley and Starr 2013). SUN (Sad1p/UNC-84 domain) proteins localized at the inner nuclear membrane (INM) interact with KASH (Klarischt/ANC-1/Syne domain) proteins at the outer nuclear membrane (ONM) (Wilhelmsen et al. 2006; Worman and Gundersen 2006; Razafsky and Hodzic 2009). Mammalian SUN proteins at the INM, such as Sun1 and Sun2, interact with the nuclear lamina and chromatin on the inside of the nucleus (Haque et al. 2006, 2010; Lei et al. 2009). Across the nuclear luminal space, the SUN proteins are

linked to domains of KASH proteins residing at the ONM (Padmakumar et al. 2005; Tzur et al. 2006). KASH proteins extend into the cytoskeleton, with mammalian proteins such as Nesprin-1 and -2 connecting to actin filaments, Nesprin-3 connecting to intermediate filaments via plakin proteins called plectins, and Nesprin-4 connecting to microtubules via motor proteins (Fridolfsson and Starr 2010; Luxton et al. 2010; Roux et al. 2009; Wilhelmsen et al. 2005; Zhang et al. 2009). The SUN–KASH coupling to diverse cytoskeleton assemblies thereby integrates the chromatin and lamina in the nuclear interior to the cell exterior.

Cytoskeleton-mediated stresses serve as a mechanistic regulator of the morphology of the nucleus, that is controlled by cell geometry and substrate rigidity (Lovett et al. 2013; Versaevel et al. 2012). The influence of an apical actin nuclear capping structure that coordinates the regulation of the nuclear shape via actomyosin and LINC complexes was demonstrated in mouse embryonic fibroblasts (Khatau et al. 2009). Further, studies have evidenced that these myosin-coupled actin stress fibers are initiated at the focal adhesions. These stress fibers extend over the nucleus to exert a contractile load on the nucleus impinging on chromatin organization (Li et al. 2014). In contrast, the microtubules which have been shown to bear considerable stresses within the cytoskeleton (Brangwynne et al. 2006) exert a compressive load on the nucleus (Mazumder and Shivashankar 2010). Additionally, intermediate filaments have also been shown to influence nuclear morphology (Toivola et al. 2005). The above experiments highlight the importance of the perinuclear cytoskeleton organization in regulating nuclear morphology.

The differential changes in actin and microtubule assemblies modulate nuclear morphology, demonstrating their coordinated physical influence on the nucleus. Depolymerization of actin filaments or stabilization of microtubule cytoskeleton results in increased nuclear height and accompanied by enhanced microtubule lateral assembly. Concomitantly, perturbation to microtubule structure results in cells and nuclei flattened by enhanced actin organization. These changes in structural organization were reflected at the nuclear envelope with alteration in lamin architecture. A targeted RNAi-mediated knockdown of specific cytoskeletal elements demonstrated a phenotypic profile of isotropic shaped nuclei with smaller projected area resulting from actin-associated perturbations (Ramdas and Shivashankar 2015). Anisotropic shaped nuclei with enlarged projected area resulted from microtubule-associated perturbations, illustrative of the dynamic actin-microtubule structural force balance. The mechanistic outcome of this physically integrated system is the existence of a nucleus that is prestressed due to a balance between chromatin entropic tension and cytoskeleton stresses (Mammoto et al. 2012; Mazumder et al. 2008; Shivashankar 2011). Nuclear prestress across multiple cell types and its emergence with the onset of cell differentiation was demonstrated via pharmacological reagents (Mazumder and Shivashankar 2010). Mechanical studies have used laser ablation experiments to demonstrate the tension inherent in the actin stress fibers (Kumar et al. 2006). Loss of this tension via perturbation to actin structure or cytoskeleton linkers enhances nuclear dynamics (Ramdas and Shivashankar 2014; Talwar et al. 2013). Consistent with this, embryonic stem cells with reduced cytoskeletal stresses exhibit enhanced nuclear dynamics and lowered nuclear rigidity (Talwar et al. 2013; Bhattacharya et al. 2009; Pajerowski et al. 2007).

The dynamic nature of cytoskeletal components also leads to the dynamic behavior of the nucleus, such as translational or rotational motion of nucleus and its plasticity (Luxton et al. 2010; Hagan and Yanagida 1997; Lee et al. 2005; Wu et al. 2014). Translational and rotational motion of the nucleus are involved in nuclear positioning, an important step for various cellular functions such as cell division, cell polarization, and migration (Levy and Holzbaur 2008). As a consequence of change in cell matrix constraints, relaxed fibroblasts on isotropic geometries show an intrinsic instability in nuclear dynamics, where the nucleus invariably undergoes rotation at about a degree per minute (Kumar et al. 2014). This nuclear rotation was shown to be caused by inherent hydrodynamic flow of orientable contractile actomyosin filaments. In migrating cells at wound edge, nuclear rotation increases dramatically, reaching speeds up to 8.5 degrees per minute (Levy and Holzbaur 2008). Microtubules and their motors kinesin and dynein have been implicated in causing such rotation of the nucleus in migrating cells (Levy and Holzbaur 2008; Brosig et al. 2010; Wilson and Holzbaur 2012). While fibroblasts exposed to large isotropic matrix constraints exhibit nuclear rotation with a timescale of few hours (~ one rotation in 6 h), stem cells or fibroblasts with reduced matrix attachment result in nuclear area fluctuations at a timescale of few minutes (frequency ~5 min).

Changes in nuclear plasticity and its deformation by cell–matrix constraints are also associated with chromatin dynamics (Banerjee et al. 2006), and reorganization, resulting in differential transcriptional programs (Li et al. 2014; Jain et al. 2013a). In terminally differentiated cells, fluorescent recovery after photobleaching (FRAP) studies showed that perturbation to actin components enhances core histone dynamics (Ramdas and Shivashankar 2014). The chromatin reorganization is brought about by a number of alterations in epigenetic modifications on histone and nonhistone proteins that package DNA. For example, cytoplasmic to nuclear shuttling of histone deacetylases (HDACs) have been implicated in tuning histone acetylation on lysine (Walkinshaw et al. 2008). In addition, various methylation modifications such as H3K9 me3 and H3K27 me3 have been shown to compact chromatin structure (Tessarz and Kouzarides 2014). Exerting forces on the cell surface receptors led to alteration in nuclear and chromatin organization (Maniotis et al. 1997). On the other hand, laser ablation of condensed heterochromatin nodes in the nucleus resulted in perturbation to focal adhesion structure (Mazumder and Shivashankar 2007). This illustrates the mechanical continuity from the cell surface to the nucleus (Shivashankar 2011; Jaalouk and Lammerding 2009; Wang et al. 2009), which could finally tune epigenetic modifications and transcription programs.

In addition to physical signals, a number of studies have suggested that chemical signals are also important in regulating gene expression programs (Shivashankar 2011; Low et al. 2014). For example, substrate-stiffness sensing of cells is associated with cytoplasmic-nuclear shuttling of YAP/TAZ (Dupont et al. 2011). Nuclear localization of myocardin-related transcription factor (MRTF-A) is regulated by actin polymerization (Miralles et al. 2003). Further, the lineage-specific transcription programs are brought about by an elaborate nonrandom spatial organization of chromosomes (Cremer and Cremer 2001; Parada et al. 2004; Bolzer et al. 2005; Fraser and Bickmore 2007; Cavalli and Misteli 2013). Chemical regulatory signals such as

transcription factors (TFs) are optimized for co-clustering of chromosomes, which is essential for the co-regulation of a specific group of genes (Schoenfelder et al. 2010; Fanucchi et al. 2013). These collectively illustrate that both physical and chemical signals are critical to regulating genome programs. However, the integrated mechanisms underlying such signaling for genome regulation remains unclear.

In this book chapter, we highlight the significance of the comprehensive cytoskeletal coupling to control nuclear morphology and dynamics. We further show the influence of this coupling to modulate chromatin epigenetic modifications, 3D chromosome organization, and modular gene expression. High resolution single cell imaging, fluorescence anisotropy imaging, microfabrication, and fluorescence in situ hybridization (FISH) were used in these studies.

10.2 Geometric Constraints Alter Actomyosin Contractility, Nuclear Morphology, and Dynamics

To understand how extracellular mechanical constraints regulate cytoskeletal-mediated nuclear morphology and dynamics, NIH3T3 fibroblasts were cultured on fibronectin-coated micropatterns of different shapes and sizes (Fig. 10.1a). Such micropatterns were molded out of polydimethylsiloxane (PDMS) that was poured and cured on silicon wafers, with microfabricated wells of desired patterns. These PDMS micropatterns were then coated with fibronectin and inverted onto untreated cell culture dishes, followed by the treatment of nonionic surfactant, pluronic acid, before seeding fibroblast cells. Cells were seeded at a proper density to ensure the high percentage of singlets on each pattern. These single cells spread over the fibronectin-coated micropatterns and adapted to the shape of the micropattern. Knowing that the fibroblast spreading area on free fibronectin-coated surface is around 1400 μm^2, we used two extreme geometries of the micropatterns: rectangles of 1800 μm^2 area and 1:5 aspect ratio to mimic the anisotropic physiological spreading status of these cells, and circles of 500 μm^2 area to constraint the spreading area of these cells.

The cytoskeletal organization in the two geometries was observed to be significantly different. Phalloidin stainings of actin in the two geometries revealed that cells on large rectangular (large anisotropic) substrates have long actin stress fibers that connect to the focal adhesions on either ends (Wang et al. 2014). On the other hand, actin in small circular (small isotropic) cells is present as small filaments or punctae (Fig. 10.1b). Such cytoskeletal reorganization in the two substrates results in different forces on the nucleus, thereby leading to different nuclear morphologies. In large anisotropic cells, the nucleus is flattened and elongated (height 6 μm and projected area 250 μm^2), while the nucleus is rounded (height 10 μm and projected area 120 μm^2) in small isotropic cells (Fig. 10.1c).

In addition to nuclear morphology, nuclear dynamics is also affected by alterations of the cytoskeletal prestress in different cell geometric constraints. While the nucleus shows translational dynamics in unconstrained migrating fibroblasts (Wu et al. 2014) and rotational dynamics when reshaped on large isotropic

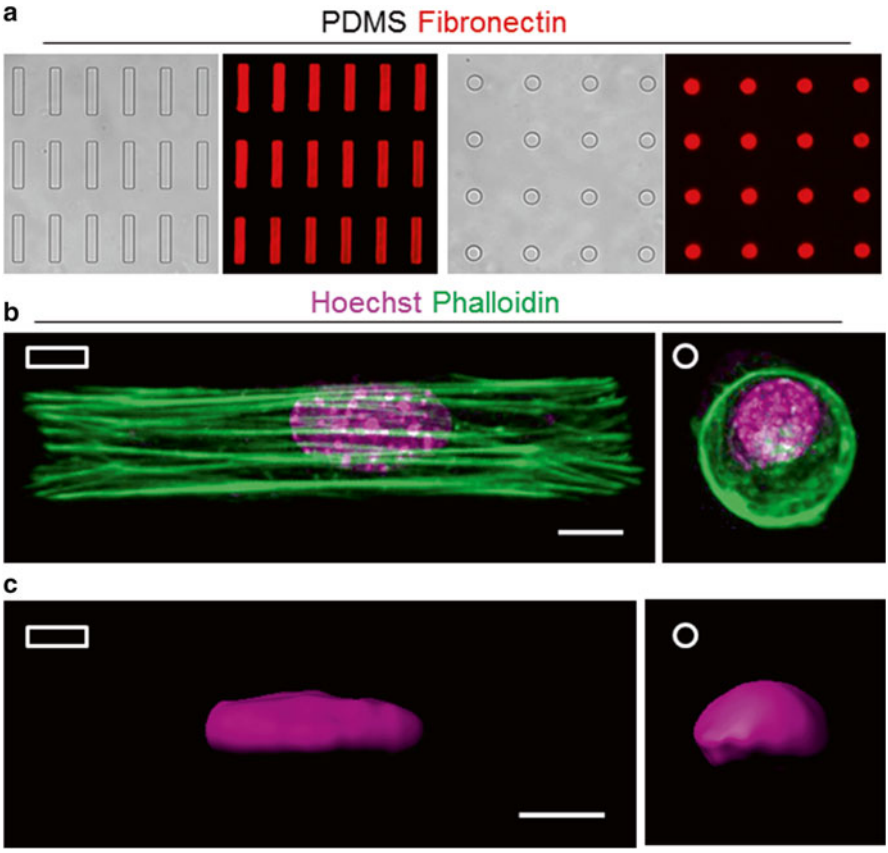

Fig. 10.1 Geometric constraints induced the cytoskeletal reorganization. (**a**) Low magnification transmitted images of microfabricated PDMS, and fluorescent images of fibronectin patterns (*red*). (**b**) Representative confocal images of NIH 3T3 cells labeled with phalloidin (*green*) and hoechst (*purple*) cultured on these patterns. Scale bar: 10 μm. (**c**) Imaris generated surface plot of representative nucleus on anisotropic vs. isotropic substrates. Scale bar: 10 μm

micropatterns (Kumar et al. 2014), it exhibits nuclear envelope fluctuations when constrained on small isotropic micropatterns. Time lapse images of the H2B-EGFP labeled nucleus in small isotropic cells show that the projected nuclear area fluctuates up to 10 % (Fig. 10.2a). The time kymograph of nuclear boundary also showed an increase in the fluctuation on small isotropic substrates (Fig. 10.2b). Such nuclear fluctuations were completely abolished upon inhibition of myosin while depolymerization of microtubules had very little influence on nuclear dynamics, suggesting a regulatory mechanism centered on actomyosin contractility in these cells.

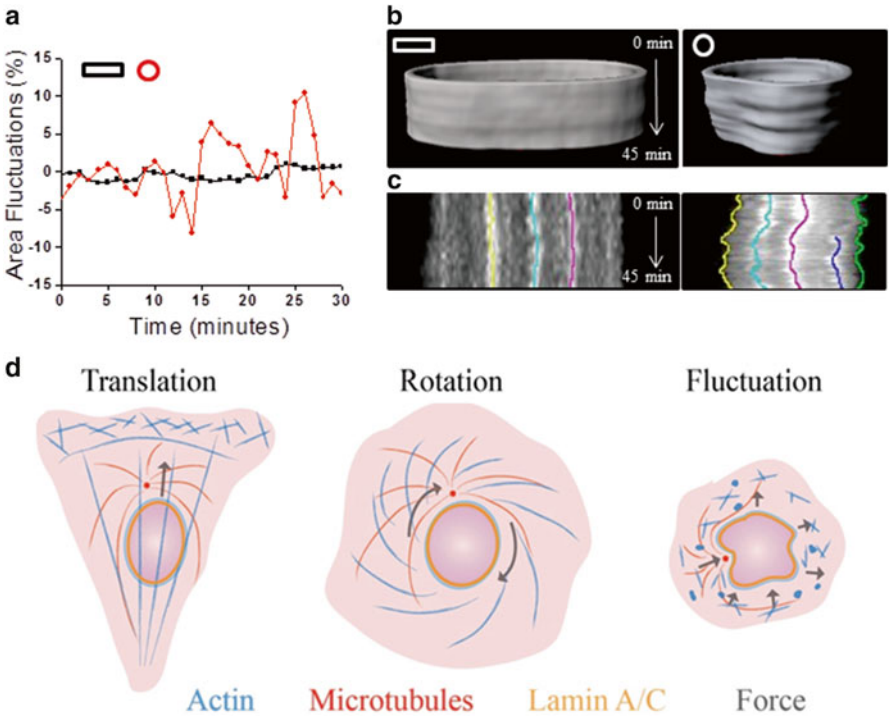

Fig. 10.2 Cell geometry modulates cytoskeletal organization and forces on the nucleus. (**a**) Projected nuclear area fluctuation vs. time traces for typical fibroblasts cultured on large rectangle or small circle micropatterns. (**b**) Nuclear periphery kymographs for typical rectangle and circle cells. (**c**) Line kymographs across H2B-EGFP time lapse images for typical rectangle and circle cells. (**d**) In migrating fibroblasts, actin is present as long stress fibers which are linked to the nucleus via nesprins. MTOC is present close to the nucleus, towards the leading edge and microtubules surround the nucleus. As the cell migrates forward, both actin and microtubules with their motors apply a net forward force on the nucleus, pulling it forward. In fibroblasts cultured on circular micropatterns, the net force from the hydrodynamic flow of actin filaments causes rotation of the nucleus. In fibroblasts constrained on small circular micropatterns or in stem cells, actin exists as meshwork of short filaments and punctae that are highly dynamic. Active forces from these dynamic actin units as well as decreased rigidity because of lower laminA/C expression levels cause fluctuations of the nuclear periphery

Cells with increased nuclear dynamics also show enhanced dynamics of the chromatin. Line kymographs across H2B-EGFP labeled nuclei show that heterochromatin foci are more dynamic in small isotropic cells as compared to cells on large anisotropic substrates (Fig. 10.2c). Actin perturbation in large anisotropic cells restores such dynamics of the heterochromatin foci, suggesting important roles for the cytoskeletal control of nuclear processes including chromosome organization and gene expression. To summarize, forces from actomyosin, which can be altered via pharmacological inhibitors or changes in cell matrix constraints, generate different dynamic behaviors of the nucleus (Fig. 10.2d).

10.3 Geometric Constraints Modulate Epigenetic State of Chromatin Assembly

The alterations in mechanical properties of the nucleus by cytoskeletal reorganization could also affect chromatin organization. Chromatin is assembled into relaxed euchromatin (gene active) and condensed heterochromatin (gene inactive) regions by posttranslational modifications on histones. These modifications on histones serve as a substrate for chromatin-modifying enzymes which either compact or relax the chromatin (Jenuwein and Allis 2001). The epigenetic landscape constitutes distinct posttranslational modifications on histones. These histone marks dictate the fate of differential chromatin compaction states and thereby gene regulation (summarized in Fig. 10.3a).

Compared to large anisotropic fibroblasts, small isotropic cells showed larger volume of condensed DNA depicted by the heterochromatin nodes observed from DAPI stain (Fig. 10.3b–d). The regions surrounding these heterochromatin nodes were enriched with acetylated histone H3 on lysine 9 (H3K9ac), which is known to represent the euchromatin region (Fig. 10.3b). Heterochromatin content is further classified into constitutive heterochromatin and facultative heterochromatin. Facultative heterochromatin is characterized by the enrichment of tri-methylation on lysine 27 of Histone H3 (H3K27me3). H3K27me3 was found to be diffused throughout the nucleus and was enriched at the nuclear periphery in both large anisotropic and small isotropic cells (Fig. 10.3c).

On the other hand, constitutive heterochromatin is represented by enrichment of tri-methylation on lysine 9 of Histone H3 (H3K9me3). In both large anisotropic and small isotropic cells, H3K9me3 was enriched in the dense DAPI-stained heterochromatin nodes. Further, larger volume of heterochromatin nodes in small isotropic cells correlated with the higher enrichment of H3K9me3 at these nodes (Fig. 10.3d). H3K9me3 further serves as a substrate for heterochromatin protein 1α (HP1α) which facilitates condensation of chromatin. Constitutive heterochromatin is known to originate from the pericentromeric regions. Centromere protein CENP-A marks the centromere and therefore the pericentromeric regions. Dense heterochromatin nodes stained by DAPI were decorated with CENP-A. In small isotropic cells, larger heterochromatin nodes was concomitant with higher enrichment of CENP-A molecules at these nodes, reconfirming that these heterochromatin nodes were constitutive heterochromatin, originating from the pericentromeric regions (Fig. 10.3e). These data demonstrates how cell–matrix interactions regulate the chromatin compaction state by modifying the epigenetic landscape. In the next section, we describe a more direct way of monitoring chromatin compaction on different substrates using fluorescence anisotropy imaging.

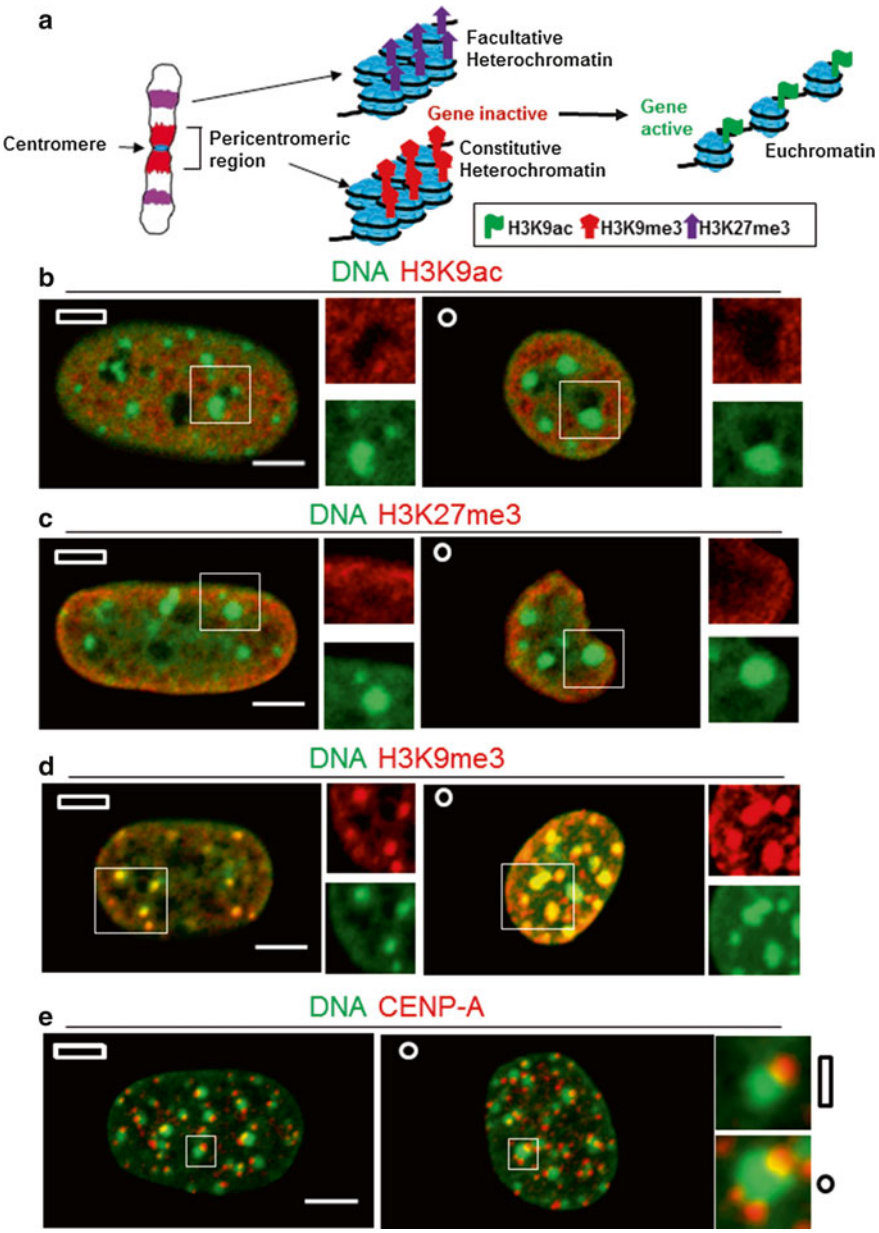

Fig. 10.3 Geometric constraints modulate epigenetic state of chromatin assembly. (**a**) Cartoon illustrating epigenetic landscape on various regions of chromosomes. (**b**) Euchromatin regions (H3K9ac). (**c**) Facultative heterochromatin (H3K27me3). (**d**) Constitutive heterochromatin (H3K9me3). (**e**) Pericentromeric regions marked with CENP-A. Scale bar: 5 μm

10.4 Direct Visualization of Chromatin Compaction States at MKL-Binding Sites

While immunofluorescence studies of the epigenetic markers depict the distribution of various chromatin binding proteins and histone modifications, a direct visualization of the chromatin compaction states can be achieved using fluorescence polarization microscopy. This technique is based on the principle that when cells with fluorescently labeled histones are excited with a polarized light, the depolarization of the emission is proportional to the rotational mobility of the histones (Lakowicz 1983; Banerjee et al. 2006). Regions with higher chromatin compaction correspond to lower rotational mobility of histones, which in turn corresponds to lower depolarization of the emission and higher anisotropy values (Fig. 10.4a).

Fluorescent anisotropy profile of cells labeled with H2B-EGFP indicates the chromatin compaction state; heterochromatin regions visualized from HP1α staining correspond to high anisotropy values (Ekta et al. 2014). A comparison with active and repressive epigenetic markers reveals that the histone anisotropy profile is also associated with the transcription state of the chromatin. Further, time series

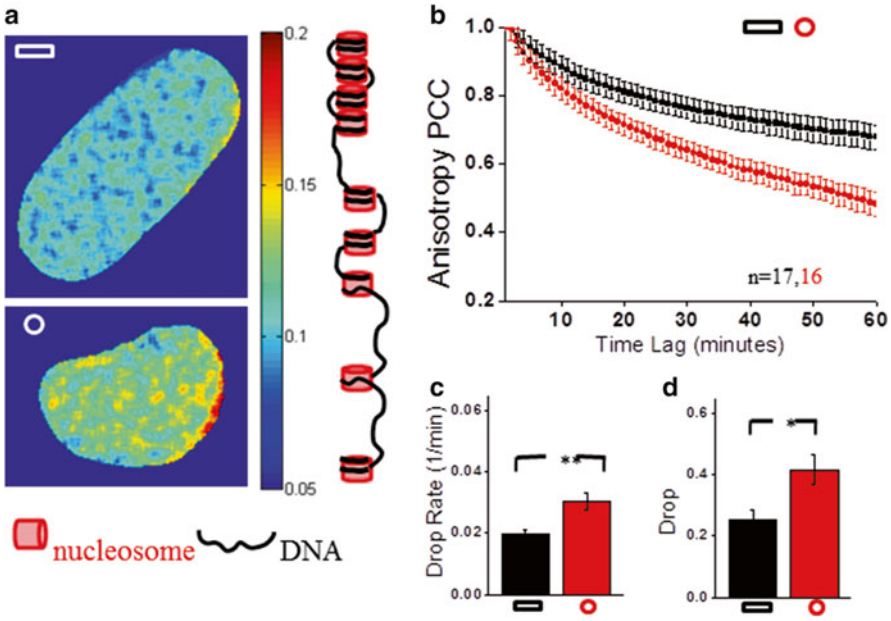

Fig. 10.4 Cell geometry modulates chromatin compaction dynamics. (**a**) Fluorescence anisotropy maps for typical cells on anisotropic and isotropic substrates. Warmer colors represent condensed chromatin while cooler colors represent decondensed chromatin. (**b**) Pearson correlation coefficient between anisotropy image series as a function of time for rectangle and circle cells. (**c**) Fraction of chromatin (α) that is permissible to change, obtained by fitting the anisotropy PCC curves to the equation $y = 1 - \alpha + \alpha e^{-(t/\tau)} - \eta$, (**d**) rate of change of chromatin compaction (α/τ)

of such anisotropy profiles, when analyzed using simple correlation techniques, allows the measurement of dynamics of chromatin structure. Pearson correlation coefficient of anisotropy image series plotted as a function of time and fitted with an exponential curve provides a measure for quantities like the rate of change of chromatin compaction pattern and the fraction of chromatin that is subject to change. Using this method, we show that in cells cultured on large anisotropic micropatterns, the compaction profiles de-correlate slower than those in small isotropic cells (Fig. 10.4b). In large anisotropic cells, the chromatin structure changes at the rate of 2 % per minute (Fig. 10.4c) and 20 % of the total chromatin is subject to change, with the compaction pattern in 80 % of the nucleus remaining constant (Fig. 10.4d). On the other hand, in small isotropic cells, the chromatin structure changes at the rate of 4 % per minute and 40 % of the total chromatin is subject to change.

Further, the technique of fluorescence polarization microscopy can also be extended to visualize the binding of a fluorescently tagged transcriptional regulator. MRTF-A, a transcription cofactor for serum responsive genes, has been shown to localize to the cytoplasm or the nucleus depending on the actin polymerization state, which is in turn governed by the mechanical state of the cell. MRTF-A binds to G-actin in the cytoplasm and shuttles to the nucleus when G-actin polymerizes to F-actin (Fig. 10.5a). In accordance with this, activity of the serum response factor and nuclear localization of MRTF-A has been observed to be enhanced in cells with stretched and polarized geometries (Jain et al. 2013b) or upon application of force (Iyer et al. 2012a).

We used fluorescence polarization microscopy of H2B and MRTF-A to simultaneously visualize chromatin compaction pattern and binding profile of the mechanoresponsive transcription regulator. Fibroblast cells co-labeled with H2B-EGFP and MRTF-A mCherry were stimulated with 15 % serum after 24 h of serum starvation. Anisotropy images in both channels were captured 15 min after serum stimulation. Zoom in of the anisotropy maps (Fig. 10.5b) and scatter plot of MRTF-A vs. H2B anisotropy (Fig. 10.5c) show an inverse relation between the two. Regions with high MRTF-A anisotropy correspond to low H2B anisotropy and vice versa. This suggests that MRTF-A binds in regions of decondensed euchromatin, while there is lower MRTF-A binding at heterochromatin regions. In the next section, we show the integration of extracellular mechanical signals with 3D chromosome organization, to differentially regulate SRF/MRTF-A targeted genes.

10.5 MKL Target Gene Expression and Transcription-Dependent 3D Chromosomes Organization with Geometric Constraints

Altering cell geometry from large anisotropic to small isotropic condition resulted in modular changes in transcriptional programs (Jain et al. 2013a). Microarray analysis of mouse fibroblasts cultured on large anisotropic vs. small isotropic substrates revealed that a group of SRF/MRTF-A targeted genes were upregulated in large

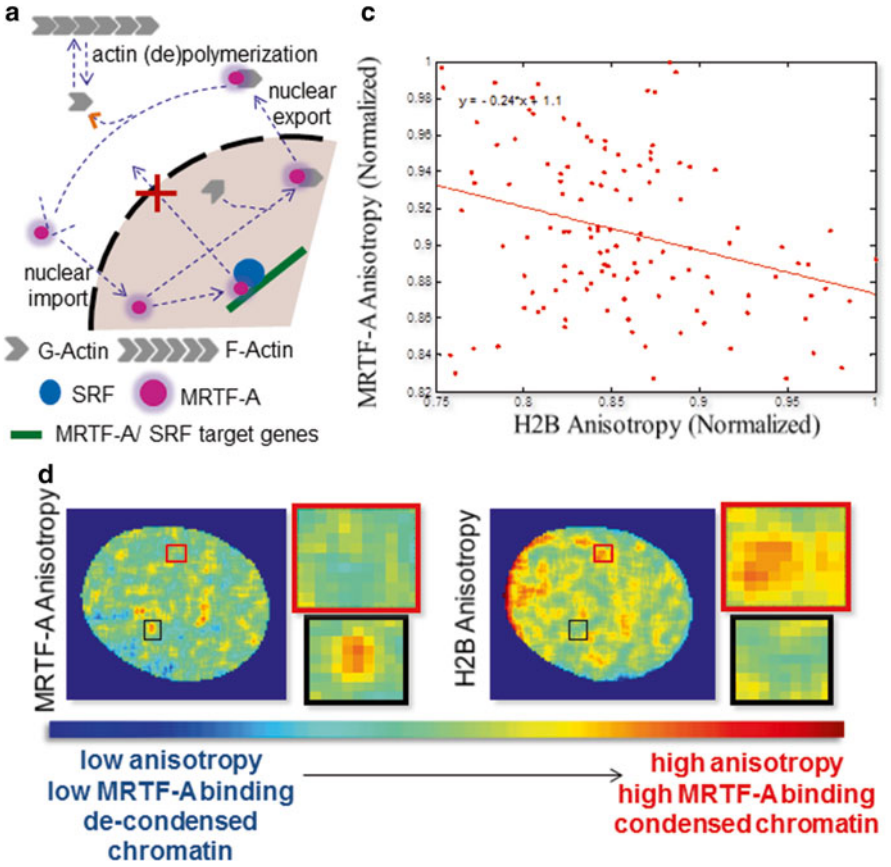

Fig. 10.5 MRTF-A binds at decondensed chromatin sites. (**a**) A cartoon summarizing the MRTF-A-SRF pathway. MRTF-A shuttles to the nucleus when G-actin polymerizes to F-actin. (**b**) MRTF-A mCherry and H2B-EGFP anisotropy images for the same nucleus. Upper zoomed region corresponds to high H2B anisotropy, i.e., heterochromatin while lower region corresponds to high MRTF-A anisotropy, i.e., MRTF-A binding. (**c**) Scatter plot for MRTF-A vs. H2B anisotropy in the zoomed regions

anisotropic cells (Fig. 10.6). Importantly, some of the genes such as *Thbs1, Myl9*, and *Acta1* were highly upregulated. *Thbs1* encodes adhesive glycoprotein that mediates the cell–matrix interactions, consistent with the larger spreading area in large anisotropic substrates. The product of *Myl9*, myosin, was shown to be more highly phosphorylated on its light chain in cells cultured on large anisotropic substrates. The upregulation of *Acta1*, whose product belongs to the actin family of proteins, correlates well with the higher level of actin on large anisotropic substrates (Jain et al. 2013a). Signals sensed at the ECM by cells not only modulate the physical links between the cytoskeleton and nucleus, but they relocate very specific transcription regulators (e.g., YAP/TAZ, MRTF-A) between cytoplasm and nucleus to regulate gene expression (Low et al. 2014; Iyer et al. 2012a). In this context,

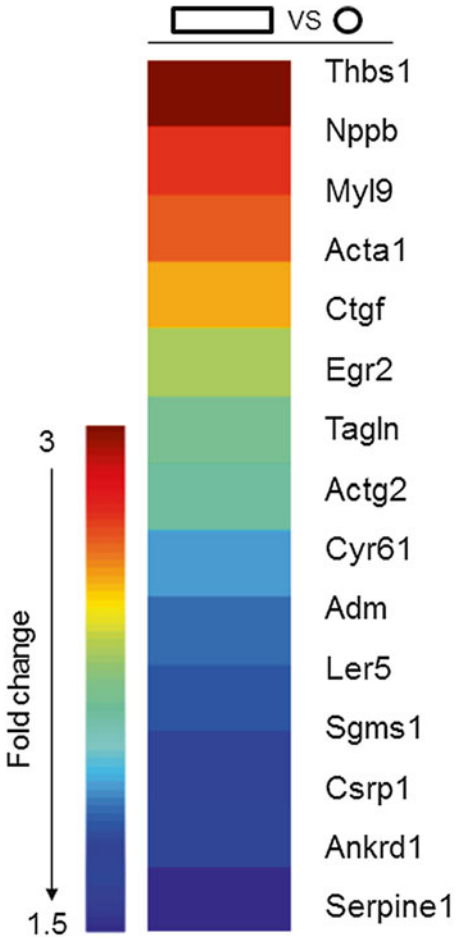

Fig. 10.6 Expression of MKL target genes in two geometry. The list of SRF/MRTF-A targeted genes that were upregulated in cells on anisotropic substrates compared to those on isotropic substrates. The larger fold change was coded with warmer colors, while the smaller fold change was coded with cooler colors

transcription cofactor MRTF-A was found to shuttle between the cytoplasm and the nucleus, with higher nuclear enrichment in large anisotropic cells. Concomitantly, reporter assay of SRF binding site SRE showed an increased activity in these cells, regulated by actomyosin contractility (Jain et al. 2013a).

These transcription factors and cofactors integrate with their target genes within the 3D chromosome organization. Cells have an elaborate nonrandom spatial organization of chromosomes to bring about lineage-specific transcription programs (Cremer and Cremer 2001; Parada et al. 2004; Bolzer et al. 2005; Fraser and Bickmore 2007; Cavalli and Misteli 2013). In flattened fibroblast cells, the radial distance of chromosomes is negatively correlated with its size (Bolzer et al. 2005), while in spherical lymphocytes the radial distance is negatively correlated with its gene density (Boyle et al. 2001). Such nonrandom organization of chromosome territories could be a reflection of gene activity (Mahy et al. 2002; Zink et al. 2004), but more than that, it could directly affect transcription (Kumaran and Spector 2008;

Reddy et al. 2008; Finlan et al. 2008). For example, when genes are relocated away from the nucleus interior to tether the inner nuclear membrane, their transcription activity was dramatically reduced (Reddy et al. 2008). In some other cases, however, the activity of genes remained the same or even increased after repositioned to the nuclear envelop (Finlan et al. 2008). Such discrepancy could be due to different regulatory mechanisms involved in the ongoing transcription at the nuclear envelope, which might be brought about by cytoskeletal to nuclear coupling, genomic environment, or different promoter properties (Cl et al. 2009; Geyer et al. 2011).

Although the correlation between the chromosome (or gene) radial position and its transcriptional activity supports the idea of nonrandom chromosome organization, it has been argued that such correlation might be oversimplified, and the radial position might not be a universal hallmark of gene activity (Takizawa et al. 2008). Instead, more evidences are suggesting that the relative position of chromosomes (or genes), controlled by cytoskeletal contractility, could be more important in genome regulation (Ragoczy et al. 2006; Brown et al. 2006, 2008). Interestingly, the negative correlation between chromosome radial position and gene density has actually implied the importance of relative position of genes for transcription (Boyle et al. 2001; Murmann et al. 2005). Tracking the transcription-dependent Uridine-50-triphosphate (UTP) compartments (Maharana et al. 2012) suggested the dynamic compartmentalization of transcription. Consistent with this, RNA polymerase II, a general transcription machinery, was found to be transiently clustered within nucleus (Cisse et al. 2013). Activation of serum response resulted in dynamic clustering of pol2 foci at a size of about 200 nm, with an average lifetime of about 5 s, as observed using superresolution microscopy in live cells.

Based on these reports, it has been hypothesized that the physical contacts formed by two or more chromosomes could play a role in genome regulation (Cremer and Cremer 2010). Chromosome conformation capture techniques such as 3C/4C have allowed the capturing and sequencing of the genome-wide chromosomal contacts (Jin et al. 2013). To test the role of such chromosomal contacts on transcription regulation, earlier studies measured the interchromosomal physical distance (IPD) based on the confocal images of fluorescence in situ hybridized (FISH) chromosomes. IPD is an indicator for the formation of chromosomal contacts: smaller IPD indicates higher possibility of contact formation, and vice versa. The differences in the activity of two chromosomes, termed as "interchromosomal activity distance (IAD)" were computed from the existing microarray data that contains the expression levels of all the genes on each chromosome. It was found that the IPD was positively correlated with IAD, suggesting chromosomes that were proximal in their 3D physical locations had similar expression patterns (Iyer et al. 2012b). Another recent study further confirmed this hypothesis, by showing that transcription activity of a group of NFkB targeted genes was maintained only when there was the co-clustering of these genes (Fanucchi et al. 2013). Such studies have highlighted the importance of the co-clustering of genes, thus the relative position of chromosomes, for specific transcription activity.

To probe the role of external mechanical signals on 3D chromosome organization, and thus gene expression, we used chromosome FISH to paint chromosome territories of mouse fibroblasts grown on large anisotropic or small isotropic

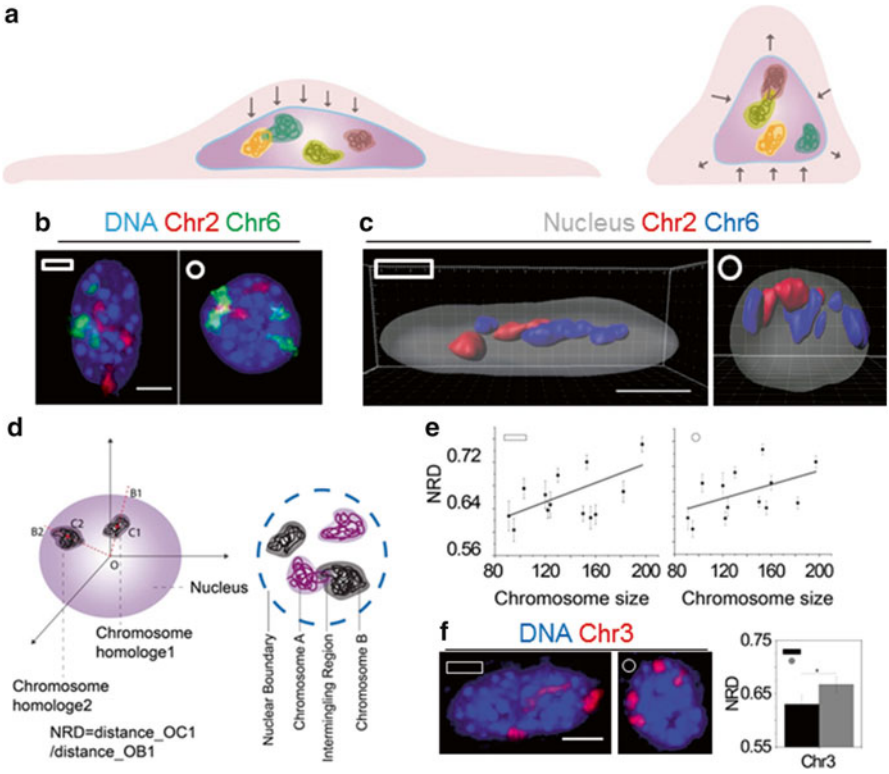

Fig. 10.7 Transcription-dependent 3D chromosome reorganization with geometric constraints. (**a**) Illustration of chromosome organization in nuclei of cells grown on anisotropic vs. isotropic substrates. (**b**) Representative confocal images of chromosome (Chr) 2 (*red*) and Chr6 (*green*) in nucleus (*blue*) of cells cultured on anisotropic (*rectangle*) and isotropic (*circle*) fibronectin-coated substrates. Scale bar: 5 μm. (**c**) Imaris generated 3D surface plot of nucleus (*gray*), Chr2 (*red*), and Chr6 on anisotropic (*rectangle*) and isotropic (*circle*) fibronectin-coated substrates. Scale bar: 5 μm. (**d**) Schematic illustration of the quantification for chromosome normalized radial distance (NRD), and the intermingling fraction between two chromosomes. (**e**) Correlation between NRD and chromosome size in cells on anisotropic (*rectangle*) and isotropic (*circle*) fibronectin-coated substrates. (**f**) Representative confocal images of Chr 3 (*red*) in nucleus (*blue*) of cells cultured on anisotropic (*rectangle*) and isotropic (*circle*) fibronectin-coated substrates. Scale bar: 5 μm. Bar graph showing the different NRD of Chr3 on the two geometric constraints

substrates. Nuclei of cells on large anisotropic or small isotropic substrates experience different forces, which results in large alterations in nuclear morphology, and possibly chromosome 3D organization (Fig. 10.7a). With the help of 3D chromosome FISH, we were able to test whether such big changes in nuclear morphology could lead to the repositioning of chromosome territories within the 3D nuclear space. The confocal images of chromosome (Chr) 2 and Chr6 showed distinct radial positions and relative positions in 2D (Fig. 10.7b). Such repositioning was also observed in the 3D imaris generated surface plot of nucleus, Chr2, and Chr6 (Fig. 10.7c). To systematically quantify the 3D position of chromosome, we defined two parameters: (a) normalized radial distance (NRD), which measures the relative

radial distance of a chromosome within the 3D nuclear volume; (b) the intermingling fraction between two chromosomes, which was determined by the overlapping volume of two chromosomes normalized by the total volume of the two chromosomes and their homologues (Fig. 10.7d). After analyzing a subset of chromosomes that have a broad range of chromosome size, and gene density, we correlated the NRD and chromosome size (Mbp). Consistent with previous studies, we found that the larger chromosomes tend to locate at the periphery of the nucleus, while the smaller ones positioned at more interior space (Fig. 10.7d). Such general correlation was not affected by different geometric constraints. However, the NRD of some specific chromosomes was affected by external mechanical signals. For instance, Chr3 moved towards interior nucleus on large anisotropic substrates, while stayed at the periphery of nucleus on small isotropic substrates (Fig. 10.7e). This repositioning was correlated with the upregulation of SRF/MRTF-A targeted genes on this chromosome (Fig. 10.6). These studies suggest that a combination of 3D organization of chromosomes and cytosolic to nuclear shuttling of transcription factors are essential to inducing modular changes in gene expression, in response to external mechanical signals.

10.6 Conclusion and Implications

In summary, this chapter describes in detail our understanding of the links between cytoskeleton and nucleus and their role on nuclear dynamics, chromatin compaction, and 3D chromosome organization in controlling differential gene expression programs. The alteration in cytoskeleton organization and nuclear morphology caused by different cell matrix constraints lead to the redistribution of TFs, accompanied by the repositioning of chromosome territories. This resulted in the dissociation of existing chromosomal contacts and the formation of new ones, to bring about differential gene expression patterns. These studies highlight the importance of freezing relative chromosome neighborhoods for optimal transcriptional network topology in specific cell types. Understanding the coupling between cell mechanics and gene expression will provide important avenues to control cellular behavior and reprogramming.

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Part IV
Applications

Chapter 11
Mechanobiological Control of Cell Fate for Applications in Cardiovascular Regenerative Medicine

Andrew J. Putnam

Abstract The burgeoning field of regenerative medicine has witnessed impressive advances over the past 25–30 years, enabling engineered tissue constructs to be translated into human patients. However, despite fundamental advances in biomaterials and stem cell biology, the goal of generating tissues at clinically relevant scale that function equivalently to the native tissues they are intended to replace remains unmet. The field of mechanobiology has similarly advanced at a rapid pace over the past three decades, leading to the emergence of an increasingly detailed network of molecular players involved in the transduction of mechanical forces and the recognition of mechanical cues may be equally as important as (or perhaps more than) biochemical cues in the determination of cellular phenotype. Exploiting mechanobiology for applications in regenerative medicine represents an exciting opportunity and an enormous challenge. There is no greater clinical need than for cardiovascular tissues, which are amongst the most widely studied in terms of mechanobiology. Furthermore, there is substantial evidence correlating alterations in tissue mechanics with the onset of cardiovascular pathologies. Therefore, the focus of this chapter is on the relationships between mechanical forces and cells of the cardiovascular system (including endothelial cells, smooth muscle cells, and cardiac myocytes), with an emphasis on translating fundamental mechanobiology insights into the control of cell fate for regenerative applications.

Keywords Stem cells • Tissue engineering • Cardiomyocytes • Endothelial cells • Smooth muscle cells

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11.1 Introduction

More than 80 million people are suffering from one or more forms of cardiovascular disease (CVD), the #1 killer in the United States. Coronary artery disease, a form of CVD, is the most common underlying cause of myocardial infarction (MI), or heart attack. A person suffers a heart attack approximately every 20 s in the USA, resulting in the death of approximately one billion cardiomyocytes (CMs) within just a few hours (Chong et al. 2014; Xu et al. 2011). The eventual clinical outcome following an MI is often heart failure, which afflicts over 14 million people worldwide and can currently only be cured via a whole heart transplant. Only a few thousand whole heart transplants are performed in the USA each year, and thus there is an enormous clinical need for new cardiac regenerative therapies.

The regenerative capacity of the human heart is limited, in contrast to some lower vertebrates like axolotls and zebrafish, which possess a much more robust regenerative response to injured limbs and organs. Studies have shown that the zebrafish heart can regenerate after surgically removing ~20 % of the myocardium at the ventricular apex (Jopling et al. 2010; Kikuchi et al. 2010; Lepilina et al. 2006; Poss et al. 2002). The regenerative process in these zebrafish is due primarily to the dedifferentiation and subsequent proliferation of preexisting CMs, rather than the generation of new CMs from an endogenous pool of progenitor cells (Jopling et al. 2010). Postnatal mammalian hearts also possess some capacity, albeit limited, for CM renewal, something that has been recognized since the 1960s. One study suggests that mammals may be capable of heart regeneration via mechanisms similar to the zebrafish, but only early in development (Porrello et al. 2011). However, genetic fate-mapping experiments in mice suggest that the limited regenerative mechanisms in the adult mammalian heart depend more on replenishment by cardiac progenitor cells than on replacement by CM proliferation (Laflamme and Murry 2011). Thus, there may be fundamental differences in the mechanisms by which lower vertebrates (reliance on dedifferentiation and proliferation) and postnatal mammals (reliance on progenitor cells) regenerate the heart. There is some CM turnover in humans due either to proliferation of existing cells or generation from progenitor cells, but this limited potential has not been successfully harnessed for cardiac regeneration.

Most cardiac regenerative strategies have focused on cell transplantation. Various cell types have been delivered to the heart and may have a significant impact on the damaged heart muscle (Nagaya et al. 2004, 2005; Silva et al. 2005; Kajstura et al. 2005). However, most transplanted cell types fail to engraft in the heart (Limbourg and Drexler 2005; Chien 2004), and instead probably exert more limited beneficial effects through the release of trophic factors, possibly enhancing neovascularization in the fibrotic myocardium (Assmus et al. 2002; Kocher et al. 2001). As a result, there is significant interest in deriving CMs from pluripotent cell types, either embryonic stem cells (ESCs) (Laflamme and Murry 2011) or induced pluripotent stem cells (iPSCs) (Takahashi et al. 2007; Takahashi and Yamanaka 2006). A recent study showed that CMs derived from human ESCs can regenerate the myocardium in nonhuman primates, which optimistically suggests the clinical scalability of stem cell therapies for eventual human applications (Chong et al. 2014).

While delivery of cells to the ischemic heart has shown some potential, approximately 90 % of cells injected directly into this harsh microenvironment do not survive beyond a few days (Qian et al. 2012; Laflamme and Murry 2005) in part because of the lack of oxygen. Thus, transplantation of functional cardiac tissue constructs undoubtedly will require a solution to the vascularization problem. The myocardium is a well-vascularized tissue, with CMs residing within ~20 μm of a capillary in order to receive critical life-sustaining oxygen (Korecky et al. 1982). Generating a functional, perfusable microvascular network therefore goes hand-in-hand with efforts to generate functional cardiac tissue replacements.

In the context of mechanobiology, all of the cells necessary to generate a functional cardiac tissue (i.e., endothelial cells, smooth muscle cells, cardiac myocytes, and cardiac fibroblasts) have been well studied for their mechanosensitivity. Using a single pluripotent cell source to generate all of these cell types is theoretically possible, and attractive for a number of reasons; however, it is clear that a multitude of physical factors can influence cell phenotype via a wide range of mechanobiological mechanisms (Fig. 11.1) (Discher et al. 2009; Yamada and Cukierman 2007). A better understanding of the physical properties of the environment (the mechanical properties in particular) that actively instruct cell fate could therefore significantly enhance ongoing efforts to develop regenerative medicine approaches to the treatment of heart failure and other CVDs. This chapter focuses on three main mechanobiological features of the cellular microenvironment that have been implicated in cell fate decisions: externally applied mechanical forces, intrinsic mechanical properties of the

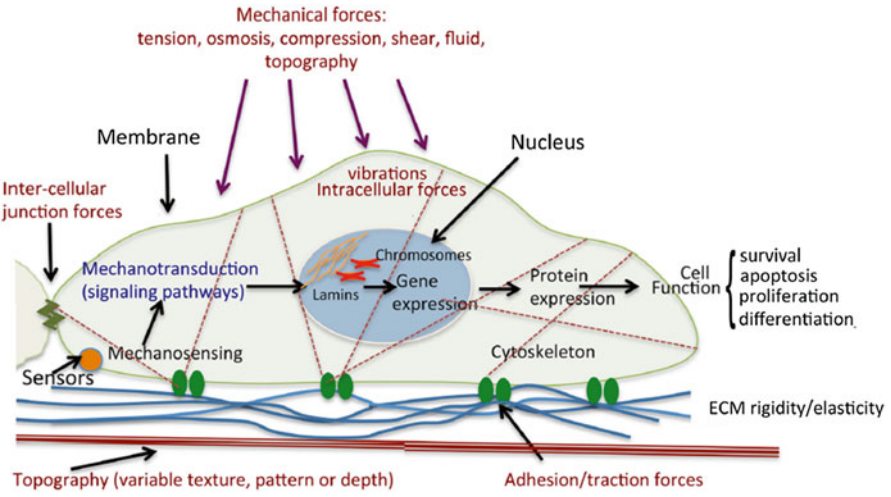

Fig. 11.1 Mechanobiological influences on cell fate. Cells receive cues from a myriad of sources in their local environment, including (among others) soluble and matrix-bound growth factors, other cells, the ECM, and physical factors, including applied mechanical forces, ECM rigidity/elasticity, and ECM topography. Mechanobiological influences are transduced by signal transduction networks and cytoskeletal elements in an integrated fashion to direct cell fate processes in the body, including proliferation, differentiation, migration, and apoptosis. (Adapted from Tsimbouri 2015 with permission granted by the Creative Commons Open Access License)

extracellular matrix (ECM), and ECM topography. I focus on how these features influence endothelial cells (ECs), smooth muscle cells (SMCs), and CMs, and try to emphasize how they have been used to control the differentiation of pluripotent stem cells towards these three lineages.

11.2 Control of Cell Fate by Externally Applied Mechanical Forces

In the late nineteenth century, Julius Wolff first proposed the idea that bone is deposited and resorbed in response to mechanical stress (Wolff 1892). This fundamental premise motivated numerous mathematical incarnations of Wolff's Law, which has had a long history of importance in orthopedic mechanobiology. Mechanical forces more broadly influence tissue development, and it is now widely believed that the application of external forces to tissues influences both developmental morphogenesis and homeostasis. A consequence of this recognition has been the extensive research focused on the development of bioreactors capable of applying mechanical forces to engineered tissues, particularly those designed for musculoskeletal and cardiovascular applications, which experience dynamic mechanical loading in vivo (Ratcliffe and Niklason 2002). Such bioreactor systems have been designed to apply mechanical strain to engineered bone (Sikavitsas et al. 2001), cartilage (Darling and Athanasiou 2003), and skeletal muscle (Powell et al. 2002). In this section, I briefly summarize the extensive bodies of literature showing that SMCs, ECs, and CMs are sensitive to applied mechanical forces, and then discuss the application of mechanical forces to induce differentiation of stem cells into these three different lineages.

11.2.1 Smooth Muscle Cells and Applied Forces

Mechanical influences on the development and function of vascular smooth muscle tissue, a major component of blood vessel walls, are widely appreciated (Lehoux and Tedgui 1998; Li and Xu 2000; Osol 1995; Williams 1998). Differentiated SMCs in normal arteries are contractile, and do not respond to growth signals under normal in vivo conditions. When removed from the body and placed in 2D cell culture, these cells revert from their contractile (differentiated) phenotype to a synthetic (proliferative) phenotype, typically seen during vascular development. This transition is influenced in culture by ECM composition, soluble factors, and mechanical stress (Thyberg 1998; Thyberg et al. 1995). A great deal of work has focused on the response of vascular SMCs and tissues to static (Putnam et al. 1998) and cyclic mechanical strain (Stegemann et al. 2005) in an effort to mimic the forces experienced by these cells that result from the pulsatile nature of blood flow. Using two-dimensional cell culture systems capable of applying uniaxial or equibiaxial strain, the transition of SMCs from the synthetic to the contractile phenotype can be

transiently induced by removal of serum components or via mechanical strain (Reusch et al. 1996; Wilson et al. 1995). Similar results have also been reported with 3D engineered smooth muscle tissues (Kim et al. 1999a; Isenberg and Tranquillo 2003). This phenotypic conversion depends on the chemical identity of the ECM, as does the phenotypic response of engineered smooth muscle tissues subjected in vitro to physiologic levels of strain (Kim et al. 1999a, b; Nikolovski et al. 2003; Stegemann and Nerem 2003). In general terms, these findings imply mechanical forces play a homeostatic role in maintaining the differentiated SMC phenotype. This argument is further supported by evidence from engineered smooth muscle tissue models, which indicate SMCs take on an osteoblast-like phenotype in the absence of cyclic mechanical strain by increasing expression of several bone-associated genes in a manner that mimics ectopic calcification (Nikolovski et al. 2003).

Cyclic strain has been used to improve the mechanical performance of small diameter tissue engineered vascular grafts. In a landmark paper in 1999, Niklason et al. subjected tubular poly(glycolic acid) scaffolds seeded with SMCs to a 5 % pulsatile radial strain at 165 beats/min (Niklason et al. 1999). After either 5 or 8 weeks, grafts were seeded with ECs and subjected to an additional 3 days of continuous perfusion. Grafts subjected to mechanical preconditioning had increased wall thickness, increased collagen content, and improved suture retention compared to constructs not subjected to mechanical loading. They also displayed functional contractile properties in response to known contractile agonists, and performed well as xenograft saphenous arteries in a Yucatan pig model. Similarly, Seliktar et al. found that 10 % cyclic strain of SMC-seeded tubular collagen constructs at a frequency of 1 Hz for 8 days led to a threefold increase in ultimate tensile strength and a 3.5-fold increase in the tensile tangent modulus (Seliktar et al. 2000). A number of other studies reported similar findings (Isenberg and Tranquillo 2003; Williams and Wick 2004; Xu et al. 2008; McFetridge et al. 2007; Iwasaki et al. 2008).

Given the importance of mechanical loading in normal vascular development and homeostasis, a number of studies have investigated the application of cyclic mechanical strain to control the differentiation of multipotent, and more recently pluripotent, progenitor cells towards a smooth muscle lineage. One such study demonstrated that cyclic uniaxial strain of marrow-derived mesenchymal stem cells (MSC) transiently increased the expression of smooth muscle markers and synthesis of type-I collagen into the SMC lineage in as short as 24 h; by contrast, equiaxial strain resulted in downregulation of SMC markers and only a transient increase in type I collagen gene expression (Park et al. 2004). A more recent study showed MSCs subjected to 10 % cyclic strain at 1 Hz frequency increased the gene expression levels of SMC markers more significantly than cells exposed to 0.1 Hz, which conversely expressed higher levels of osteoblast-specific markers (Yao and Wong 2015). Smooth muscle-like cells have also been derived from either human embryonic stem cells (ESCs) (Vo et al. 2010) or induced pluripotent stem cells (iPSCs) (Wanjare et al. 2013) via 6 days of culture on collagen type IV in a differentiation medium consisting of alpha-MEM, 10 % serum, and 0.1 mM β-mercaptoethanol, followed by 6 additional days of culture in 10 ng/mL platelet-derived growth factor (PDGF-BB) and 1 ng/mL transforming growth factor-beta (TGF-β1). Subjecting

these cells to uniaxial cyclic strain in 2D resulted in changes in cell orientation and gene expression in a manner that depended on the maturation state of the cells and the serum content. By comparison, the application of circumferential strain to iPSC-derived SMCs in tubular collagen-based constructs induced an increase in elastin production, a significant increase in collagen III gene expression, and a significant decrease in fibronectin gene expression (Wanjare et al. 2015). While much work remains, especially with respect to the mechanical regimen and the integration with soluble and ECM cues, these promising results have exciting potential for translational impact as they suggest the application of mechanical forces may yield more homogenous populations of functional SMCs from pluripotent cell sources for cardiovascular applications.

11.2.2 Endothelial Cells and Applied Forces

ECs line the lumens of blood vessels and are subjected to shear stresses induced by the flow of blood. They are amongst the most widely studied cells in terms of mechanobiology, with 40+ years of investigation demonstrating that shear stress is a critical regulator of vascular homeostasis and development. Comprehensive reviews of EC mechanobiology can be found elsewhere (Davies 1995; Shyy and Chien 2002; Zhou et al. 2014), but perhaps the most compelling piece of evidence supporting mechanobiology's importance is the clinical observation that atherosclerotic lesions tend to preferentially occur in regions of low or disturbed shear stress at vessel branch points, bifurcations, and regions of high curvature, whereas high laminar shear stress is atheroprotective (Davies 1995). Flow-induced shear stresses are also essential to remodel the primitive vascular plexus following vasculogenesis and play a key role in arterial versus venous specification during development (le Noble et al. 2004). Increasing shear stress artificially during development by increasing blood viscosity has also been shown to rescue vessel formation, even when flow is reduced (Lucitti et al. 2007).

Given the important role of shear stress in vascular development, it has been hypothesized that the application of shear stresses may influence the EC differentiation of ESCs and iPSCs (Stolberg and McCloskey 2009). Nevertheless, even recent papers on the generation of ECs and SMCs from iPSCs have focused exclusively on soluble factors, with no consideration of mechanical forces (Patsch et al. 2015). Previous studies have shown that EC derived from both mouse and human ESCs in static conditions exhibit only a subset of the characteristics of mature EC (McCloskey et al. 2006; Metallo et al. 2008). When exposed to flow-induced shear stress, both mouse ESC-ECs and human ESC-ECs aligned in the direction of flow (McCloskey et al. 2006; Metallo et al. 2008), while the human cells also changed their expression patterns of a number of markers consistent with the response of HUVECs and microvascular ECs (Metallo et al. 2008). Similar results in other studies reinforce this paradigm (Yamamoto et al. 2005; Nsiah et al. 2014; Sivarapatna et al. 2015; Wolfe and Ahsan 2013).

ECs within large arteries and arterioles are exposed to flow-induced shear stresses, but such stresses are quite small or even negligible in parts of the microvasculature where blood flow is near zero. Therefore, other mechanical forces likely play more significant roles in the formation and remodeling of the microvasculature. For example, Helm et al. demonstrated that slow interstitial flow of the magnitudes that typically exist between the blood and the lymphatic microvasculature in vivo biases the distribution of cell-secreted proteases and thereby creates a gradient of VEGF originally bound to the ECM, which in turn induces directional capillary morphogenesis parallel to the interstitial flow (Helm et al. 2005). Kilarski et al. showed that mechanical strain generated by endogenous fibroblasts on the ECM promote the development and growth of new vessels during wound healing in vivo (Kilarski et al. 2009). By contrast, Boerckel et al. showed that externally applied mechanical loads actually reduce vessel invasion in a bone injury model when applied at early time points, but enhance vascular remodeling when the load is applied after a delay (Boerckel et al. 2011). Research from my own laboratory has shown that ECs subjected to cyclic strain form capillary networks to the same extent as those cultured in static conditions (Ceccarelli et al. 2012), similar to a study by Krishnan et al. which found no differences in segment length distribution in strained and unstrained collagen gels (Krishnan et al. 2008). On the other hand, capillary sprouting occurred parallel to the direction of applied strain in both 3D collagen gels (Krishnan et al. 2008) and fibrin gels (Fig. 11.2) (Ceccarelli et al. 2012). Similarly, endothelial sprouting from spheroids occurred in a directional fashion in fibrin gels that had been aligned via an applied magnetic field (Morin and Tranquillo 2011). Collectively, these studies suggest mechanical strain and interstitial flow may be useful methods to orient microvasculature. A more ordered and regularly spaced microvasculature may in turn help the survival and function of other parenchymal cell types in cell transplantation applications (Baranski et al. 2013).

11.2.3 Cardiomyocytes and Applied Forces

Cardiac muscle, much like skeletal muscle, responds to changes in load; perhaps the clearest demonstration is pathological cardiac hypertrophy and eventual heart failure in human patients with hypertension-induced CVD. Mechanical forces also play critical roles in cardiac development and function. Using the zebrafish heart as a model system, Hove et al. demonstrated the importance of hemodynamic forces on cardiac development; they showed that flow occlusion at either cardiac inflow or outflow tracts resulted in hearts with an abnormal third chamber, diminished looping, and impaired valve formation (Hove et al. 2003).

In the context of cardiac tissue engineering, Zimmerman et al. created engineered heart tissue (EHT) by seeding rat neonatal CMs in a mixture of collagen I and Matrigel into ring-shaped molds, and subsequently subjected the EHTs to unidirectional cyclic strain (Zimmermann et al. 2002). The resulting constructs displayed interconnected, longitudinally oriented cardiac muscle bundles reminiscent

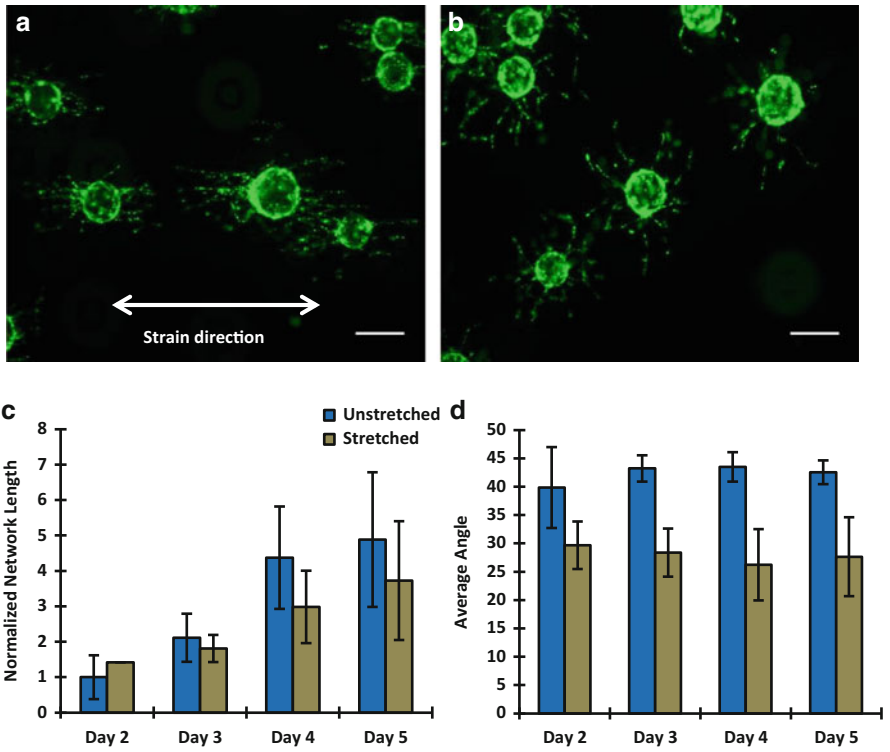

Fig. 11.2 Cyclic strain induces directional angiogenic sprouting. (**a**, **b**) Representative pictures of HUVEC-coated dextran microcarrier beads at day 5 in 3D angiogenesis assay under strained (*left*) and static (*right*) conditions. (**c**) Normalized network lengths of strained and unstrained capillaries. Data for each condition were normalized to the day 2 unstrained condition. No significant differences were observed between the strained and unstrained conditions at each time point. (**d**) A significant decrease in average sprout angle was observed in the strained conditions (relative to unstrained) for all time points. Random alignment corresponds to an average angle of 45°, while perfect alignment in the direction of strain corresponds to 0°. Data reported as mean +/− S.E.M. Scale bars 100 μm. (Adapted from Ceccarelli et al. 2012 with permission from Springer: *Cellular and Molecular Bioengineering*, 5;4, copyright 2012)

of native heart muscle from adult rats, including highly organized sarcomeres. Furthermore, the EHTs displayed contractile characteristics and action potentials of native myocardium (Zimmermann et al. 2002). A subsequent study showed that these EHTs could improve cardiac output following MI in rat hearts (Zimmermann et al. 2006). Other studies have also examined the effects of applied mechanical loading on tissue engineered cardiac muscle constructs (Salazar et al. 2015; Birla et al. 2007). Electrical stimulation of cardiac tissue constructs has been used to induce synchronous contractions of CMs, resulting in the formation of a cardiac muscle tissue with elongated cells aligned in parallel (Radisic et al. 2004).

Despite the recognition of the importance of mechanobiology in the bioengineering community, current state-of-the-art differentiation protocols to derive CMs

from hESCs typically involve culturing the stem cells on Matrigel-coated plates and subjecting them to a soluble factor cocktail consisting of activin A and bone morphogenetic protein (BMP)-4 for more than 2 weeks (Chong et al. 2014; Laflamme et al. 2007). However, even with this established protocol, the CM yield is only 30 % (70 % of the cells are something else), and the CMs must be purified via fluorescence-activated cell sorting (FACS). Considering other features of the cellular microenvironment, mechanical forces in particular may affect the differentiation process and perhaps significantly increase the efficiency with which bona fide CMs are generated from pluripotent progenitors. Supporting this concept, there is evidence that CMs derived from ESCs acquire characteristics of a more mature phenotype when mechanically stimulated, in comparison to those cultured statically (Shimko and Claycomb 2008; Gwak et al. 2008). In one such case, engineered cardiac patches were made by culturing ESCs within an elastic polymer scaffold, subjecting them to cyclic mechanical strain for 2 weeks, and then implanting them in a rat MI model (Gwak et al. 2008). The mechanically stimulated patches exhibited enhanced CM differentiation, reduced apoptosis, elevated VEGF expression, and increased angiogenesis compared to unstrained controls, and led to reduced fibrosis and improved myocardial regeneration. (For further discussion of mechanical forces and other factors important in myocardial regeneration, readers are referred to some recent review papers on the topic (Parsa et al. 2016; Stoppel et al. 2016).)

11.3 Control of Cell Fate by ECM Mechanics

11.3.1 Evidence Supporting the General Principle

While it is clear that cells in the cardiovascular system (and elsewhere in the body) respond to applied forces, cardiovascular tissues are also influenced by the baseline mechanical stress in the cardiovascular system. Hypertensive conditions effectively increase the "zero stress state" (the residual intrinsic stress in the absence of blood pressure), and induce the phenotypic transition in SMCs, characterized by a decrease in the expression of smooth muscle myosin heavy chain isoforms, α-actin, h-caldesmon, and calponin (Li and Xu 2000; Aikawa et al. 1995, 1997). SMCs in hypertensive tissues respond, in part, by remodeling their ECM in response to the altered mechanical load, as evidenced by changes in elastin deposition (Keeley and Bartoszewicz 1995). Similar behavior is observed in bone remodeling and other connective tissues, suggesting more generally that cells may respond to changes in their mechanical microenvironment by remodeling their ECM (Chiquet 1999; Chiquet et al. 1996, 2003).

An essential and conserved element of the mechanism by which cells sense the intrinsic mechanical properties of their environment is via the generation of tractional forces. Nearly all cells in the human body use their actin-myosin machinery in a "tug-of-war" fashion to interrogate the resistance of their surroundings to deformation. This mechanical resistance, typically characterized by the elastic modulus

of the tissue, varies greatly from one tissue to another in vivo. For example, soft neurological tissues (e.g., brain) possess an equilibrium elastic modulus on the order of 1 kPa, whereas hard connective tissue (e.g., bone) can have elastic moduli values in excess of 1 MPa (Fung 1993). Do these different mechanical properties actively instruct cells? The first insights into this question came in the late 1980's, when the differentiated phenotype of epithelial cells in vitro was obtained in a soft, deformable 3-D matrix, but not on a rigid glass substratum with comparable ligand density (Opas 1989). Subsequent studies on ECM elasticity focused on its influence on cell adhesion, spreading, and migration (Pelham and Wang 1997). Pelham and Wang first demonstrated that 3T3 fibroblasts become less motile as substrate rigidity increased (Pelham and Wang 1997). A subsequent study identified a phenomenon called durotaxis (or mechanotaxis) by showing that 3T3 fibroblasts migrate in a directional fashion from softer substrates to stiffer substrates, but not vice-a-versa, indicating a dependence on the mechanical properties of the substrate in the absence of any soluble chemical stimuli (Lo et al. 2000).

Two subsequent and particularly important papers helped catapult the importance of ECM elasticity and cell shape into the scientific mainstream consciousness. First, McBeath et al. demonstrated the critical role for cell spreading on the control of cell fate (McBeath et al. 2004). In that study, the authors used fibronectin stamped on PDMS as adhesive islands of controlled area to reveal that MSCs differentiated along an osteogenic lineage when allowed to spread; when spreading was restricted, they differentiated along an adipogenic lineage. Furthermore, the authors showed that RhoA/ROCK-mediated contractile forces were mechanistically at the heart of this lineage regulation by cell shape (McBeath et al. 2004). Since one of the most widely observed manifestations of changes in ECM elasticity is a change in cell shape, these findings suggested that ECM elasticity would influence cell fate in 2D via similar (if not identical) mechanisms. Validating this concept, Engler et al. demonstrated that MSC differentiation depends directly on ECM elasticity, with compliant matrices mimicking the elasticity of brain supporting characteristics of neuronal cells and stiff substrates consistent with a pre-mineralized osteoid matrix supporting the expression of osteoblastic markers (Engler et al. 2006). Substrates with intermediate stiffness supported a skeletal muscle-like phenotype. Engler et al. also investigated the effects of matrix elasticity on the differentiation of multinucleated skeletal muscle myotubes (Engler et al. 2004), and subsequent studies by others showed that ECM elasticity can regulate the differentiation state of other stem cell populations as well (Saha et al. 2008).

It is worth noting there has been some debate regarding the concept that ECM elasticity directly controls cell fate. Trappmann et al. argued that changing polyacrylamide gel formulations to change ECM elasticity simultaneously altered the presentation of collagen tethered to the gels via sulfo-SANPAH (Trappmann et al. 2012). Due to the porous nature of polyacrylamide gels, the authors suggested that collagen tethering to the gels changed as gel elasticity was varied, and attributed subsequent changes in MSC fate to changes in ligand tethering rather than ECM elasticity. Reinforcing this argument, the authors showed that PDMS gels of varied elasticity did not alter the differentiation status of MSCs (Trappmann et al.

2012). However, by way of rebuttal, Wen et al. performed a set of experiments in which polyacrylamide porosity was modulated without altering stiffness, and demonstrated that MSC differentiation was not affected (Wen et al. 2014). The authors also increased the concentration of sulfo-SANPAH 50-fold to affect ECM ligand tethering to the underlying polyacrylamide, and again found no effects on MSC differentiation. These findings reinforced the earlier paradigm originally supported by Engler et al. (2006).

Going a step further, Yang et al. used a different material platform to demonstrate that MSCs apparently have mechanical memory, and that memory depends on the activity of the Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ) (Yang et al. 2014). Using PEG hydrogels, the authors reported that MSCs exhibit characteristics of a pre-osteogenic phenotype when cultured on soft (2 kPa elastic modulus) substrates after previous culture time on rigid tissue culture polystyrene (TCPS, elastic modulus of ~3 GPa), and that the extent of the osteogenic phenotype depended on the duration of the pre-culture period on TCPS. They also used an innovative photodegradable PEG platform to dynamically alter substrate mechanical properties (from initially stiff to soft) to show that the activation of YAP/TAZ and Runx2 (an osteogenic transcription factor) was reversible, until a threshold mechanical dose was surpassed (Yang et al. 2014).

Whether ECM elasticity affects cells in 3D materials has been a more difficult question to address, due in large part to the coupling of ECM mechanics, chemistry, and microstructure in most hydrogel platforms. However, this is an important question since 3D materials are widely considered to more accurately mimic the native microenvironment of many cell types in the human body. In natural protein-based hydrogels (e.g., collagen, fibrin, Matrigel), increasing protein concentration affects elastic modulus but simultaneously alters the number of binding sites available for cell adhesion and can disrupt the diffusive transport of soluble morphogens (Ghajar et al. 2008). A 2010 paper by Huebsch et al. tackled this question using RGD-modified alginate gels, demonstrating that osteogenesis of MSCs was best supported by gels of intermediate elasticity in 3D (Huebsch et al. 2010). This was distinct from the 2D case, for which MSC differentiation towards an osteogenic lineage was positively correlated with increasing elastic modulus (Engler et al. 2006). The various formulations of alginate exploited in the 3D study permitted equal levels of diffusive transport, and also inhibited the ability of the MSCs to spread.

Using a dynamic hyaluronic acid hydrogel platform, Khetan et al. demonstrated that the relationship between ECM elasticity and MSC differentiation in 3D is perhaps more complex (Khetan et al. 2013). The authors showed that MSCs capable of spreading and generating relatively high levels of traction force on their adhesive contacts undergo osteogenesis; however, when the gel substrates were effectively locked into place on the fly through a secondary cross-linking strategy, traction forces were suppressed, gel degradation was impeded, and the cells differentiated into an adipogenic fate, despite being spread. The authors concluded that neither cell shape nor ECM elasticity per se were the underlying determinants of the cell fate decision, but rather the ability of the cells to generate tractional forces was key (Khetan et al. 2013). Recent studies have implicated the viscous properties of the

ECM in the control of cell spreading (Chaudhuri et al. 2015a) and MSC fate (Chaudhuri et al. 2015b), but cell sensing of creep and stress relaxation likely still requires cell-generated traction forces. If viscous features of the ECM influence cells of cardiovascular lineage remains to be determined.

11.3.2 Smooth Muscle Cells and ECM Mechanics

There is compelling evidence that SMCs sense and respond to their mechanical microenvironment. In a variety of cardiovascular pathologies, such as hypertrophy, hypertension, and atherosclerosis, the mechanical properties of blood vessels and small arteries are dramatically altered. However, it remains to be seen whether or not these mechanical alterations are solely a downstream consequence of earlier causal events, or whether or not they actively contribute to the onset of disease.

Using ECM-functionalized polyacrylamide substrates, SMC spreading has been shown to increase quantitatively with substrate elasticity (Engler et al. 2004; Peyton and Putnam 2005). The magnitude of spreading depended strongly on ECM ligand density (fibronectin) for SMCs cultured on soft substrates (polyacrylamide), but was invariant to these changes on rigid polystyrene controls, suggesting that matrix elasticity may override ligand density after some threshold is surpassed (Peyton and Putnam 2005). Beyond spreading, Wong et al. demonstrated that the direction of SMC migration could be controlled via patterned gradients in ECM elasticity (Wong et al. 2003), thereby exploiting the previously observed phenomenon of durotaxis (Lo et al. 2000). Prior work from my own laboratory demonstrated that SMC migration speeds depend on ECM elasticity in a nonlinear (i.e., biphasic) manner (Fig. 11.3) (Peyton and Putnam 2005). The value of the optimal substrate stiffness at which cell migration speed was maximized depended on the density of immobilized ECM ligand (fibronectin), suggesting a tight coupling between ECM chemistry and mechanics to control cell migration. While higher density of adhesive ligand shifted the optimal ECM elasticity to lower values, lower densities required higher elastic moduli to achieve maximal migration speeds (Peyton and Putnam 2005).

How SMCs respond to changes in ECM mechanical properties in 3D has been harder to assess, due to the coupling of ECM mechanics, chemistry, and microstructure in natural protein-based hydrogels noted previously. To overcome this limitation, my laboratory initially adapted synthetic hydrogels based on peptide-modified PEG gels, widely used and characterized as synthetic ECM analogs for tissue engineering applications. Due to their excellent protein resistance and the facile manner with which they can be covalently modified, PEG-based gels provide an ideal blank-slate template upon which key functionalities of the ECM can be conferred. However, SMCs do not survive long-term in nondegradable PEG gels, due in part to the very small pore size (Peyton et al. 2006).

To circumvent this issue, PEG hydrogels have been modified with proteolytically sensitive peptide sequences (Gobin and West 2002; Zisch et al. 2003; Raeber et al. 2005) or hydrolytically sensitive polymer blocks (Bryant and Anseth 2003).

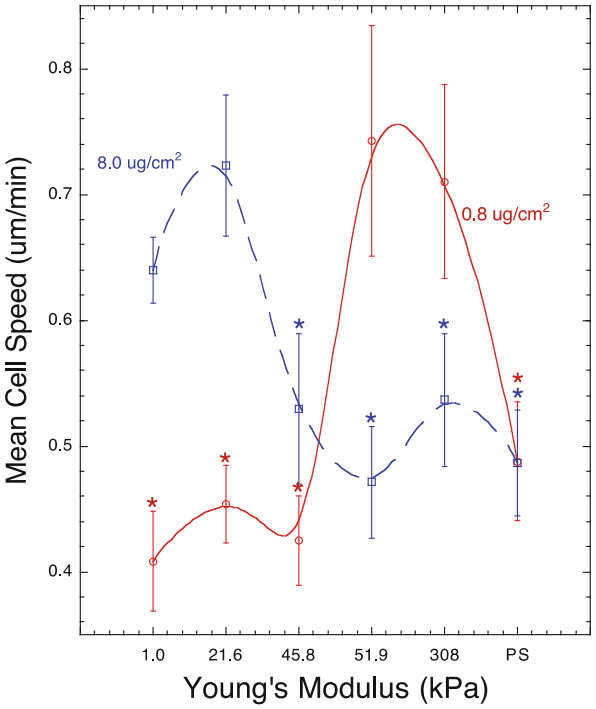

Fig. 11.3 Cell motility depends on ECM rigidity in a biphasic manner. In 2D culture, SMCs reach a maximum migration speed on substrates of intermediate stiffness. The value of this intermediate stiffness shifts to softer substrates as ligand density is increased. The *solid curve* shows migration speed on a polyacrylamide gel with 0.8 μg/cm^2 fibronectin, and the *dashed curve* represents a concentration of 8.0 μg/cm^2. *Asterisks* denote statistical significance from the maximum migration speed on respective fibronectin concentration. (Figure adapted from Peyton and Putnam 2005 with permission from John Wiley and Sons, Inc.: *Journal of Cellular Physiology*, 204;1, copyright 2005)

We adopted a previously described biosynthetic hydrogel platform (Almany and Seliktar 2005), in which full-length fibrinogen is reacted with PEG-diacrylate via thiol groups present in cysteine residues within the protein to enable independent control over the initial mechanical properties and adhesion ligand density presented to cells (Peyton et al. 2008). Using this material system, we demonstrated that SMC proliferation does not significantly depend on ECM compliance in 3D, which was somewhat surprising, given the prior results in 2D (Peyton and Putnam 2005). However, when SMCs were induced to express constitutively active RhoA, thereby increasing their traction forces, proliferation was attenuated significantly in 3D across all stiffness conditions in concert with distinct changes in the SMC cytoskeleton. The levels of smooth muscle-specific differentiation proteins were also found to depend on ECM rigidity in 3D, but only when RhoA was constitutively active (Peyton et al. 2008). Combined, these data implicate both ECM mechanics and RhoA-mediated tractional stresses in the cooperative regulation of SMC phenotype in a 3D environment.

In a subsequent study, we reported that SMCs cultured in 3D in this PEG-fibrinogen material adopted a contractile phenotype in the less cross-linked, more compliant gels (Kim et al. 2009a). When cultured in the PEG-fibrinogen gels in the presence of a myosin II inhibitor (blebbistatin), SMCs failed to spread and failed to express α-SMA, calponin, and smoothelin. These findings together confirm SMCs use traction forces to probe their mechanical microenvironment in 3D, but must be able to actively deform the ECM in order to upregulate the expression of hallmark markers of the contractile SMC phenotype (Kim et al. 2009a). These observations are consistent with the hypothesis SMC differentiation is favored by a more compliant vessel wall in vivo. However, for the application of a vascular graft, compliance has to be achieved in concert with sufficient strength to withstand the high pressures associated with the hemodynamic forces of the cardiovascular system.

Harnessing the SMCs' ability to generate traction forces and remodel their ECM has been exploited as a very effective strategy in vascular graft applications by enabling the cells to compact the ECM around a central mandrel to positively improve their mechanical properties (L'Heureux et al. 1993; Girton et al. 2000; Isenberg et al. 2006). The presence of the mandrel led to greater mechanical stiffness in the circumferential direction by inducing an increasing circumferential alignment of the SMCs and collagen, and by inducing a large stress on the SMCs, resulting in secretion and accumulation of stiffening components. This approach was subsequently applied to fibrin-based smooth muscle tissues, with similar improvements in mechanical properties (Grassl et al. 2002), and represents an excellent example of how fundamental mechanobiology understanding has been used to improve outcomes for engineered tissue constructs.

11.3.3 Endothelial Cells and ECM Mechanics

The ECM's mechanical influence on capillary morphogenesis is an area in which my laboratory and many others are interested (Califano and Reinhart-King 2010). Ingber and Folkman were arguably the first to propose a critical role for ECM mechanics on EC differentiation into tubular networks (Ingber and Folkman 1989; Ingber 2002). This work built on earlier 2D studies demonstrating that while rigid substrates resisted cell-generated forces and encouraged cell spreading, more malleable substrates facilitated cell-rounding (Ingber et al. 1985). They made the correlation to EC differentiation based on matrix density; by culturing capillary ECs on a rigid substrate, they were able to induce rapid tube formation by coating the cell culture plates with intermediate concentrations of either fibronectin or collagen type IV. High protein density led to cell spreading while low ECM density resulted in less attachment (and hence apoptosis) and decreased cell–cell contact, which is necessary for tube formation to occur (Ingber and Folkman 1989).

The role of substrate mechanical properties has also been more explicitly investigated by culturing human umbilical vein endothelial cells (HUVEC) on fibrin hydrogels of varying physicochemical properties. These studies showed that

HUVECs only form tube-like structures on fibrin gels of very low density (0.5 mg/mL), while bovine retinal ECs required a far more dense substrate to form capillary-like structures (8.0 mg/mL) (Vailhe et al. 1998). These findings suggest that the native microenvironment of a particular EC is a determinant in its behavior. Similar studies using ECM-modified polyacrylamide gels demonstrated that keeping the adhesive ligand density constant while increasing the stiffness of the substrate was sufficient to drive HUVEC away from a tube-like phenotype and towards a proliferative phenotype (Deroanne et al. 2001). Many subsequent studies have demonstrated that ECM mechanics influence EC phenotype and morphogenesis in 2D (Califano and Reinhart-King 2009; Pompe et al. 2009).

To more appropriately mimic the 3D microenvironment experienced by cells in vivo and explore the effect of matrix mechanical properties on capillary morphogenesis, Nehls and Herrmann created a system in which microcarrier beads coated with ECs are embedded within fibrin gels. By varying the physicochemical properties of the matrix via pH changes, they demonstrated that relatively rigid gels supported uncoordinated EC migration while the malleable gels, polymerized at higher pH, supported formation of microvessels (Nehls and Herrmann 1996). Subsequent studies support the notion that increasing fibrin gel stiffness by increasing cross-linking density via Factor XIII results in reduced capillary network formation (Urech et al. 2005). Others have used collagen to determine whether floating gels are better able to support capillary morphogenesis than constrained gels. While mechanically constrained collagen gels led to a slightly lower length of the capillary-like structures that formed, the constrained gels demonstrated significantly higher average lumen area, which was attributed to the inability of ECs to remodel and contract these matrices vs. their floating counterparts (Sieminski et al. 2004). Collagen hydrogels have also been used to demonstrate that neighboring colonies of ECs produce tractional stresses that remodel the fibrillar network and cause directional sprouting of neighboring colonies towards each other (Korff and Augustin 1999). Similar studies using floating versus attached 3D gels have shown that fibroblast proliferation depends on gel contraction in part due to changes in the activity of the PDGF receptor, suggesting cross-talk between substrate mechanics and growth factor signaling (Lin et al. 1998).

Studies in my laboratory using 3D fibrin gels demonstrated that increasing ECM density significantly attenuates capillary sprouting in the in vitro model system originally pioneered by Nehls and Hermann (Fig. 11.4a) (Ghajar et al. 2006, 2008). We subsequently showed similar effects in vivo (Kniazeva et al. 2011). While these increases in fibrin density trigger significant changes in ECM mechanical properties, they also significantly alter permeability and thereby influence the diffusive transport of soluble morphogens (Ghajar et al. 2008). It is thus impossible to isolate mechanical effects in a protein-based hydrogel. Nevertheless, we have shown that EC-generated traction forces are required for capillary morphogenesis in 3D (Kniazeva and Putnam 2009), and that capillary growth in 3D is optimized in fibrin gels that can be maximally deformed by the ECs (Kniazeva et al. 2012). Our initial efforts to link ECM mechanical properties, EC traction forces, and morphogenesis relied on bulk mechanical measurements due to the inability to quantify mechanical

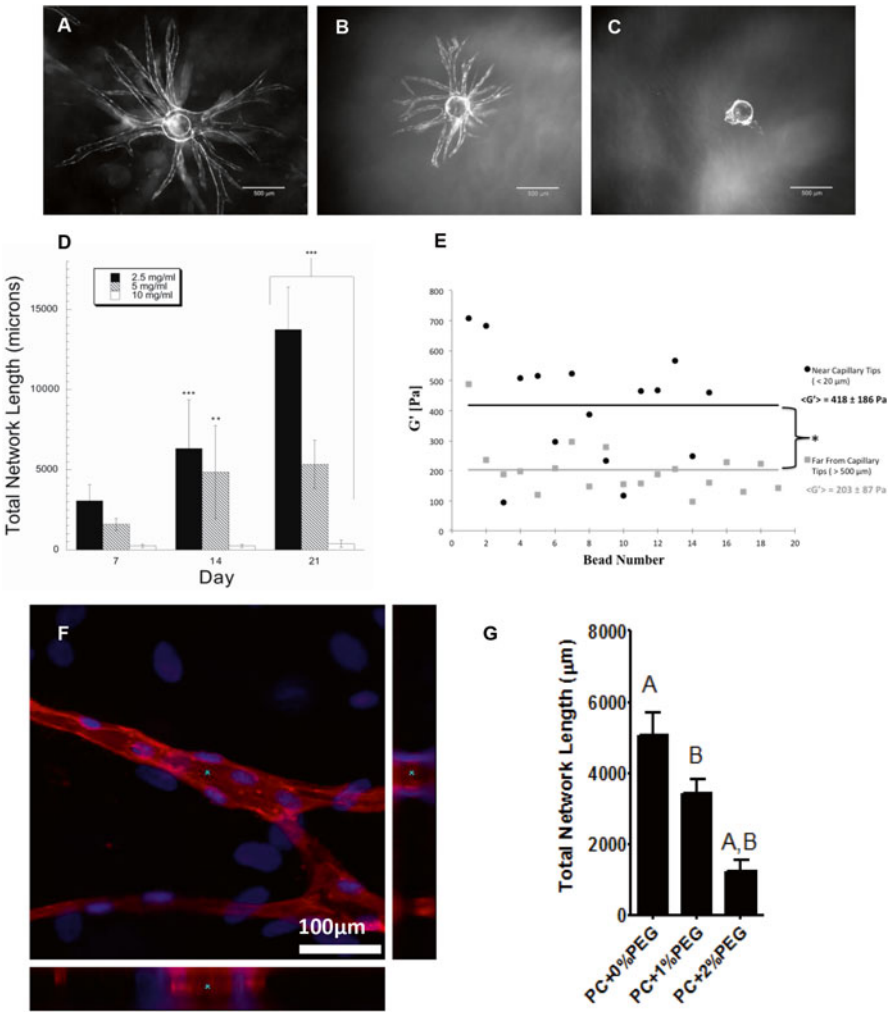

Fig. 11.4 ECM mechanical properties influence endothelial sprouting in 3D. (**a**–**d**) In a 3D model of capillary morphogenesis, the extent of EC differentiation into capillary-like tubes decreases with increasing fibrin concentration. Shown are representative images after 14 days of culture in (**a**) 2.5, (**b**) 5, and (**c**) 10 mg/mL fibrin (Scale bar, 500 mm), along with (**d**) quantification over a 21 day time course. (Adapted from Ghajar et al. 2006 with permission from Mary Ann Liebert, Inc: *Tissue Engineering*, 12;10, copyright 2006.) (**e**) Active microrheology measurements in the same sprouting assay show that ECs local stiffen the ECM by a factor of two at the tips of sprouts. (Adapted from Kniazeva et al. 2012 with permission from the The Royal Society of Chemistry: *Integrative Biology*, 4;4, copyright 2012.) (**f**, **g**). Similar experiments in synthetic PEG-based gels demonstrate that increased cross-linking, which results in an increased elastic modulus, significantly impedes EC morphogenesis. (Adapted from Singh et al. 2013 with permission from Elsevier: *Biomaterials*, 34;37, copyright 2013)

properties on a length scale relevant for cells. However, even macroscopically homogeneous protein hydrogels exhibit significant mechanical heterogeneities (Kotlarchyk et al. 2011), which may be important in directing morphogenesis. Therefore, using active microrheology methods, we have demonstrated that ECs locally stiffen the ECM by a factor of two as they sprout in fibrin (Fig. 11.4b) (Kniazeva et al. 2012).

Given the difficulty in isolating the ECM's mechanical influence in natural protein-based hydrogels, my research group (Singh et al. 2013; Vigen et al. 2014) and others (Hanjaya-Putra et al. 2011, 2012; Sokic and Papavasiliou 2012; Turturro et al. 2013; Moon et al. 2010; Miller et al. 2010) have adopted synthetic materials. These platforms enable independent control over mechanical properties, adhesive ligand type and identity, and proteolytic susceptibility, and thereby permit each of these parameters and their influence on cell fate to be examined in a systematic fashion. However, it is important to note these types of synthetic materials are amorphous, and lack the fibrillar architecture of native matrices which appears to be important in mechanosensing (Baker et al. 2015). Furthermore, increasing cross-linking to increase the mechanical properties of these gels can also influence swelling and diffusive transport, and thus it is important to define the range of properties for which transport is unaffected when attempting to isolate mechanobiological events. In our studies, increasing the cross-linking of PEG-based hydrogels without significantly affecting diffusive transport led to significant reductions in capillary morphogenesis (Fig. 11.4c, d) (Singh et al. 2013; Vigen et al. 2014). When combined with observations from natural protein hydrogels, these data strongly support a role for EC-generated tractional forces, and the ECM's mechanical resistance to those forces, in capillary morphogenesis.

11.3.4 Cardiomyocytes and ECM Mechanics

CMs also respond to the intrinsic mechanical properties of their microenvironment, at least in vitro (Tallawi et al. 2015). One study demonstrated that ECM elasticity altered the transmission of contractile work from embryonic CMs (from quail) to the ECM (Engler et al. 2008). This is particularly relevant in the context of cardiac regenerative medicine as it suggests that ECMs that mimic the elasticity of the developing myocardium are optimal for transmitting work from the contractile cells to their microenvironment and for promoting a beating phenotype. Both beating frequency and the number of beating cells dropped off precipitously when ECM elasticity was increased beyond the ~10 kPa values of native myocardium. Jacot et al. reported similar findings, showing that neonatal rat ventricular CMs cultured on collagen-coated polyacrylamide substrates with an elastic modulus of 10 kPa developed aligned sarcomeres, generated greater mechanical force than on stiffer or softer substrates, and had the largest calcium transients, sarcoplasmic calcium stores, and sarcoplasmic/endoplasmic reticular calcium ATPase-2a expression (Jacot et al. 2008). Collectively, these studies suggest that a biomaterial whose

elasticity matches that of the myocardium may support a functional CM phenotype better than stiffer or softer materials. A role for ECM mechanics in cardiac development and regeneration in vivo has been more difficult to discern, but it is clear that cell-generated forces are involved in cardiogenesis (Patwari and Lee 2008). Defects in the force-generating machinery of CMs manifest as developmental defects both in model organisms and in humans (Granados-Riveron and Brook 2012). Furthermore, it has also been hypothesized that the altered mechanical environment of the fibrotic scar impedes cell-based regenerative strategies (Berry et al. 2006).

While most cardiac regenerative approaches involve delivery of cells, an exciting recent development in the literature is the concept of direct lineage reprogramming, which would conceivably enable scar-forming cardiac fibroblasts to be transdifferentiated into functioning CMs. This type of cell reprogramming was once thought to be impossible since normal adult somatic cells have reached the end of the developmental process and are extremely stable. However, in the 1960s, it was shown that transplantation of an amphibian adult cell nucleus into the cytoplasm of an unfertilized egg could restore the adult cell's nucleus to a pluripotent state, a process known as somatic cell nuclear transfer (Gurdon and Uehlinger 1966). A simpler strategy published in 1996 showed that fibroblasts could be transdifferentiated into skeletal myoblasts in vitro or in the injured heart by overexpressing a single gene encoding for MyoD, a potent myogenic transcription factor (Murry et al. 1996). Yamanaka and colleagues showed that pluripotency could be induced in somatic cells via reprogramming (Takahashi et al. 2007; Takahashi and Yamanaka 2006), a discovery which led to the 2012 Nobel Prize in Physiology or Medicine and laid the foundation for the current explosion in reprogramming research. Transdifferentiation has now been demonstrated in several cell types, including the reprogramming of pancreatic exocrine cells to β-cells (Zhou et al. 2008), fibroblasts to muscle cells (Choi et al. 1990), lymphocytes to macrophages (Xie et al. 2004), and fibroblasts to neurons (Vierbuchen et al. 2010). Reprogramming adult cells appears to require reactivation of embryonic genes that are expressed during early organ development, a conserved principle also exhibited in regeneration in lower vertebrates (Tanaka 2003; Brockes and Kumar 2002).

In the case of cardiac reprogramming, several recent papers suggest reason for optimism. In 2010, Ieda et al. used a creative approach to screen 14 candidate transcription factors to activate a cardiac-specific transgene in cardiac fibroblasts (Ieda et al. 2010). A systematic approach narrowed the original 14 factors down to just 3 (Gata4, Mef2c, and Tbx5; GMT), which when delivered to fibroblasts via viruses generated induced cardiomyocytes (iCMs) after 2–4 weeks in culture (markers of CMs appeared in <1 week). The iCMs possessed a CM-like gene expression profile and beat like functional CMs. A 2011 paper by Efe et al. reported a multi-step method to reprogram mouse embryonic fibroblasts into iCMs by starting with the Yamanaka factors to initiate reprogramming, followed by the addition of the cardiogenic morphogen BMP-4 (Efe et al. 2011). This approach yielded only minimal iPSCs, minimizing any potential teratogenic effects of the cells, and generated beating iCM colonies within just 2 weeks in culture. In 2012, back-to-back papers in *Nature* showed that mouse cardiac fibroblasts could be directly reprogrammed in

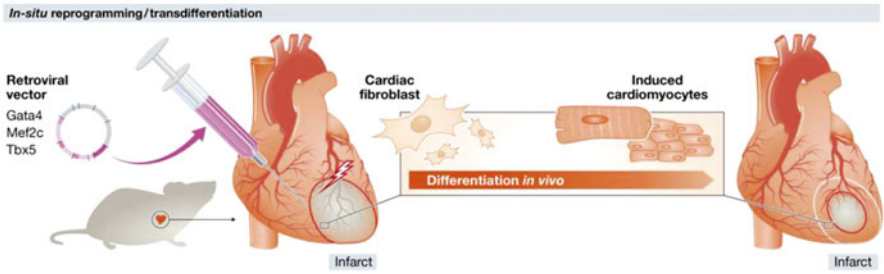

Fig. 11.5 Reprogramming in situ as a strategy for cardiac regeneration. This cartoon schematic depicts the concept of direct lineage reprogramming, in which cardiac regulator genes are delivered in vivo directly to cardiac tissue surrounding an infarct. In this strategy, cardiac fibroblasts express the transgenes and transdifferentiate into functional cardiomyocytes, thereby reducing the size of the original infarct and partially (or perhaps completely) restoring cardiac function. A role for the microenvironment in this process has been suggested, but not fully investigated. (Reproduced from DeWitt and Trounson 2012 with permission from John Wiley and Sons: *EMBO Journal,* 31;10, copyright 2012)

situ following an MI via viral delivery of either GMT or GMT+HAND2, yielding significant functional benefits in cardiac work (Fig. 11.5) (Qian et al. 2012; Song et al. 2012).

While most of the attention in the reprogramming literature has focused on the role of specific genetic factors and the epigenetic state of the cells, it was observed that reprogramming efficiencies are significantly higher in vivo than in vitro (Qian et al. 2012), suggesting that elements of the cardiac microenvironment influence the reprogramming process. To better understand the key features of the cellular microenvironment that influence this process, a study from my laboratory by Kong et al. used simple hydrogel substrates made from naturally derived proteins and an indirect cardiac reprogramming protocol that utilizes the same 4 factors originally used to create iPS cells (Kong et al. 2013a). We discovered fibrin and fibrin-collagen composite substrates supported cardiac reprogramming much more efficiently than either pure type-I collagen or Matrigel substrates, yielding beating cardiomyocyte-like cells from fibroblasts with greater efficiency (Fig. 11.6). Cell-generated traction forces also played major roles in reprogramming the cells into CMs, with large traction forces induced through the expression of constitutively active RhoA leading to decreased dedifferentiation and cardiogenic efficiency, while reduced traction forces through the forced expression of dominant-negative RhoA increased dedifferentiation and led to more progenitor-like colonies. Systematically varying the concentration of the protein matrices and characterizing their physical properties revealed that the ECM's mechanical properties and microstructure play lesser roles in the reprogramming process, as does the degree of cell proliferation. These findings suggest that optimization of the mechanical microenvironment may influence the efficiency of reprogramming in situ, an idea supported by recent findings that inhibition of the RhoA/ROCK pathway significantly improved the efficiency of reprogramming fibroblasts into CMs (Zhao et al. 2015).

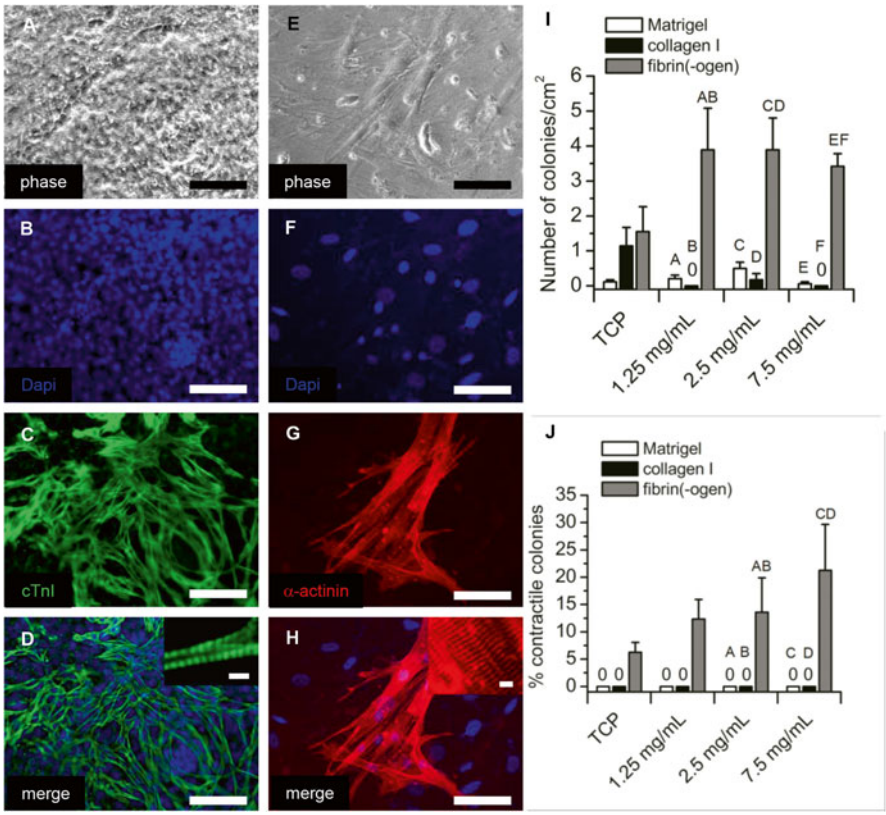

Fig. 11.6 Fibrin gels support indirect cardiac reprogramming better than Matrigel or type-I collagen I gels. (**a**–**h**) Mouse embryonic fibroblasts (MEFs) were reprogrammed using lentiviral delivery of an inducible polycistronic vector encoding for Oct4, Sox2, Klf4, and cMyc (OSKM) and then treated with BMP-4. Within a period of 14 days, the MEFs cultured on compliant fibrin gels showed morphological changes (**a**, **e**) consistent with a mesenchymal-to-epithelial transition and formed colonies capable of significant contraction with much higher efficiency than did those cultured on Matrigel, type-I collagen, or tissue culture plastic. Nuclei in representative colonies (**b**, **f**) were observed by staining with DAPI. Reprogrammed colonies stain positive for cardiac troponin I (**c**, **d**) and α-actinin (**g**, **h**), and show a characteristic striation pattern consistent with cardiomyocytes. [Scale bars = 100 μm (5 μm for the *insets* in panels (**d**) and (**h**)).] (**i**, **j**) Quantification of the total numbers of colonies per cm^2 (**i**) and percentage of contractile colonies (**j**) obtained from indirect cardiac reprogramming on Matrigel, type-I collagen, and fibrin gels of various protein concentrations shows that fibrin better supports reprogramming. (Figure adapted from Kong et al. 2013a with permission from Macmillan Publishers Ltd.: *Scientific Reports*, 3, copyright 2013)

11.4 Control of Cell Fate by ECM Topography

In addition to the instructive role of applied mechanical forces and intrinsic mechanical properties, a large number of studies over the past 10–15 years have investigated the effects of physical topographical features (e.g., lines, gratings, holes,

pillars) and/or chemical topographical features (e.g., "tracks" or "islands" of printed or adsorbed ECM proteins). The common rationale to pursue this line of inquiry is that native ECM contains nanoscale topographies, and thus features of similar size on engineered substrates may better mimic the native ECM (Kim et al. 2012). In many cases, cells sense topographic cues in the cellular environment via the same mechanisms as mechanical content. Given the overlap with mechanobiology, I have elected to give this topic some treatment here in this chapter.

Numerous studies linking nanoscale physical topographies with cell adhesion and morphology have appeared in the literature in the past decade (Biela et al. 2009; Diehl et al. 2005; Janson et al. 2014; Karuri et al. 2004; Kim et al. 2009b; Teixeira et al. 2004; Yim et al. 2005). Early examples from the literature used substrates with various nanoscale features to investigate the adhesive characteristics of fibroblasts and ECs (Curtis et al. 2001; Dalby et al. 2002a, b, c). An abundance of evidence has shown that nanotopography can influence cell shape/morphology, for example, with cells cultured on nanogrooves (often called nanoridges or nanogratings) that have large axial dimensions (~ mm) and nanoscale lateral dimensions, typically with periodic patterns of variable ridge height and width. Cells of many different origins readily align parallel to these grooved substrates (Janson et al. 2014; Kim et al. 2009b, 2010, 2013; Yim et al. 2005; Curtis et al. 2001; Jain and von Recum 2003; Lamers et al. 2010; Watari et al. 2012). One study suggested that groove depth plays a central role in cells' sensitivity to nanotopographic ridges (Teixeira et al. 2003). However, whether such physical nanotopographic cues can be more important than chemical cues remains unknown. In the context of microtopography, a prior study created both physical and chemical features to investigate pre-osteoblast alignment using a polymeric base surface coated with titanium and gold with micron sized gratings. Microcontact printing was utilized to imprint fibronectin lanes either parallel or perpendicular to the underlying physical surface. Despite a perpendicular adhesive protein cue, cells in this case preferentially aligned with the underlying physical topography (Charest et al. 2006).

There is also increasing evidence that micro- and nanotopographies influence cell migration (Diehl et al. 2005; Brammer et al. 2008; Ranucci and Moghe 2001; Mello et al. 2003; Tan and Saltzman 2002). One study demonstrated that nanogratings alter the polarization of SMCs in a wound healing migration assay, with orientation of the microtubule-organizing center towards the wound on unpatterned surfaces and along the axis of cell alignment in cells cultured on patterns (Yim et al. 2005). Another study used micropatterned chemical topography to compare the responses of multiple cell types in 3D matrices, on 2D surfaces, and on "1D" lines (1–10 μm width) coated with various ECM proteins (fibrinogen, vitronectin, and fibronectin) (Doyle et al. 2009). Fibroblast adhesion and spreading on the 1D lines were similar to their behavior in 3D. Knockdown of the small GTPase Rac in cells cultured in 2D produced an elongated cell morphology similar to that observed on the 1D substrates. However, the migration speeds of the Rac knockdown cells did not increase, and vinculin staining of these cells revealed that their adhesions were still distinct from those observed for the 1D and 3D cases.

Physical nanotopography may also influence cell proliferation and differentiation. One of the earliest and most highly cited papers reported enhanced alkaline phosphatase activity and extracellular calcium deposition for rat osteoblasts cultured on nanophase ceramics (Webster et al. 2000). Experiments documenting MSC response to nanotopographies appeared a few years later (Dalby et al. 2006a, b). In one study with MSCs, arrangements of 120-nm diameter, 100-nm deep nanopits in PMMA that were asymmetric and more disordered (i.e., deviated from perfectly square or hexagonal arrays) were found to enhance the expression of osteogenic genes and proteins, even in the absence of soluble osteogenic supplements (Dalby et al. 2007). The same group of investigators later demonstrated that regular square arrays of these nanopits embossed in polycaprolactone promote MSC stemness (McMurray et al. 2011). MSCs grown on gelatin-coated poly(urethane acrylate) nanogratings also reportedly upregulate osteogenic gene expression compared to cells on control surfaces (You et al. 2010), as do titanium oxide nanotubes (Oh et al. 2009). An ambitious study recently described an approach to fabricate a library of 2176 distinct, randomly designed surface topographies on poly(DL-lactic acid) and used high-content imaging to identify formerly unknown surface nanotopographies capable of inducing MSC proliferation or alkaline phosphatase (ALP) expression (as a surrogate for osteogenic differentiation) (Unadkat et al. 2011). Others have recently shown that spatial patterning of different nanotopographies on the same surface can be used to spatially control the switch between adipogenesis and osteogenesis in MSCs (Ahn et al. 2014). SMC shape and proliferation have similarly been regulated by micropatterning approaches (Thakar et al. 2003, 2009).

Several recent studies examined the role of nanotopography in the maintenance of human embryonic stem cells (hESCs) (Chen et al. 2012; Kong et al. 2013b; Lapointe et al. 2013; Lu et al. 2014). One study found that hESCs better retained their expression of Oct3/4 (a transcription factor and characteristic marker of undifferentiated ESCs) when cultured on smooth surfaces than nanoroughened ones (Chen et al. 2012). However, another study showed that hESC expression of Oct4 was better maintained by culturing the cells on polystyrene nanopillar arrays with either regular hexagonal or honeycomb lattice arrangements relative to those cultured on smooth surfaces (Kong et al. 2013b). Another recent study supported the former idea that nanoscale topography can reduce Oct4 expression and drive differentiation of ESCs (Lapointe et al. 2013).

CMs have also been shown to be responsive to ECM topography. In one particular example, PEG hydrogels were patterned with nanotopography via a UV-assisted lithography method, and covalently functionalized with fibronectin (Fig. 11.7a) (Kim et al. 2010). Neonatal rat ventricular myocytes cultured on these nanotopographic substrates not only aligned parallel to the topography (Fig. 11.7b, c), but impressively displayed anisotropic action potential propagation reminiscent of native myocardium to a greater degree than cells cultured on unpatterned substrates and elevated connexin-43 expression. The authors also showed evidence that the cells penetrated into the nanogratings (Fig. 11.7d, e), and attributed the enhanced myocyte function in part to the increased adhesion between cells and the patterned

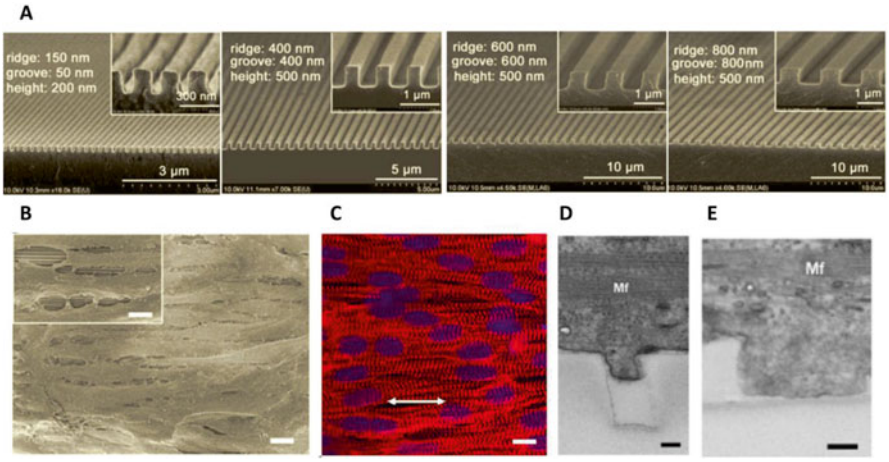

Fig. 11.7 Topography influences alignment of cardiac myocytes. (**a**) SEM micrographs of poly(ethylene glycol) (PEG) gels with nanotopography ranging from 50 to 800 nm in size. (**b**) SEM image of neonatal rat ventricular cardiac myocytes grown on fibronectin-coated nanopatterned PEG gel substrates show cells aligned with the underlying nanotopography. *Inset* shows transverse intercellular connections (scale = 5 μm). (**c**) Immunofluorescent image of sarcomeric α-actinin (*red*) and nuclei (*blue*) observed in cardiac myocytes grown on nanopatterned PEG gel substrates (scale = 10 μm). (**d**, **e**) SEM micrographs illustrate that cells penetrate into nanometer grooves; "Mf" depicts myofilaments (scale = 200 nm). (Adapted from Kim et al. 2010 with permission from the National Academy of Sciences: *PNAS*, 107;2, copyright 2010)

substrates. When the patterned PEG hydrogels were embedded with beads used as fiduciary markers to characterize cell-generated traction forces, the CMs generated contractile forces that were highly aligned with the topography. As the feature size became smaller and the substrates approached a non-patterned environment, the beneficial effects of topography disappeared (Kim et al. 2010). Similarly, Feinberg et al. used micropatterned surfaces to create regularly spaced (20 μm), parallel arrays of multicellular cardiac muscle fibers (Feinberg et al. 2012). Aligning sarcomeres via this substrate patterning led to increased peak systolic stress, suggesting that CM force generation can be controlled via topographic patterning. Another innovative approach used hydrogel-molding techniques to template the 3D geometry of cardiac tissue patches by controlling the spatial pattern of mechanical tension (Bian et al. 2009). A subsequent study demonstrated that patches fabricated in this manner enabled human ESC-derived CMs to attain a more mature and functional phenotype after 2 weeks of culture, with significantly higher conduction velocities, longer sarcomeres, and enhanced expression of genes encoding for cardiac contractile proteins compared to ESC-CMs cultured in 2D (Zhang et al. 2013). Finally, Morez et al. recently reported that parallel microgroove topography significantly enhanced reprogramming of cardiac progenitor cells into CM-like cells via lentiviral delivery of myocardin, Tbx5, and Mef2c (Morez et al. 2015). Collectively, these studies indicate that ECM topography as a way to control cell shape and geometry provides a powerful instructive cue to CMs.

11.5 Conclusions and Perspective

Slowly but surely, the application of fundamental mechanobiology knowledge is beginning to have clinical impact in cardiovascular medicine, and will undoubtedly influence the implementation of regenerative medicine in the future. A multitude of instructive signals within the cellular microenvironment, including applied mechanical forces, intrinsic ECM mechanical properties, and topography, offer potential knobs that can be tuned to achieve better functional outcomes through mechanotransduction. The consensus regarding ECM elasticity is that biomaterials whose mechanical properties more closely match those of native cardiovascular tissue are likely to achieve better functional outcomes. Similarly, applying physiologic mechanical forces, such as cyclic strain and fluid-induced shear, to better mimic the native tissue environment appears to be a generalized theme. Finally, controlling cell shape or creating anisotropy in cardiac tissue constructs via material topography in a manner that more closely mimics native cardiac tissue may support electrical coupling and achieve superior outcomes.

Nevertheless, many of the important papers cited here related to applications of mechanobiology have focused on somatic cells or MSCs; far fewer studies have investigated the role(s) of these microenvironmental features on human ESCs, iPSCs, or other progenitor cells of cardiovascular relevance. Such studies are needed to better define the parameter space available for cardiovascular applications, especially if pluripotent cell types are eventually translated into human applications. Furthermore, it remains unclear if parameters like ECM elasticity and topography do in fact have significant impact upon cell fate decisions in vivo as they do in vitro.

Current challenges in vascularization continue to limit nearly all tissue engineering strategies, and represent a particularly acute problem in cardiac applications, given the high metabolic demands of CMs. Strategies to preform vascular networks within cardiac patches have shown promise in small animal models (Stevens et al. 2009), but the clinical scalability of such approaches for humans must be addressed. Beyond the need to provide the oxygen and nutrients required for proper cardiac function, vasculature may also provide key angiocrine signals to actively maintain and/or instruct progenitor cells resident in the heart and elsewhere in the body.

Other significant challenges must be overcome to translate fundamental mechanobiology discoveries into clinical products. Bioreactors that apply appropriate mechanical stimuli have yet to be standardized, which poses challenges from the perspective of regulatory and manufacturing considerations for engineered tissues. For example, will dozens (perhaps hundreds or even thousands) of tissue engineered vascular grafts need to be mechanically stimulated in parallel via a standardized bioreactor for weeks or months in an FDA-approved manufacturing process for widespread use in humans? Will these processes be largely automated, in much the same way that automobiles are manufactured? Process development and scale-up represented a huge piece of the successful commercialization of biotechnology products in the 1980s, with entire facilities devoted to scaling fermentation and cell

culture processes from the bench (<1 L) to manufacturing sizes (>10,000 L), enabling for the manufacture of reproducible products that withstand rigorous quality controls and assurances. The burgeoning regenerative medicine industry has yet to significantly address the scale-up challenge, instead mostly relying on contract manufacturing organizations to date. Similarly, the wide variety of boutique biomaterials, and the lack of standard synthesis protocols and characterization metrics, continues to provide a barrier to scale-up and GMP-compliant manufacturing. Three-dimensional printing strategies have also generated a great deal of enthusiasm and buzz, but significant hurdles associated with this approach must also be overcome to translate the technique into clinical products.

Beyond these technical challenges, the appetite of investors (e.g., large biotech and pharmaceutical companies, venture capitalists) for regenerative medicine product development remains a challenge, especially since many of the needs are not particularly headline grabbing or sexy from an investment perspective. Increasingly the burden is on academic investigators to translate their fundamental science discoveries into products tested in human clinical trials, traversing the entire spectrum from in vitro cell culture models, small animals, large animals, and human patients before attracting enough interest from potential investors for a new technology to be developed and/or licensed. Most universities lack GMP-qualified facilities for scale-up and commercialization of new technologies, and thus the bootstrap efforts of academia often fail to bring promising technologies across the so-called valley of death. Nevertheless, we are beginning to see exciting successes from industry start-ups in the cardiovascular space, including companies like Humacyte, Cytograft, and Lone Star Heart, among others. Thus, despite the significant challenges, there is reason to be optimistic that mechanobiology will impact clinical applications in cardiovascular medicine in the coming decades.

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Chapter 12
Heart Valve Mechanobiology in Development and Disease

Aileen Zhong and Craig A. Simmons

Abstract Heart valves reside in one of the most mechanically demanding environments within the body, experiencing over 100,000 cycles daily of a combination of biomechanical and hemodynamic forces. The forces applied to heart valves are critical for proper valvulogenesis and normal valve function and maintenance, but disruptions in the mechanical environment can lead to developmental defects and disease. In this chapter, we review current understanding of the roles of hemodynamic forces in valve development, from the initiation of valvulogenesis by cardiac jelly formation, to the invasion of cells into the cardiac cushion through the process of endothelial-to-mesenchymal transition (EndMT) and subsequent remodeling of the extracellular matrix to give rise to the tri-layered structure of developed valves. We also review growing evidence that implicates shear stress, cyclic strain, and matrix mechanics in regulating the initiation and progression of calcific aortic valve disease (CAVD), the most common adult valve disease for which there currently is no medical therapy. An improved understanding of how mechanical forces regulate valve development and disease is expected to help identify therapeutic targets for the treatment of adult valve diseases and to guide the design of living tissue replacement valves for patients with congenital valve defects or diseased valves.

Keywords Cardiac valve • Hemodynamics • Valvulogenesis • Aortic sclerosis • Aortic valve • Bicuspid valve

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12.1 Introduction

The human heart contains four valves that passively open and close with every heartbeat to maintain unidirectional blood flow within the heart. While their role is purely mechanical and relatively simple, it is essential to efficient heart function and cardiac health. To accomplish this for a lifetime is no small feat: heart valves open and close approximately 100,000 times per day while withstanding blood flow-induced shear stresses and tissue deformations that, for the aortic valve, are among the most extreme of any tissue in the body. Unsurprisingly, these forces can influence the function of the cells that produce valves during embryogenesis, repair and maintain the valve throughout life, and contribute to valve disease. Insights from mechanical regulation of valve development and disease may suggest therapeutic targets and inform strategies to engineer living tissue replacements for congenitally defective or diseased heart valves. In this chapter, we review the current understanding of valve mechanobiology, with focus on the role of hemodynamic forces in regulating valve development and the most common heart valve disease, calcific aortic valve disease (CAVD).

12.2 Mechanobiology of Heart Valve Development

In vertebrate embryos, the heart is the first organ to function. The vertebrae heart starts off as a linear tube of myocardial cells lined on the inner surface with a layer of endocardial cells. The tube undergoes several folding steps and remodeling to form the mature chambered heart (Butcher and Markwald 2007). In human embryos, the myocardium initiates heart beating at approximately 3 weeks to generate blood flow to deliver nutrients to surrounding tissues and to drive morphogenesis of tissues (Hinton and Yutzey 2011). As the structure of the heart becomes more defined, valve development is necessary to maintain unidirectional flow.

Notably, proper valve morphogenesis depends on the biomechanical and hemodynamic forces generated by the pumping of the heart and the resultant flow of blood. This was shown by Hove et al., who analyzed the effects of blood flow-related mechanical forces on cardiogenesis in zebrafish, which are transparent to enable blood flow visualization in vivo (Hove et al. 2003). Blood flow was blocked by surgically placing microbeads at either the inflow or the outflow tract (OFT) of the heart tube. This manipulation, which reduced wall shear stress by ~10-fold, disrupted valvulogenesis, cardiac morphogenesis, and cardiac function, suggesting a role for blood flow-induced shear stress in development (Fig. 12.1). In an alternate approach, Bartman et al. studied the effect of myocardial contraction on valvulogenesis by inhibiting myocardial function pharmacologically or by using silent heart ($sih^{-/-}$) mutant mice, which have a mutation in the cardiac troponin T (*tnnt2*) gene (Bartman et al. 2004). When the contraction of myocytes was blocked, valvulogenesis failed. Whether valvulogenesis is regulated mainly by shear stress or

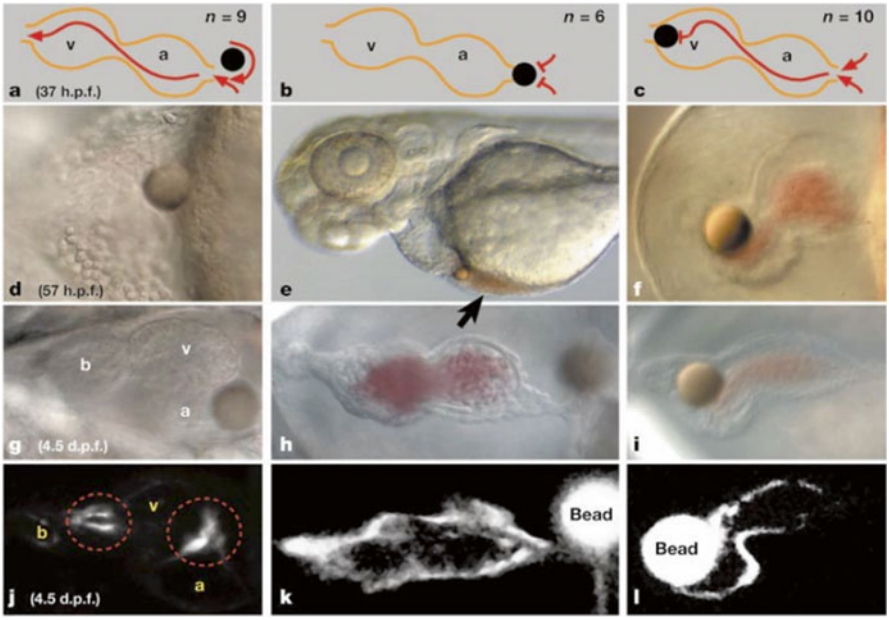

Fig. 12.1 Blood flow is critical in cardiogenesis and valvulogenesis. Microbeads were surgically placed near the inflow tract as control (**a**) and at the inflow tract (**b**) or outflow tract (**c**) of the heart to impair blood flow. (**d–f**) Blood accumulation was assessed by the presence of erythrocyte accumulation. Accumulation of erythrocytes was visible in front of the atrium (**e**) and inside the chambers of the heart (**f**) in embryos with successful microbead implantation. (**g**, **j**) Heart chamber and valve development was unaffected in control embryos where blood flow was undisrupted. Implantation in both the inflow (**h**, **k**) and outflow (**i**, **l**) tracts of the heart disrupted normal blood flow and resulted in abnormal heart chamber and valve development. From Hove et al. (2003)

tissue contraction could not be determined by these studies, as both manipulations induced confounding effects: blocking flow with beads affected myocardial function and prevented heart looping, and inhibition of myocardial function reduced wall shear stresses. Nonetheless, together they clearly demonstrated that valve development is mechanically regulated.

Subsequent insight into mechanoregulation of valve development has been enabled by a variety of model systems, including mice, chicks, and zebrafish (Lindsey and Butcher 2011) and cell culture models. As mentioned previously, zebrafish and chick models are particularly valuable to study the developmental role of hemodynamics since they provide high optical advantages and accessibility (Groenendijk et al. 2005; Vermot et al. 2009). When combined with tools like in vivo imaging of blood flow and computational fluid dynamics (CFD) modeling (Hove et al. 2003; Peterson et al. 2012), characterization of the dramatic changes in embryonic hemodynamics that occur during the course of development as the heart tube changes its structure to a four-chambered heart and the heart rate and cardiac output increase to accommodate the increasing needs of the embryo becomes

possible. As summarized below, spatial and temporal correlation of hemodynamic microenvironments and with key events during valve morphogenesis has provided insights into the cellular and molecular factors involved in mechanical regulation of the cardiac jelly formation to initiate valvulogenesis; invasion of cells into the cardiac cushion through endocardial-to-mesenchymal transition (EndMT); and synthesis and remodeling of the ECM to the laminar structure of fully developed valves.

12.2.1 Initiation of Embryonic Valve Formation

Valvulogenesis initiates approximately 3 weeks after conception in humans (Hinton and Yutzey 2011), embryonic day 8.5 (E8.5) in mice (Savolainen et al. 2009), Hamburger-Hamilton (HH) stage 16 in chicks (Yalcin et al. 2011), and 37 h post fertilization (hpf) in zebrafish (Beis et al. 2005). The initiation of valvulogenesis begins with the secretion by the myocardium of cardiac jelly, a glycosaminoglycan (GAG)-rich extracellular matrix (ECM) with an abundance of hyaluronan, between the myocardial and endocardial layers. Local secretion of cardiac jelly creates swellings known as the endocardial cushions (Person et al. 2005). The cushions occur in regions of the OFT and the atrioventricular canal (AVC) that mark the position of future semilunar valves and atrioventricular valves, respectively.

Notably, the location of the endocardial cushions correlates spatially with distinct hemodynamic environments flow within the developing heart tube (Banjo et al. 2013; Hove et al. 2003; Peterson et al. 2012; Vermot et al. 2009). Focal disturbances of the hemodynamic environment within the heart tube are due in part to contraction of the myocardium producing local constrictions of the heart tube prior to presence of the valves. Contractions cause regions of high wall shear stress (2.5–10 dynes/cm^2) within the AVC (Hove et al. 2003) and outer curvature of the OFT (Peterson et al. 2012). In zebrafish, unsynchronized contraction waves have also been shown to produce oscillatory flow around the region in the AVC where the cushion forms (Heckel et al. 2015; Hove et al. 2003). A variety of mechanosensitive molecules have been implicated in regulating shear-dependent cushion formation, including microRNA (*miR*)-*21* (Banjo et al. 2013) and the shear-sensitive genes Krüppel-like lung factor 2a (*klf2a*) (Heckel et al. 2015; Vermot et al. 2009) and endothelial nitric oxide synthase (*NOS3*/eNOS) (Groenendijk et al. 2005; Richards et al. 2013), which are elevated in regions of high shear stress, and endothelin-1 (*edn1*), which is downregulated in high shear stress regions (Groenendijk et al. 2005). For example, in zebrafish *miR-21* expression is present at 36 hpf in the AVC and at 48 hpf in the OFT and the level of expression increases as development progresses; however, when blood flow is inhibited, *miR-21* expression is not present at 48 hpf in the AVC or OFT and the valves failed to develop (Banjo et al. 2013). Perhaps the more potent mechanostimulus for valve development is not the magnitude of the wall shear stress, but rather its oscillatory nature. This was elegantly addressed using *gata1* and *gata2* zebrafish mutants (Heckel et al. 2015; Vermot et al. 2009) (Fig. 12.2). The *gata1* mutant zebrafish has no circulating blood cells,

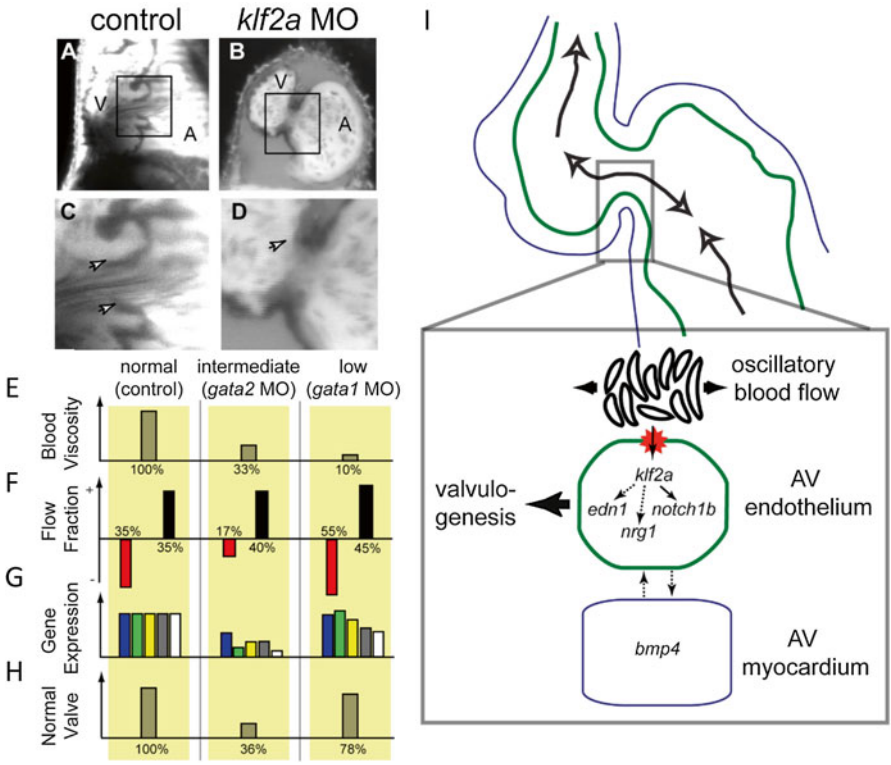

Fig. 12.2 *klf2a* mediates oscillatory flow-regulated valvulogenesis. Control embryos (**a**, **c**) had normal valve development whereas *klf2a* morpholino (MO)-injected embryos (**b**, **d**) had impaired valvulogenesis. The relative roles of oscillatory/retrograde flow versus shear stress magnitude in valvulogenesis were tested in control and *gata1* and *gata2* mutant zebrafish. (**e**) *gata1* and *gata2* mutations dose-dependently reduced the blood viscosity. (**f**) Percentage of forward flow (*black bars*) and retrograde flow (*red bars*). *gata2* mutants had decreased retrograde flow fraction and *gata1* mutants had increased retrograde flow fraction. (**g**) Gene expression profile of flow-sensitive genes: *bmp4* (*blue bar*), *notch1b* (*green bar*), *edn1* (*yellow bar*), *neuregulin1* (*grey bar*), and *klf2a* (*white bar*). (**h**) Percentage of zebrafish embryos with normal valves. *gata2* mutants had impaired valvulogenesis. (**i**) Proposed signaling mechanism during valvulogenesis. Oscillatory flow triggers downstream signaling through *klf2a* to regulate the expression of target genes (*edn1*, *neuregulin1*, and *notch1b*) in the endocardial cushion cells. *klf2a* also regulates bmp4 expression possibly through endocardial cell-myocardium crosstalk. Adapted from Vermot et al. (2009)

which reduces their blood viscosity by ~90 % relative to wild-type controls, while the *gata2* mutant zebrafish has 72 % fewer circulating blood cells than wild types, resulting in a ~70 % reduction in blood viscosity (Vermot et al. 2009). The magnitude of the wall shear stress is proportional to blood viscosity, and thus if high shear stress magnitude was required for valve development, one would expect the *gata1* mutants to demonstrate greater valve defects. However, this is not the case: *gata2* mutants have more severe valve defects. They also exhibit less flow reversal in the AVC than *gata1* mutants, suggesting that the absence of flow reversal plays an

important role in the development of valve defects and not the reduction in shear stress alone. Interestingly, in *gata2* mutants, the expression of *klf2a* and *notch1b* genes was significantly downregulated. The Notch signaling pathway is essential for valve development and is mechanosensitive (Wirrig and Yutzey 2014). *miR-21* expression was also lost in the *gata2* mutants but not in the *gata1* mutants (Banjo et al. 2013). A recent study showed that oscillatory shear-induced *klf2a* expression is upregulated through Trpv4 and Trpp2, membrane-bound mechanosensitive channels (Heckel et al. 2015).

The studies to date indicate that the initiation of valve development and the positioning of the valves is hemodynamically regulated, and disruptions to flow during this phase of development can cause valve malformations similar to congenital heart defects (Combs and Yutzey 2009; Hove et al. 2003). This is notable in the context of congenital heart valvular defects, and particularly bicuspid aortic valves (BAVs), which have two leaflets instead of the normal three. BAVs are the most common congenital valve defect, occurring in 1–2 % of the adult population (Hoffman and Kaplan 2002). While numerous genetic and microenvironmental factors likely contribute to valve developmental defects, it is notable that several of the mutations in mice that lead to BAVs are mutations of shear- or mechanosensitive genes and pathways, including eNOS (Mohamed et al. 2012), the C-type natriuretic peptide (CNP) pathway (Blaser et al. 2015), and the NOTCH pathway (Garg et al. 2005).

12.2.2 Endocardial-to-Mesenchymal Transition

At approximately HH17 in chick development, E9 in mice, and day 20 in humans, the endocardial cells lining the cardiac cushions delaminate, downregulate their endocardial markers, transition to mesenchymal phenotypes, and invade the cushions (Butcher and Markwald 2007; Lindsey and Butcher 2011). This process of EndMT is critical for proper valve development (Riem Vis et al. 2011; Yalcin et al. 2011) as the invading cells are the precursors to valvular interstitial cells (VICs) that remodel the cushions to the thin, pliable leaflets that are required for proper valve function.

The extent to which EndMT is mechanically regulated during valvulogenesis has not been determined primarily due to the lack of good experimental models. Signaling pathways involved in EndMT, including BMP, TGFβ, Notch, and Wnt pathways, are known to be shear- and stretch-sensitive, suggesting the potential for mechanical regulation. There is some evidence of mechanically regulated EndMT in adult valves. In adult mitral valves surgically constrained to increase tissue strains, there was a significant increase in endothelial cells expressing α-smooth muscle actin (α-SMA), indicative of increased EndMT (Dal-Bianco et al. 2009). Additional support comes from studies in which cyclic strain promoted EndMT of adult valvular endothelial cells (VECs) relative to static controls in vitro (Balachandran et al. 2011a). Interestingly, low strain (10 %) EndMT was regulated by TGF-β1 signaling, whereas under high strain (20 %), EndMT was more depen-

dent on Wnt/β-catenin signaling in this in vitro model system. While these data suggest the possibility of mechanically regulated EndMT, their applicability to valve development has yet to be determined.

12.2.3 Extracellular Matrix Remodeling

After EndMT and cell invasion, the cardiac cushions are remodeled into thin fibrous leaflets. The mesenchymal cells mediate this process by remodeling the hyaluronan ECM of the cardiac cushions and synthesizing new ECM containing collagens, elastin, and proteoglycans (Lindsey 2014). Ultimately, this leads to thinned, elongated leaflets that are stiffer than the cushion precursor ECM, but remain pliable. This remodeling process coincides with when the heartbeat and blood pressure start to increase (Butcher et al. 2007; Lindsey 2014).

Mature valves have distinct layers through the thickness of the leaflets (see Sect. 12.3.1). The laminar structure of the leaflets of the aortic and pulmonary valves only emerges postnatally, coincidental with changes in blood flow patterns and transvalvular pressures that occur with the lungs becoming functional and the foramen ovale and ductus arteriosus shunts in the fetal heart closing at birth (Aikawa et al. 2006) (Fig. 12.3). Under postnatal hemodynamic forces, the aortic and pulmonary valve leaflets remodel to have a tri-laminar structure consistent with the functional loads they experience. Interestingly, valve cells may adapt mechanically as well: VICs from the aortic valve on the higher pressure, left side of the heart are twice as stiff as VICs from the pulmonary valve on the lower pressure, right side of the heart (Merryman et al. 2006). Stratification of valve leaflets and valve-specific cell mechanical properties likely represent functional adaptations that occur in response to the changes in hemodynamics that occur postnatally, although causality has yet to be demonstrated explicitly.

12.3 Mechanobiology of Calcific Aortic Valve Disease

CAVD is the most common valve disease in adults, affecting approximately 25 % of North Americans over the age of 65 and with increasing prevalence as the population ages (Lindroos et al. 1993). CAVD is a disease spectrum that encompasses initial cellular changes to aortic valve sclerosis (thickening of the valve) to aortic valve stenosis (calcification and functional impairment of the heart valves) (Stephens et al. 2008). As disease progresses and the leaflets thicken and stiffen, there is a decrease in the efficacy of valve mobility leading to aortic regurgitation due to left ventricular pressure overloading and other complications. Key characteristics of CAVD include the activation of osteogenic gene networks (Mohler et al. 2001; Rajamannan et al. 2003), inflammation (Mathieu et al. 2014), and maladaptive ECM remodeling including tissue fibrosis by myofibroblasts (Chen and Simmons 2011). Notably, several lines of evidence point to a role for mechanics in regulating

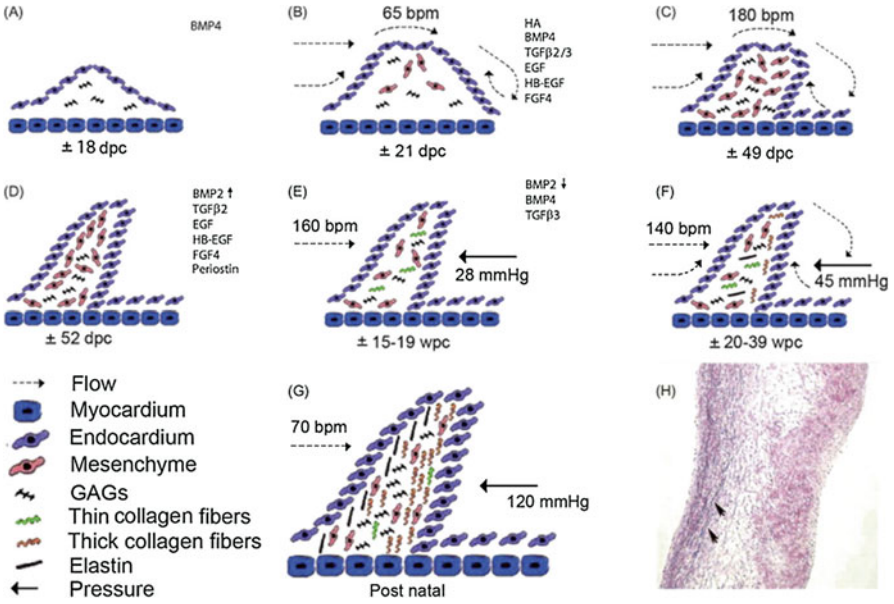

Fig. 12.3 Hypothetical timeline for valve development and the role of hemodynamic forces. (**a**) Formation of the cardiac jelly characterized by the secretion of GAGs. (**b**) Initiation of unidirectional blood flow through cardiomyocyte contraction promotes EndMT leading to mesenchymal cell invasion into the cardiac jelly. Reversal flow occurs at this stage. (**c**) Blood flow velocity increases and side-specific flow patterns initiate valvular morphogenesis. (**d**) Valve elongation phase. (**e**) The presence of transvalvular pressure correlates with the production of thin collagen fibers. (**f**) Transvalvular pressure gradient increases and elastin fibers are present. (**g**) Tri-layered ECM composition is present postnatally. (**h**) Histological staining of aortic valve showing the three ECM layers. From Riem Vis et al. (2011)

the disease. For example, fibrotic and calcific lesions occur focally in regions of aortic valve leaflets associated with distinct hemodynamic and biomechanical microenvironments (Bäck et al. 2013); hypertension and BAVs, both of which alter mechanical loading on the aortic valve, are risk factors for CAVD (Sider et al. 2011); and mechanosensitive developmental processes appear to be recapitulated in disease (Li et al. 2013). Here we review aortic valve structure and function and the evidence for mechanobiological regulation of CAVD.

12.3.1 Aortic Valve Structure and Composition

The three leaflets of the normal aortic valve are thin (~1 mm in humans (Otto et al. 1994)), multilayer structures designed to provide both flexibility so as not to impede blood flow from the heart during systole and the durability required to withstand cyclic mechanical loading with every heart beat (Fig. 12.4). To accomplish these

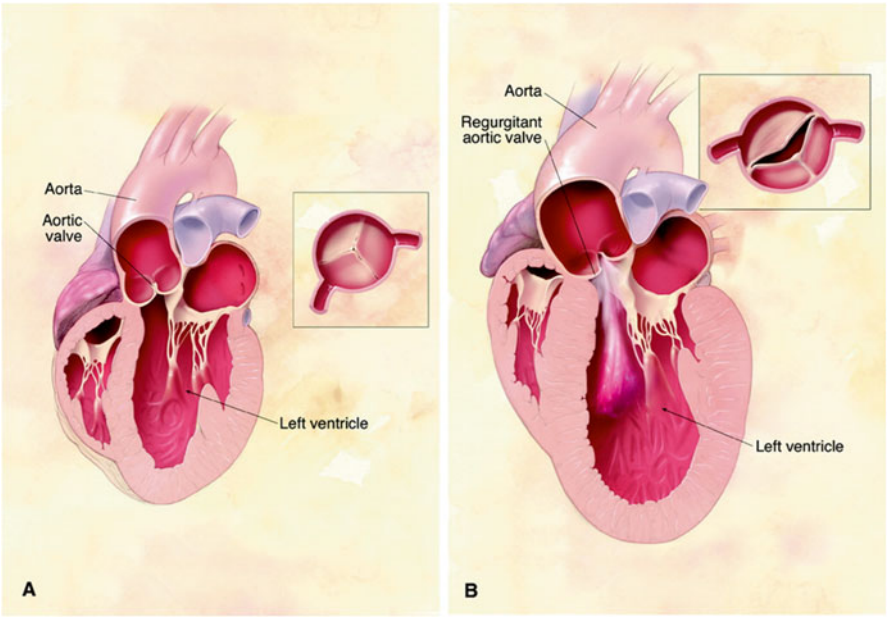

Fig. 12.4 The aortic valve. (**a**) The normal aortic valve contains three cusps or leaflets that open fully during systole to permit flow of blood from the left ventricle to the aorta and systemic circulation. During diastole, the leaflets coapt (*inset*) to prevent retrograde flow. (**b**) Bicuspid valves are a common congenital defect in which two leaflets are fused, which alters the biomechanical and hemodynamic functions of the valve, potentially leading to retrograde flow and accelerated calcific aortic valve disease. From Nishimura (2002)

functions, the leaflets have three distinct layers—the fibrosa, spongiosa, and ventricularis—each with distinct ECM composition, organization, and mechanical properties (Fig. 12.5). The fibrosa faces the aorta and is made up of circumferentially oriented type I collagen fibers that are important for its load-bearing properties. The spongiosa is primarily made up of GAGs, which cushion and lubricate the relative movement of the outer two layers of the valve. The ventricularis faces the left ventricle and is rich in elastin, which is required for recoiling the leaflets at the start of diastole. The different compositions of the layers result in layer-specific mechanical properties, as measured by tensile testing of isolated layers, micropipette aspiration of intact leaflets, and atomic force microscopy of tissue sections, with the fibrosa layer being stiffer than the ventricularis (Sewell-Loftin et al. 2012; Vesely and Noseworthy 1992; Zhao et al. 2011).

Mature heart valve leaflets are lined by a layer of VECs and populated within all three ECM layers by VICs (Fig. 12.5). Both cell types are mechanosensitive. VECs are exposed to blood flow-induced shear stress in vivo and demonstrate some shear-dependent responses that differ from those of vascular endothelial cells (Butcher et al. 2006). VICs are a heterogeneous population of primarily quiescent fibroblasts in normal adult valves. However, VICs exhibit with high plasticity during development,

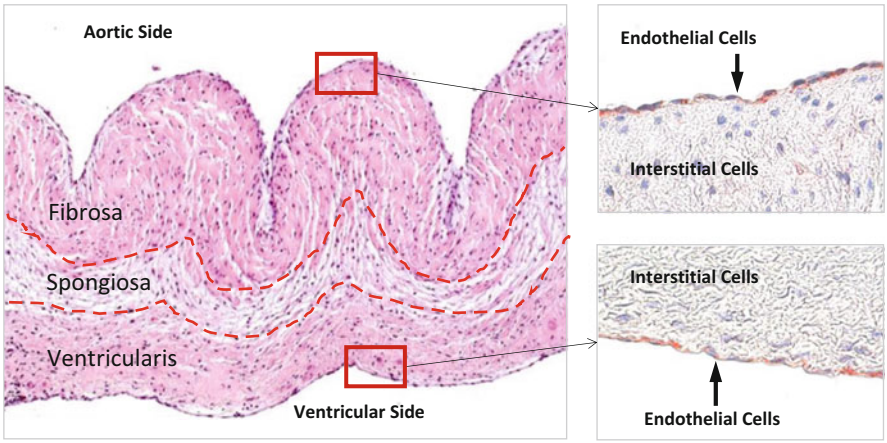

Fig. 12.5 Aortic valve leaflet cross section. Histological staining of the aortic valve reveals three ECM layers: fibrosa, spongiosa, and ventricularis. Endothelial cells line the surface of the aortic valve on the fibrosa and ventricularis sides. Interstitial cells reside within all three layers of the leaflet. Adapted from Simmons et al. (2005)

repair, and regeneration (Aikawa et al. 2006). They also demonstrate mesenchymal stromal cell-like properties, with the ability to differentiate to multiple lineages, including osteoblasts, adipocytes, chondrocytes, and myofibroblasts (Chen et al. 2009). The calcification and fibrosis that occur in CAVD are likely primarily the consequence of dysregulated differentiation of VICs to ectopic mesenchymal lineages. For example, activation of VICs to myofibroblasts is important for normal tissue repair and remodeling, but if unchecked can lead to fibrosis. VIC injury or apoptosis, which is often associated with myofibroblastic VICs (Hutcheson et al. 2013; Rajamannan et al. 2001), can lead to dystrophic calcification of the leaflets. Valve calcification can also occur through osteogenic differentiation of VICs and the secretion and remodeling of bone-like deposits within the leaflet interstitium (Chen et al. 2009; Yip et al. 2009).

12.3.2 Adult Aortic Valve Hemodynamics

The aortic valve opens during systole in response to contraction of the left ventricle and closes during diastole, when the ventricle relaxes and low inertial flow in the sinuses of the aortic root force the leaflets to close by coapting with each other. As a result, the aortic valve is subjected to a variety of hemodynamic and biomechanical forces throughout the cardiac cycle, including blood flow-induced shear stresses, hydrostatic pressures, tensile stretch, and bending (Fig. 12.6). Due to the rapid movements of the valve leaflets during the cardiac cycle, accurate quantification of

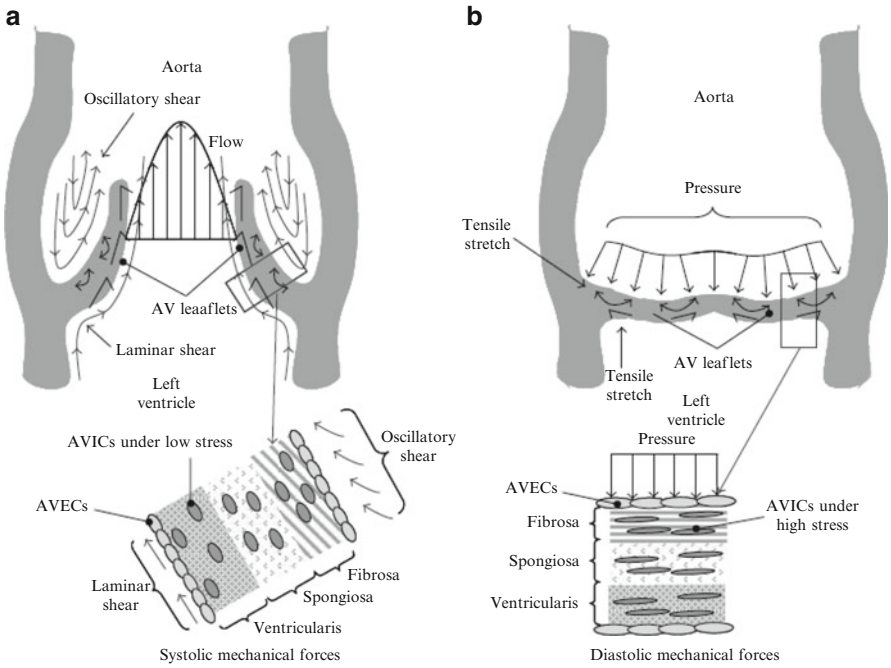

Fig. 12.6 Hemodynamic and biomechanical loading of the aortic valve. Mechanical forces experienced by the aortic valve at the tissue and cellular level during (**a**) systole and (**b**) diastole. From Balachandran et al. (2011c)

shear stress and tissue deformation at the level of the valve leaflet in vivo is a challenge. These challenges have led researchers to estimate shear stresses on the leaflet surface using computational modeling and ex vivo validation studies using particle image velocimetry (Weston et al. 1999; Yap et al. 2012b). These studies confirm that different shear stresses are experienced on the two sides of the leaflets, with high levels of unidirectional shear stress on the ventricular surface (up to 70 dynes/cm^2) and disturbed, oscillatory, low magnitude shear stress on the aortic surface (magnitudes <20 dynes/cm^2) (Yap et al. 2012b) (Fig. 12.7).

The leaflets also bear the transvalvular pressure, which peaks at ~80 mmHg during diastole, and decreases to zero during systole (Thubrikar 1990). Pressure loading during diastole causes the leaflets to lengthen, which is critical to ensure valve closure and the formation of a coaptive seal. Measurements made in canines indicate elongation of 31 % in the radial direction and 11 % in the circumferential direction (Thubrikar 1990); similar anisotropic stretching was observed in ex vivo studies with porcine valves, with large dependency on the transvalvular pressure gradient (Yap et al. 2010) and regional heterogeneity (Weiler et al. 2011). The difference in stretch in the radial versus circumferential direction is due to intrinsic alignment of the leaflet ECM: the circumferential organization of collagen fibers in the fibrosa

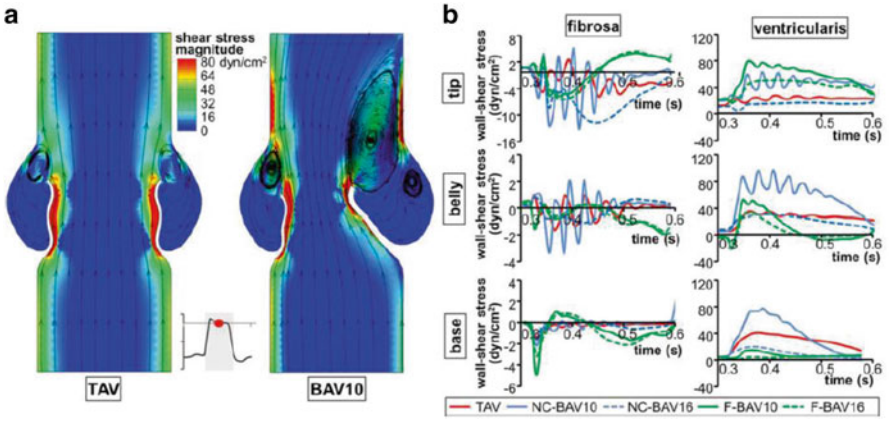

Fig. 12.7 Side-dependent leaflet wall shear stresses in tricuspid and bicuspid aortic valves. Predictions by computational fluid dynamics of the shear stress on the fibrosa and ventricularis sides of the aortic valve. (**a**) Snapshot at peak systole of the shear stress fields in a tricuspid (*left*) and bicuspid with a 10 % eccentricity (BAV-10), where eccentricity was defined as the distance between the centerline of the aortic root and the leaflet coaptation line, expressed as a percentage of the aortic root diameter. (**b**) Temporal variations in the tip, belly, and base regions for tricuspid (TAV) and bicuspid valves with 10 % (BAV-10) or 16 % (BAV-16) eccentricity for the noncoronary (NC) or fused (F) leaflet. From Chandra et al. (2012)

makes aortic valve leaflets less compliant in the circumferential direction than the radial direction.

The hemodynamics and biomechanics of BAVs are quite different from those of tricuspid aortic valves, owing to their significant anatomic and functional differences. Computational models of blood flow through BAVs and tricuspid aortic valves predict that because of a smaller effective orifice, the velocity, vorticity, and transvalvular pressure gradient are greater in BAVs (Yap et al. 2012a). This is predicted to alter the wall shear stress magnitudes and temporal and spatial gradients in some regions of the valve leaflets (Chandra et al. 2012) (Fig. 12.7) and to increase tissue strains (Szeto et al. 2013). It is widely assumed, but not yet proven, that these alterations in mechanical loading are responsible for the higher and earlier incidence of CAVD in patients with BAVs.

12.3.3 Mechanobiological Regulation of Aortic Valve Pathobiology

In the initial stages of CAVD, cellular phenotypic changes and lesion formation occur in distinct regions of the valve leaflet, including in the fibrosa layer at the coaptation line and leaflet base, and more frequently in patients with high blood pressure or BAVs. These regions and conditions are all associated with altered

hemodynamics and biomechanics, suggesting roles for various types of mechanical forces, including shear stress, stretch, and ECM stiffness, in CAVD initiation and development, as discussed below.

12.3.3.1 Shear Stress

Blood flow-induced shear stress is predominantly sensed by the VECs on the surface of the valve leaflets. VECs help maintain valve homeostasis, and their dysfunction or damage can lead to disruption of this homeostasis and initiate disease. Even in normal aortic valves, differences in the hemodynamic environment on the aortic vs. ventricular sides of the valve can influence side-dependent VEC biology. Side-dependent VEC phenotypes may explain, in part, why CAVD initiates in the fibrosa layer of the valve leaflets, while the ventricularis and spongiosa are relatively disease-protected (Chen and Simmons 2011; Gould et al. 2013; Yip and Simmons 2011). Support for this hypothesis was first demonstrated by microarray analysis of VECs isolated from the fibrosa vs. ventricularis sides of normal porcine aortic valves (Simmons et al. 2005). The transcriptional profile of the VECs from the disease-prone fibrosa layer suggested that they were more permissive to calcification and fibrosis than ventricularis-side VECs. This was demonstrated by significantly higher expression of osteochondral genes, such as bone morphogenetic protein (BMP)-4, and significantly lower expression of paracrine factors known to protect against calcification and fibrosis, including osteoprotegerin, CNP, chordin, and parathyroid hormone. Notably, the fibrosa-side permissiveness to calcification and fibrosis appeared to be balanced by an enhanced antioxidative state in normal valves. In a follow-up study, pigs were fed a high cholesterol diet for 2 weeks to induce early aortic valve disease (Guerraty et al. 2010). Unexpectedly, systemic insult resulted in induction and persistence of a protective endothelial phenotype on the pathosusceptible aortic side. Further evidence for compensatory mechanisms to protect the fibrosa side includes lower expression of *miR-370* on the fibrosa side of aortic valves from human patients undergoing heart transplantation (Holliday et al. 2011). *miR-370* is upregulated during EndMT, a developmental process that can occur in adult valves (Balachandran et al. 2011a) and contribute pathological VICs in CAVD.

It is likely that the differential shear stresses on opposite sides of the leaflets regulate, in part, side-dependent VEC phenotypes. Indeed, relative to static controls, shear stress suppresses osteochondral gene expression in porcine VECs (Butcher et al. 2006) and promotes anti-osteogenic and anti-inflammatory gene expression in induced pluripotent stem cell (iPSC)-derived endothelial cells (Theodoris et al. 2015), consistent with in vivo transcriptional profiles of VECs from the high shear stress ventricularis side of aortic valve leaflets (Simmons et al. 2005). Notably, exposure of fibrosa-side VECs to ventricularis-like flow (unidirectional, high shear stress) ex vivo increased the expression of the inflammatory and pro-fibrocalcific proteins VCAM-1, ICAM-1, BMP-4, and TGF-β1, suggesting that aortic and ventricular side VECs exhibit different shear responsiveness (Sucosky

et al. 2008). The sensitivity of fibrosa-side VECs to altered flow may explain in part the propensity for BAVs to calcify and fibrose more rapidly than normal tricuspid valves. In support of this hypothesis, BAV leaflet wall shear stress profiles applied to aortic valve leaflets ex vivo promoted the expression of proteins associated with inflammation, matrix degradation, and osteogenesis (Sun et al. 2012). It is also notable in the context of BAVs that shear stress upregulates genes involved in osteogenesis, inflammation, and oxidative stress in iPSC-derived endothelial cells with *NOTCH1* haploinsufficiency (*NOTCH1*$^{+/-}$), in contrast to the protective effect of shear stress in *NOTCH1*$^{+/+}$ iPSC-derived endothelial cells (Theodoris et al. 2015). The NOTCH signaling pathway is known to be shear sensitive and mutations in the *NOTCH* gene are involved in BAV and CAVD development (Garg et al. 2005; Masumura et al. 2009; Theodoris et al. 2015); it is intriguing to consider that accelerated disease in BAVs may be explained by *NOTCH1* haploinsufficiency abolishing the protective effect of shear stress in the aortic valve.

A mechanism by which shear stress regulation of VECs contributes to valve homeostasis or disease development is likely via paracrine signaling to the underlying VICs. VECs protect co-cultured VICs from myofibrogenesis and osteogenesis in vitro (Gould et al. 2014; Richards et al. 2013), and more so when the VECs are subjected to unidirectional, high magnitude shear stress (Butcher and Nerem 2006; Chen et al. 2013). A few paracrine signaling molecules have been identified. Ex vivo leaflet studies suggest that physiological shear stress suppresses BMP4 and TGF-β1 levels, which may promote VIC osteogenic and myofibrogenic differentiation, respectively (Sun et al. 2012). Nitric oxide (NO), a potent shear-dependent vasodilator that regulates vascular tone and homeostasis of the vasculature, may also regulate VIC phenotypic expression in the aortic valve. NO inhibits VIC activation, osteogenic differentiation, and calcification in vitro (Kennedy et al. 2009; Richards et al. 2013). eNOS expression is more abundant in ventricularis-side aortic VECs than fibrosa-side VECs further suggesting a role of shear stress in regulating eNOS signaling (Richards et al. 2013). Exposure of whole porcine aortic leaflets to unidirectional flow increased cGMP levels, a downstream effector in the NO signaling pathway, compared with oscillatory flow. Inhibition of cGMP using a soluble guanylyl cyclase inhibitor caused increased osteogenic marker expression and calcified nodules. Another candidate for shear stress-dependent VEC paracrine regulation of VICs is CNP. Similar to NO, CNP expression by vascular endothelial cells is stimulated by shear stress (Zhang et al. 1999) and CNP binding to its primary receptor, natriuretic peptide receptor (NPR)-B, potentiates intracellular synthesis of cGMP and downstream signaling events. In normal aortic valves, CNP expression by VECs is higher on the ventricular side leaflets (Simmons et al. 2005; Yip et al. 2011), consistent with it being hemodynamically regulated. In vitro, CNP inhibits pathological differentiation of VICs to the myofibroblasts and osteoblast lineages (Yip et al. 2011), and emerging evidence suggests that CNP is protective against CAVD in mice (Blaser et al. 2015). Together, these studies suggest that cGMP-dependent pathways may have therapeutic value for the treatment of CAVD, although this has yet to be tested.

12.3.3.2 Mechanical Strain

Aortic valve cells experience cyclic stretch during diastole as the transvalvular pressure increases, the valve leaflets close, and blood pools in the cusps of the valve leaflets aiding in the closure of the valves. Risk factors such as BAV and hypertension increase the amount of mechanical strain experienced by the valve, potentially leading to accelerated calcification (Katayama et al. 2013; Siu and Silversides 2010; Yap et al. 2010). As the valve stenoses, the effective orifice area is reduced, which increases the transvalvular pressure gradient to further increase the mechanical strain on the aortic valve and potentially establish a positive feedback loop.

The effects of strain have been investigated using aortic valve tissue ex vivo and aortic VICs in vitro. Supraphysiological levels of strain have numerous effects on VICs and valve tissue that are putatively relevant to CAVD. At the cellular level, cyclic stretch activates VICs to myofibroblasts, which express more α-SMA, are more contractile and stiffer (Wyss et al. 2012), and more synthetic than fibroblastic VICs (Balachandran et al. 2006; Merryman et al. 2007a). As a result, activation of VICs to myofibroblasts at supraphysiological levels of strain leads to increased synthesis of collagen and GAGs (Balachandran et al. 2006, 2011b; Ku et al. 2006). The expression and activity of the proteolytic enzymes matrix metalloproteinase (MMP)-1, MMP-2, and MMP-9 and cathepsins S and K are also increased with pathological strain (Balachandran et al. 2009), consistent with observations from diseased valves. Strain-dependent VIC myofibroblast differentiation is modulated by other factors. For example, TGF-β1 and cyclic strain act synergistically to enhance VIC myofibroblast differentiation and collagen synthesis (Merryman et al. 2007b; Moraes et al. 2013) and VIC response to cyclic strain is ECM protein-dependent (Moraes et al. 2013). Intriguingly, VICs from the fibrosa layer of the aortic valve are less sensitive to pro-fibrotic cues, including cyclic strain, than those from the ventricularis layer (Moraes et al. 2013).

In addition to inducing a fibrotic response, excessive mechanical strain is associated with increased calcification by VICs. In ex vivo tissue and in vitro VIC experiments, strain can induce VIC apoptosis (Balachandran et al. 2010; Bouchareb et al. 2014; Hutcheson et al. 2012), which is associated with dystrophic calcification in vivo. This may occur in part because mechanical strain can promote the transport of ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)-containing vesicles to the plasma membrane in a stretch-mediated RhoA/ROCK-dependent manner, where they are involved in mineralized microparticle production and accumulation (Bouchareb et al. 2014). These observations are consistent with increased ENPP1 expression and mineralized particles in regions of stenotic BAVs predicted to be under high strain (Bouchareb et al. 2014). Strain may also contribute to valve calcification by regulating osteogenic differentiation of VICs. In the presence of osteogenic media, calcified nodules form in aortic valve tissue exposed to 10 or 15 % strain ex vivo (Balachandran et al. 2010). This was accompanied by increases in *BMP-2*, *BMP-4*, and *Runx2* expression in a strain magnitude-dependent manner. Upregulation of osteogenic genes, including alkaline phosphatase (*ALPL*) and *BMP2*, by pathological levels of strain may be regulated through the Wnt/β--

catenin signaling pathway and the long-coding RNA, *HOTAIR* (Carrion et al. 2014). The expression of *HOTAIR* is reduced in BAVs and in mechanically strained VICs. Repression of *HOTAIR*, which associates with elevated levels of β-catenin under mechanical strain, leads to increased expression of target calcification genes, including *ALPL* and *BMP2*.

Stretch may also play a role in driving inflammation, which is a key component of CAVD pathogenesis. Stretch of VICs in vitro induces the expression of inflammatory genes (Patel et al. 2015; Warnock et al. 2011). This may be due in part to strain-dependent suppression of the expression of microRNA *miR-148a-3p*, which normally represses NF-κB signaling and inflammatory gene expression (Patel et al. 2015; Warnock et al. 2011). Thus, pathological levels of strain may disrupt the repressive effect of *miR-148a-3p* to permit the expression of inflammation-related genes. Notably, *miR-148a-3p* expression is also repressed in BAVs, perhaps due to elevated tissue strains.

12.3.3.3 Matrix Stiffness

The differences in the structure and composition of the different layers of the aortic valve give rise to intrinsic differences in tissue stiffness, with the fibrosa layer being the stiffest (Zhao et al. 2011). It has been speculated that the intrinsic ECM stiffness of the fibrosa layer may modulate local VIC phenotypes and thereby contribute to the fibrosa being disease prone (Chen and Simmons 2011; Yip and Simmons 2011). This idea is supported by in vitro studies that use biomaterial substrates with tunable mechanical properties (e.g., collagen, polyacrylamide, and poly(ethylene glycol) gels), which clearly demonstrate that VIC plasticity is regulated by matrix stiffness. For example, aortic VICs only robustly express and incorporate α-SMA into stress fibers when cultured on substrates with elastic moduli >15 kPa (Chen et al. 2011; Pho et al. 2008; Yip et al. 2009), even in the presence of TGF-β1, the myofibrogenic effects of which are mediated via Wnt/β-catenin signaling (Chen et al. 2011). VIC osteogenic differentiation is also modulated by matrix elasticity. Similar to bone marrow-derived mesenchymal stromal cells (Engler et al. 2006), stiffness-dependent osteogenic differentiation of VICs is biphasic: on very soft substrates, VICs remain fibroblastic; intermediate stiffness substrates (~25 kPa) promote osteogenic differentiation and the formation of bone nodules; and stiffer substrates promote myofibroblast differentiation, leading to the formation of calcified aggregates through apoptosis (Yip et al. 2009). Strikingly, the threshold stiffness for the transition from quiescent to pro-osteogenic substrates corresponds to the upper end of stiffnesses measured in the fibrosa layer (Chen et al. 2011), supporting the idea that the intrinsic stiffness of the fibrosa makes it permissive to pathological differentiation and disease development, which would be exacerbated as the tissue stiffens as it calcifies and fibroses.

12.4 Perspectives for Clinical Applications

The aortic valve must function in arguably one of the most biomechanically demanding microenvironments of the body. Wear-and-tear from the mechanical loads applied to the valve was traditionally thought to lead to valve damage and failure. However, valve disease is now recognized as an active cell-mediated pathology involving inflammation and unchecked matrix dysregulation leading to fibrosis, calcification, and impaired function (Rajamannan et al. 2011). Currently, there are no effective medical therapies for CAVD, leaving surgical intervention as the only treatment option. The lack of medical therapies stems from our incomplete understanding of CAVD pathobiological mechanisms and failure to identify effective therapeutic targets to date (Yutzey et al. 2014). As summarized in this chapter, there is ample evidence that many of the pathological processes in CAVD are mechanobiologically regulated, and in some cases recapitulate developmental processes, which themselves are critically influenced by mechanical factors. There, insights gained from understanding mechanobiological regulation of valve development and disease may identify molecular and cellular mechanisms that can be targeted therapeutically. An interesting example of this is CNP: we first identified CNP in the valve in a microarray analysis of side-dependent (and putatively hemodynamically regulated) endothelial genes (Simmons et al. 2005). Its higher expression on the high shear stress ventricularis side of the leaflets, which is typically spared from disease, suggested that CNP was protective against disease development. We confirmed this in subsequent in vitro studies (Yip et al. 2011) and more recently, in mice (Blaser et al. 2015), motivating future studies to test the therapeutic potential of CNP and its downstream effectors in CAVD. Other mechanoregulatory signaling pathways in the valve, including the Wnt/β-catenin, TGF-β superfamily, NOTCH1, and NF-κB pathways, may represent alternative targets. Challenges moving forward for all test therapies include identifying appropriate animal models (Sider et al. 2011), developing early detection methods so that treatment can initiate before the "point-of-no-return," and addressing how the heterogeneity of CAVD manifestation and progression impacts treatment strategies (Yutzey et al. 2014).

In addition to providing insights into disease mechanisms, a promising clinical application of heart valve mechanobiology is towards engineering of living replacement tissue. Currently, surgical replacement is the primary option for the treatment of valvular disease and congenital defects. Mechanical, bioprosthetic, or cryopreserved homograft valves enhance survival and quality of life for many patients. However, no current prosthetic valve has the capacity for growth, repair, and adaptation, features that are essential for pediatric patients with congenital defects, children and young adult rheumatic fever patients, and active adult patients with valve disease. Heart valve tissue engineering (HVTE) may address this unmet need, particularly for pediatric patients who would benefit the most from a living replacement valve. Many HVTE strategies use biomechanical conditioning of valves

in vitro to promote differentiation of progenitor cells to myofibroblasts and synthesis of mechanically robust tissue that can withstand the mechanical loading experienced in vivo (Parvin Nejad et al. 2016). However, mechanical stimulation can also induce fibrotic cell phenotypes and maladapted tissue synthesis that lead to valve regurgitation and prevent long-term success. The solution to the challenge of these competing objectives remains to be identified, but may be informed by fundamental knowledge of mechanobiological and homeostatic systems in the native valve, very few of which have been considered for translation to tissue-engineered heart valves to date (Parvin Nejad et al. 2016).

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Chapter 13
Molecular and Cellular Mechanobiology of Cancer

Laurent Fattet and Jing Yang

Abstract Besides biochemical interactions between the primary tumor and its microenvironment, the mechanical involvement of tumor–stroma interactions has recently emerged as an important contributor for tumor progression. Indeed, solid tumors often initiate by a fibrotic state associated with increased matrix deposition and remodeling, an important contributor of tumor progression. Specifically in breast cancer patients, the presence of dense clusters of collagen fibrils, or fibrotic foci, associated with significant increased tissue rigidity, is a prognostic marker of distant metastasis and correlates with poor survival. Clinical and experimental data have shed light on the role of extracellular matrix stiffness during progression to invasive and metastatic tumors, especially regarding breast cancer. Here we describe how extracellular matrix stiffness contributes to tumorigenesis, through different roles on tumor cells or stromal cells, at the primary tumor and at metastatic sites. Eventually we discuss the latest and most promising therapeutic approaches targeting or taking advantage of this newly defined implication of mechanoregulation in cancer progression.

Keywords ECM • Stiffness • Mechanosensing • Microenvironment • EMT • Metastases

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Abbreviations

βAPN	β-Aminopropionitrile
BMC	Bone marrow-derived cells
CAF	Cancer-associated fibroblasts
CAM-DR	Cell adhesion-mediated drug resistance
DTC	Dormant tumor cells
DXR	Doxorubicin
ECM	Extracellular matrix
EGFR	Epithelial growth factor receptor
EMT	Epithelial-to-mesenchymal transition
FAK	Focal adhesion kinase
FN1	Fibronectin 1
G3BP2	GTPase activating protein (SH3 domain) binding protein 2
GBM	Glioblastoma multiform
HA	Hyaluronic acid
HCC	Hepatocellular carcinoma
LOX	Lysyl oxidase
LOXL	Lysyl oxidase-like
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
MMP	Matrix metalloproteinase
NSCLC	Non-small-cell lung cancer
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
ROCK	Rho-associated protein kinase
SMA	Smooth muscle actin
TGFβ	Transforming growth factor beta
TNC	Tenascin C
VEGF	Vascular endothelial growth factor
YAP	Yes-associated protein

13.1 Importance of Mechanoregulation During Tumor Progression

Biochemical interactions between the primary tumor and its microenvironment have been extensively studied over the past decades, as reviewed in the updated Hallmarks of Cancer: The Next Generation (Hanahan and Weinberg 2011). The mechanical involvement of tumor–stroma interactions has recently emerged as an important contributor for tumor progression. Often a tumor is initiated by a desmoplastic response, a fibrotic state characterized by increased matrix deposition and

remodeling and enhanced posttranslational modifications of the ECM proteins (Lu et al. 2012). In breast cancer patients, manual palpation is used to detect tumor tissues based on their increased density or stiffness compared to a more compliant healthy surrounding tissue. Indeed, both tumor cells and surrounding stroma cells display increased stiffness compared to normal tissues. Increased mammographic density is correlated to a two- to six-fold increase in tumor susceptibility, thus representing a major risk factor for breast cancer (Boyd et al. 2002). Specifically, a fibrotic focus is histologically composed of fibroblasts and variable amount of collagen fibers. In breast cancer patients, the presence of fibrotic foci, associated with a 10- to 50-fold increase in tissue rigidity, is a prognostic marker of distant metastasis and correlates with poor survival (Colpaert et al. 2001; Hasebe et al. 2002; Mujtaba et al. 2013). Moreover, shear-wave elastography is being increasingly used as a diagnosis tool as it allows quantitative and noninvasive measurement of local stiffness and correlates with subtypes of breast cancer (Chang et al. 2013). Along with such clinical data, several experimental studies have shown that increasing ECM (extracellular matrix) stiffness without altering biochemical components induce a malignant and invasive phenotype in mammary epithelial cells (Paszek et al. 2005; Levental et al. 2009). Therefore, this chapter mainly focuses on mechanoregulation of breast tumors, but also highlights the latest and most interesting findings related to others types of cancer.

This chapter describes how mechanical properties of tumor ECM contribute to tumorigenesis, by acting directly on tumor cells or on stromal cells, at the primary tumor site and at metastatic niches. We then focus on the implication of mechanoregulation of cancer progression in cancer therapeutics.

13.1.1 Mechanoregulation in the Primary Tumor

Most of the mechanotransduction pathways discussed above have been identified or involved in the regulation of tumor cells responses to the ECM mechanical properties. However, some recent and promising results presented below show that ECM stiffness could also have an impact on the regulation of stromal cells behavior and interaction with the primary tumor cells.

13.1.1.1 Effects of ECM Stiffness on Tumor Cells

ECM stiffness can affect many aspects of tumor cell properties through regulation of apoptosis, cell proliferation, cell adhesion, epithelial-to-mesenchymal transition (EMT), and cell migration and invasion (Table 13.1). In this chapter, we discuss the most direct evidence in the literature demonstrating that mechanical cues, either alone or in combination with biochemical cues, promote tumor progression.

Unlimited proliferation is a major hallmark of tumor cells. Using human glioblastoma cell models, a recent study showed that increasing ECM stiffness can

Table 13.1 Role of ECM stiffness on tumor cells

Hallmarks of cancer targeted	Effect of ECM stiffness	Cell type, organ	Signaling pathway involved	Type of data	Reference
Sustained proliferation	Promotes EGFR mitogenic signaling	Glioma cells	EGFR, PI3K, Akt	in vitro	Umesh et al. (2014)
	Promotes integrin-dependent cell growth	Mammary epithelial cells	MUC1, integrins, FAK	in vitro	Paszek et al. (2014)
	Transduces into cellular stiffness and increases Cyclin D1	Mammary epithelial cells	FAK, Cas, Rac, Lamellipodin, Cyclin D1	in vitro, in vivo	Bae et al. (2014)
Resistance to cell death	Controls switch from TGFß-induced apoptosis to TGFß-induced EMT	Mammary epithelial cells	TGFß, PI3K/Akt	in vitro	Leight et al. (2012)
	Promotes integrin-dependent cell survival	Mammary epithelial cells	MUC1, integrins, FAK	in vitro	Paszek et al. (2014)
	Promotes anchorage-independent cell survival through Rac	Mammary epithelial cells	Laminin V, integrins, Rac, NF_KB	in vitro	Zahir et al. (2003)
	Promotes resistance to anoikis	Squamous cell carcinoma	Fibronectin, FAK	in vitro	Zhang et al. (2004)
EMT, invasion and metastasis	Disrupts Twist1/G3BP2 interaction and triggers nuclear translocation of Twist1	Mammary epithelial cells, Breast cancer	ß1-integrin, Twist1, G3BP2	in vitro, in vivo, human samples	Wei et al. (2015)
	Uses Vinculin to transmit force and activate FAK and Akt signaling	Mammary epithelial cells, Breast cancer	Vinculin, Actin, Talin, FAK, Akt	in vitro, in vivo, human samples	Rubashkin et al. (2014)
	Induces miR-18a expression to inhibit PTEN and activate PI3K	Mammary epithelial cells, Breast cancer	HOXA9, PTEN, FAK, Akt	in vitro, in vivo, human samples	Mouw et al. (2014)
	Increases MT1-MMP activity to activate MMP2 and MMP9	Panc-1, Pancreatic cancer	HA, MMPs	in vitro	Haage et al. (2014)

increase the expression and activation of epithelial growth factor receptor (EGFR) and resulted in phosphorylation of EGFR downstream effectors (Shinojima et al. 2003; Umesh et al. 2014). Thus, increasing ECM stiffness can promote glioma cell proliferation through activation of the EGFR-dependent mitogenic signaling, and also promotes glioma cells motility through intracellular pathways involving ROCK and the nonmuscle myosin II (Ulrich et al. 2009). Moreover, the glycocalyx formed by large glycoproteins in the microenvironment of solid tumors has been shown to confer a growth advantage in tumor cells (Paszek et al. 2014). Eventually, Bae and colleagues identified a FAK/p130^{Cas} signaling, activated in response to ECM stiffness in MEFs (mouse embryonic fibroblasts), resulting in an intracellular stiffening-dependent upregulation of cyclin D1, thus increasing cell proliferation. Furthermore, in an in vivo model of inducible vascular injury, where arterial stiffness is known to increase locally, the FAK/p130^{Cas}/cyclin D1 signaling node is shown to be essential for stiffness-induced cell cycling (Bae et al. 2014).

ECM stiffness has also been shown to regulate apoptosis resistance in tumor cells. In an in vitro study on mammary epithelial cells, ECM stiffness is shown to regulate a switch in TGFβ-induced cell responses. Specifically, TGFβ induces cell apoptosis on a compliant ECM; in contrast, increasing ECM stiffness triggers the pro-invasive function of TGFβ by activating EMT through a PI3K/Akt signaling (Leight et al. 2012). This study suggests for the first time that ECM stiffness could explain in part the double-edged sword effects of TGFβ in antitumor growth and pro-invasion observed during tumor progression (Akhurst and Derynck 2001). In addition, several studies have shown that increased production of ECM components such as laminin V or fibronectin can help tumor cells to escape loss of adhesion-induced cell death termed anoikis, respectively, through Rac or FAK signaling pathways (Zahir et al. 2003; Zhang et al. 2004).

Eventually, the best-documented role for ECM stiffness on tumor cells is the regulation of cell adhesion, epithelial–mesenchymal transition (EMT), migration, and invasion. A pioneer study by Paszek and colleagues described that in the 3D culture of mammary epithelial cells, a malignant phenotype could be driven by high stiffness substrate, corresponding to the average elastic modulus measured in mouse breast tumor tissues (Paszek et al. 2005). This work has identified an ECM stiffness-induced integrin clustering in cooperation with growth factor-dependent ERK activation to drive malignant transformation of mammary epithelial cells. Using 3D culture of mammary epithelial cells on polyacrylamide hydrogels with elastic moduli ranging from 150 Pa (normal mammary tissue) to 5700 Pa (average breast tumor tissue), a recent study identified a mechanotransduction signaling pathway that activates the EMT-inducing transcription factor Twist1 to promote EMT, invasion, and metastasis. Specifically, increasing ECM stiffness can signal through integrins to release Twist1 from its cytoplasmic anchoring protein G3BP2, thus allowing Twist1 translocation into the nucleus to act as a transcription factor and activate the EMT program (Wei et al. 2015). This mechanotransduction pathway is important for promoting tumor progression and metastatic dissemination in vivo, and importantly downregulation of G3BP2 combined to increased ECM stiffness synergistically predict poor outcome in breast cancer patients. This study provides the first evidence for a direct role of ECM stiffness on EMT transcription factor, leading to increased tumor cell invasive properties.

Modifications of the ECM stiffness could also impact tumor invasion via additional mechanisms. The focal adhesion component vinculin can act as a molecular bridge transmitting mechanical cues, through a talin-actin scaffolding complex, to promote tumor cell invasive phenotype. Interestingly, at the invasive border of human breast tumors, ECM stiffness regulates vinculin-dependent activation of focal adhesion and correlates an increased mechanosignaling, as shown by elevated FAK and Akt signaling (Rubashkin et al. 2014). ECM stiffness can also induce certain microRNAs, such as miR-18a, to repress the tumor suppressor PTEN, and lead to a PI3K-dependent malignant progression of tumor cells (Mouw et al. 2014). Using pancreatic cancer cell lines, Haage and colleagues have demonstrated that ECM stiffness increases Membrane Type 1-Matrix MetalloProteinase (MT1-MMP or MMP14) activity to promote invasion through the ECM (Haage and Schneider 2014; Haage et al. 2014). Remodeling and stiffening of ECM by LOX enzymes produced by stromal cells facilitate tumor growth, dissemination, and metastasis in vivo (Erler et al. 2006; Levental et al. 2009). Interestingly, secretion of TGFβ by myeloid cells from the microenvironment activates the cancer-associated fibroblasts to produce elevated amounts of LOX. LOX in turn increases ECM cross-linking and stiffening to promote tumor cell metastasis in vivo through activation of FAK signaling (Pickup et al. 2013).

13.1.1.2 Effect of ECM Stiffness on Stromal Cells

Tumor-associated stromal cells are embedded in the collagen-rich ECM, making them the front line to sense and modify matrix stiffness during many pathological conditions. Using primary dermal human fibroblasts cultured in three-dimensional alginate/collagen-I interpenetrating networks, ECM stiffness was shown to increase wound healing properties. Increasing matrix stiffness, independently of gel architecture, polymer concentration, or adhesion ligand density, directly regulates elongated, spindle-like fibroblasts morphology and triggers wound healing gene expression program (Branco da Cunha et al. 2014).

In tumors, recent evidence also shows that ECM stiffness directly acts on stromal cells (Table 13.2), especially cancer-associated fibroblasts (CAFs), to modify tumor–stroma interactions. CAFs are found in many solid tumors and promote invasion and metastasis through the production of soluble factors and matrix remodeling (Kalluri and Zeisberg 2006). Using mouse mammary cancer cells 4T1 xenograft model, expression levels of lysyl oxidase-like 2 (LOXL2) in tumors were correlated with the levels of α-smooth muscle actin (α-SMA, a marker of the myofibroblast subpopulation of CAFs). Interestingly, results showed that tumor-secreted LOXL2 is required for the ability of fibroblasts to invade though ECM, contract collagen, and express markers of activated CAFs such as fibronectin or stromal cell-derived factor 1. This study identified a β3-integrin and FAK-dependent mechanotransduction signaling pathway linking the tumor-derived LOXL2 to the activation of myofibroblasts in the tumor microenvironment (Barker et al. 2013). Calvo and colleagues isolated and characterized fibroblasts from different stages of breast tumor progres-

Table 13.2 Role of ECM stiffness on stromal cells

Hallmark of cancer targeted	Effect of ECM stiffness	Cell type, organ	Signaling pathway involved	Type of data	Reference
Angiogenesis	Increases VEGFR2 expression	Endothelial cells	GATA2, TFII-I	in vitro	Mammoto et al. (2009)
	Triggers VEGFR2 translocation to promote endothelial cells growth	Endothelial cells	VEGFR2, PI3K, Akt, GSK3b, Cyclin D1	in vitro	Liu and Agarwal (2010)
EMT, invasion and metastasis	Promotes CAFs activation	Fibroblasts, Breast cancer	LOXL2, integrin, FAK	in vitro, in vivo	Barker et al. (2013)
	Triggers YAP activation to maintain CAF identity	Fibroblasts, Breast cancer	YAP, Src, ROCK, actomyosin cytoskeleton	in vitro, in vivo, human samples	Calvo et al. (2013)
	Favors tumor cell invasion through increased Caveolin-1 secretion by CAFs	Fibroblasts, Breast cancer	Caveolin-1	in vitro, in vivo, human samples	Goetz et al. (2011)

sion and further demonstrated that generation and maintenance of CAFs relies on a prerequisite YAP activation in response to ECM stiffening. This model involves a positive feedback loop to maintain YAP activation, sustained by changes in the actin cytoskeleton contractility, and the CAF identity (Calvo et al. 2013). Furthermore, actomyosin contractility, Src and ROCK kinases are all required for stiffness-induced YAP activation leading to maintenance of CAFs critical contribution in breast tumor progression. Finally, Goetz and colleagues elegantly showed that ECM stiffness can also be mediated by CAFs through caveolin-1 (Cav1)-dependent regulation of Rho GTPase activity, leading to increased metastatic potential in vivo (Goetz et al. 2011). These studies unravel close mechanical crosstalks between CAFs and ECM, and such remodeling of the tumor microenvironment by CAFs is critical for tumor invasion and metastasis.

ECM stiffness has also been reported to act on additional tumor stromal cell types such as endothelial cells to promote tumorigenesis. Neo-angiogenesis is an essential step during tumor progression to provide the growing tumor mass with the oxygen and nutrients required. Mechanical cues can impact angiogenesis via integrins on the endothelial cells during angiogenesis (Avraamides et al. 2008). ECM stiffness was also found to regulate, though an unknown upstream mechanism, the balance between GATA2 and TFII-I transcription factors to activate the expression of VEGF receptor 2 (VEGFR2) and thus promote endothelial cell survival and

proliferation (Mammoto et al. 2009). Another study found that VEGFR2 itself could translocate into the nucleus in response to increasing ECM stiffness in human dermal microvascular endothelial cells (HDMECs) and could also trigger a PI3K/Akt/GSK3β mechanoresponsive cascade to promote endothelial cell growth and survival by preventing the degradation of cyclin D1 (Liu and Agarwal 2010).

In summary, these data suggest that various tumor stromal cells can sense the mechanical properties of tumor microenvironment and then feed back to the tumor cells to promote tumor progression.

13.1.2 Mechanoregulation of Metastatic Niche Establishment

Over a century ago, Stephen Paget's study of breast cancer biopsies led him to propose the "seed and soil" hypothesis, where the "seeds" (the tumor cells) specifically colonize the "soil" of distant organs presenting a microenvironment suitable for survival and growth (the metastatic niche). The requirement of biochemical cues in this process has been extensively studied and is reviewed in Joyce and Pollard (2008). In addition to the impact of matrix stiffness in the primary tumor site discussed above, growing evidence show that ECM remodeling and stiffening at the secondary site—during the establishment of the metastatic niche—is a critical step to promote metastatic outgrowth.

Using orthotopic implantation of mammary cancer cells in mice, two studies demonstrated that primary tumor-secreted LOX proteins, including LOX, LOX-like 2, and LOX-like-4, are secreted by primary tumor cells and accumulate in the pulmonary parenchyma to increase collagen IV cross-linking, thus promoting the recruitment of bone marrow-derived cells (BMCs) (Wong et al. 2011). Adhesion of BMCs to cross-linked ECM increases MMP-2 activity to strengthen local recruitment of stromal cells, which in turn further modify the premetastatic niche to enhance metastatic outgrowth (Erler et al. 2009). Moreover, in hepatocellular carcinoma (HCC), induction of LOXL2 expression by hypoxia and TGFβ in the primary tumor promotes collagen cross-linking, leading to metastatic niche formation (Wong et al. 2014). In this system, LOXL2-mediated metastatic niche formation favors intrahepatic local invasion of tumor cells and extrahepatic metastatic colonization and outgrowth in the lungs through the recruitment of BMCs.

The critical involvement of ECM remodeling and stiffening in the establishment of metastatic niche raises the possibility that ECM stiffness could contribute to reactivation of dormant tumor cells (DTCs) leading to metastatic outgrowth. The microenvironment of secondary site can control the fate of early-disseminated DTCs lacking genetic alterations conferring metastatic advantage (Páez et al. 2012); the pre-metastatic niche could help maintaining the dormant state of disseminated tumor cells until the soil is suitable for the seed to grow and form recurrent metastatic lesions. Depending on the primary cancer type, this metastatic outgrowth could be reactivated months, years, or decades after treatment of surgical removal of the primary tumor.

13.2 Effect of Treatment on Microenvironment Organization/Relationship Between Mechanics and Resistance to Chemotherapy

While most tumors are intrinsically sensitive to chemotherapeutic treatments, after an unpredictable period, tumor cells develop mechanisms of chemoresistance. Tumor cells can activate several chemoresistance mechanisms at different stages of tumor progression, including alteration of drug metabolism or rewiring of intracellular signaling pathway (Wilson et al. 2006; Groenendijk and Bernards 2014). The ability of tumor cells and stromal cells to remodel their biochemical and physical microenvironment following drug treatment provides another mechanism for tumors to acquire chemoresistance.

Using several breast cancer cell models, Pupa and colleagues showed that doxorubicin (DXR) treatment of xenografted tumor upregulated the expression of fibulin-1, a protein thought to function as a bridge in the organization of ECM supramolecular structures (Argraves et al. 2003), fibronectin (FN1) and laminin-1 in vivo (Pupa et al. 2007). More interestingly, DXR-induced inhibition of tumor growth was significantly greater in tumors grown in the absence of Matrigel compared with Matrigel-mixed tumors, suggesting that ECM can somehow protect tumor cells form DXR-induced cell death. This work provides evidence that fibulin-1 acts as an additional ECM components providing prosurvival function to promote chemoresistance in breast cancer cells. A similar approach identified the serpin-like serine protease inhibitor Maspin as an important regulator of ECM-dependent tumor resistance to DXR. In vivo, Maspin expression is correlated to enhanced collagen fiber accumulation and decreased diffusion of the chemotherapeutic drug (Triulzi et al. 2013). Furthermore, Gemcitabine (Gem), the main drug currently used in the treatment of advanced pancreatic cancers, has shown disappointing results in clinical trials due to high intrinsic resistance of pancreatic tumor cells, which could be induced by ECM-integrin interactions, named cell adhesion-mediated drug resistance (CAM-DR) (Damiano et al. 1999). Huanwen and colleagues have found that laminin-induced FAK phosphorylation contributes to Gemcitabine CAM-DR through the regulation of the pro-apoptotic protein Bad, the anti-apoptotic protein survivin, and Akt (Huanwen et al. 2009). Those results show that ECM production and remodeling could contribute to chemoresistance in various human cancers.

In addition to conventional chemotherapy, ECM remodeling has also been shown to contribute to targeted therapy resistance. In Her2+ breast tumors, disruption of the Laminin-5/CD151/Integrin/FAK mechanotransduction axis sensitizes tumor cells to ErbB2 antagonists trastuzumab or lapatinib (Yang et al. 2010). Despite very promising results on human non-small cell lung cancer cells (NSCLC) in vitro, anti-EGFR agents (such as cetuximab) have failed to improve the outcome when combined to conventional chemo- and radiotherapy in patients. By merging NSCLC patients data with in vitro and in vivo approaches, a recent study found that increased expression of FN1 and enhanced adhesion of tumor cells to FN1 through $\alpha 5\beta 1$ integrins reduce

cetuximab's cytotoxic and radiosensitizing efficacy (Eke et al. 2013). Mechanistically, the increased expression of FN1 by cetuximab-treated cells depends on the activation of the p38 MAPK-ATF2 signaling and the inhibition of the MEK/ERK pathway, and depletion of FN1 sensitized NSCLC cells to cetuximab efficacy.

13.3 Targeting Mechanotransduction Pathways for Cancer Therapy

As presented above, ECM composition and stiffness can promote both primary tumor growth and metastatic progression. Based on these knowledge, several strategies can be employed to counteract the tumor-promoting effects by matrix stiffening (Jung et al. 2015). More specifically, targeting ECM mechanotransduction players have shown promises for adjuvant therapies in combination with traditional and targeted therapies.

One mechanism for tumor cells to develop resistance to chemotherapy is thought to be caused by the presence of high pressure, physical barriers, and immature blood vessels that interfere with the proper delivery of the drug to the tumor, as first shown by the enhanced chemotherapy efficacy observed after depletion of stromal tissue in a mouse model of pancreatic ductal adenocarcinoma (PDAC) (Olive et al. 2009). Thus, one interesting strategy is to target microenvironmental ECM to facilitate antitumor immune responses and drug treatment by locally increasing intra-tumor penetration and accessibility to target tumor cells (Choi et al. 2013). Indeed, disruption of ECM integrity has shown encouraging results in preclinical studies. For example, methylumbelliferone, a hyaluronic acid (HA) synthesis inhibitor, was effective in inhibiting bone metastasis in lung cancers in mice (Futamura et al. 2013). Another approach is to target enzymes secreted by stromal or tumor cells in the microenvironment. As discussed, above, the LOX family of collagen cross-linking enzymes has been implicated in the regulation of metastatic progression of tumors and fibrotic diseases (Levental et al. 2009; Cox et al. 2013; Pickup et al. 2013). Chen and colleagues found a direct effect of LOX inhibitor β-aminopropionitrile (βAPN) in preventing human breast cancer cell invasion through downregulation of focal adhesion signaling pathways (Chen et al. 2012). Thus, LOX inhibitors have been brought as anti-metastatic drugs (Nishioka et al. 2012). In particular, LOXL2 inhibitory monoclonal antibody AB0023 is shown to impact tumor microenvironment, including reduction of growth factors contents and stromal cells recruitment, to significantly impede progression of tumors and fibrotic diseases (Barry-Hamilton et al. 2010).

In the case of pancreatic cancer however, most recent data showed that depletion of the stromal compartment instead favors the progression to a more vascularized and more aggressive tumor, leading to a decreased survival in a mouse model of PDAC (Özdemir et al. 2014; Rhim et al. 2014). Thus, instead of directly targeting microenvironmental ECM, another approach is to use various components of ECM

as vehicles for targeted drug delivery. A successful clinical trial for patients with glioblastoma multiforme (GBM) has used tumor-secreted ECM as scaffolding targets to achieve proper drugs delivery. Zalutsky and colleagues engineered radioactive particles coupled to an anti-Tenascin C (TNC) antibody to specifically target tumor cells. This approach is shown to increase GBM patients benefit and overall outcome with minimal toxicity (Zalutsky et al. 2007). This TNC-based targeting of tumor microenvironment could be likely to bring further promising utilities as TNC has been shown to be an important signature of pre-metastatic niche establishment in breast cancer metastasis (Oskarsson et al. 2011). Recent studies also employed HA conjugates to ensure proper delivery of poorly soluble drugs, such as Paclitaxel, to tumors and have shown encouraging results in significantly improving overall survival of animals with breast cancer-induced brain metastasis (Mittapalli et al. 2013; Arpicco et al. 2014).

Finally, a possible strategy would be to target integrins, a key sensor of matrix stiffening. Interestingly, Hendersson and colleagues have found that depletion of α4-integrin on myofibroblasts inhibits the pathological fibrosis in multiple solid organs (Henderson et al. 2013). With better understanding how integrins sense and transmit mechanical cues from the tumor microenvironment, new strategies could be developed to specifically targeting the mechano-sensing properties of integrins to disrupt the ECM stiffness-driven tumor progression.

In summary, this chapter covers current development in understanding how mechanical properties of the tumor microenvironment contribute to tumor development and progression. Such information holds the promise to develop new therapeutic approaches to target the mechanotransduction pathways for cancer therapeutics.

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