THE ANATOMICAL RECORD 297:1758–1769 (2014)

The Role of Mechanotransduction on Vascular Smooth Muscle Myocytes Cytoskeleton and Contractile Function

GEORGE J.C. YE, ALEXANDER P. NESMITH, AND KEVIN KIT PARKER*
Disease Biophysics Group, Wyss Institute for Biologically Inspired Engineering and the School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts

ABSTRACT

Smooth muscle (SM) exhibits a highly organized structural hierarchy that extends over multiple spatial scales to perform a wide range of functions at the cellular, tissue, and organ levels. Early efforts primarily focused on understanding vascular SM (VSM) function through biochemical signaling. However, accumulating evidence suggests that mechanotransduction, the process through which cells convert mechanical stimuli into biochemical cues, is requisite for regulating contractility. Cytoskeletal proteins that comprise the extracellular, intercellular, and intracellular domains are mechanosensitive and can remodel their structure and function in response to external mechanical cues. Pathological stimuli such as malignant hypertension can act through the same mechanotransductive pathways to induce maladaptive remodeling, leading to changes in cellular shape and loss of contractile function. In both health and disease, the cytoskeletal architecture integrates the mechanical stimuli and mediates structural and functional remodeling in the VSM. Anat Rec, 297:1758–1769, 2014. © 2014 Wiley Periodicals, Inc.

Key words: mechanotransduction; tissue mechanics; smooth muscle; cytoskeleton

Smooth muscle (SM) structure and function interact over many orders of spatial magnitude, ranging from the centimeter-length scale of vessels to the nanometer-length scale of cytoskeletal proteins. These relationships are maintained *in vivo* in several ways, one of which is the dynamic responsiveness to mechanical forces at the tissue, cellular, and subcellular spatial scales through mechanotransduction, the translation of mechanical stimuli into biochemical reactions within a cell. In one example, cyclic cardiac pumping exposes vascular SM (VSM) (Fig. 1) to a number of mechanical stimuli, such as transmural pressure, vascular shear strain induced by pulsatile pressure, and circumferential wall tension (Osol, 1995). The resultant changes within the VSM are cytoskeletal remodeling (Hayakawa et al., 2001; Cunningham et al., 2002; Gunst and Zhang, 2008), altered membrane conductance (Sparks, 1964; Kirber et al., 1992; Langton, 1993), and biochemical signal activation (Mills et al., 1990; Kulik et al., 1991; Pirola et al., 1994; Cattaruzza et al., 2004), that ultimately lead to functional changes in VSM tone. A well-studied example of this process is the myogenic response, where small arteries contract to counteract increased intraluminal pressure, protecting the blood vessel from potential hypertensive injury (Davis, 2012). Hence, the ability of VSM to sense and respond to mechanical forces experienced in normal physiology and in injury is critical for proper regulation of vascular tone.

It is now widely accepted that the cellular cytoskeleton plays a critical role in mediating mechanotransduction (Wang et al., 1993b; Alenghat and Ingber, 2002;

*Correspondence to: Kevin Kit Parker, Harvard School of Engineering and Applied Sciences, 29 Oxford St, Pierce Hall 321, Cambridge, MA 02138. Fax: 617-496-1793. E-mail: kkparker@seas.harvard.edu

Received 24 May 2014; Accepted 6 June 2014.

DOI 10.1002/ar.22983

Published online in Wiley Online Library (wileyonlinelibrary.com).

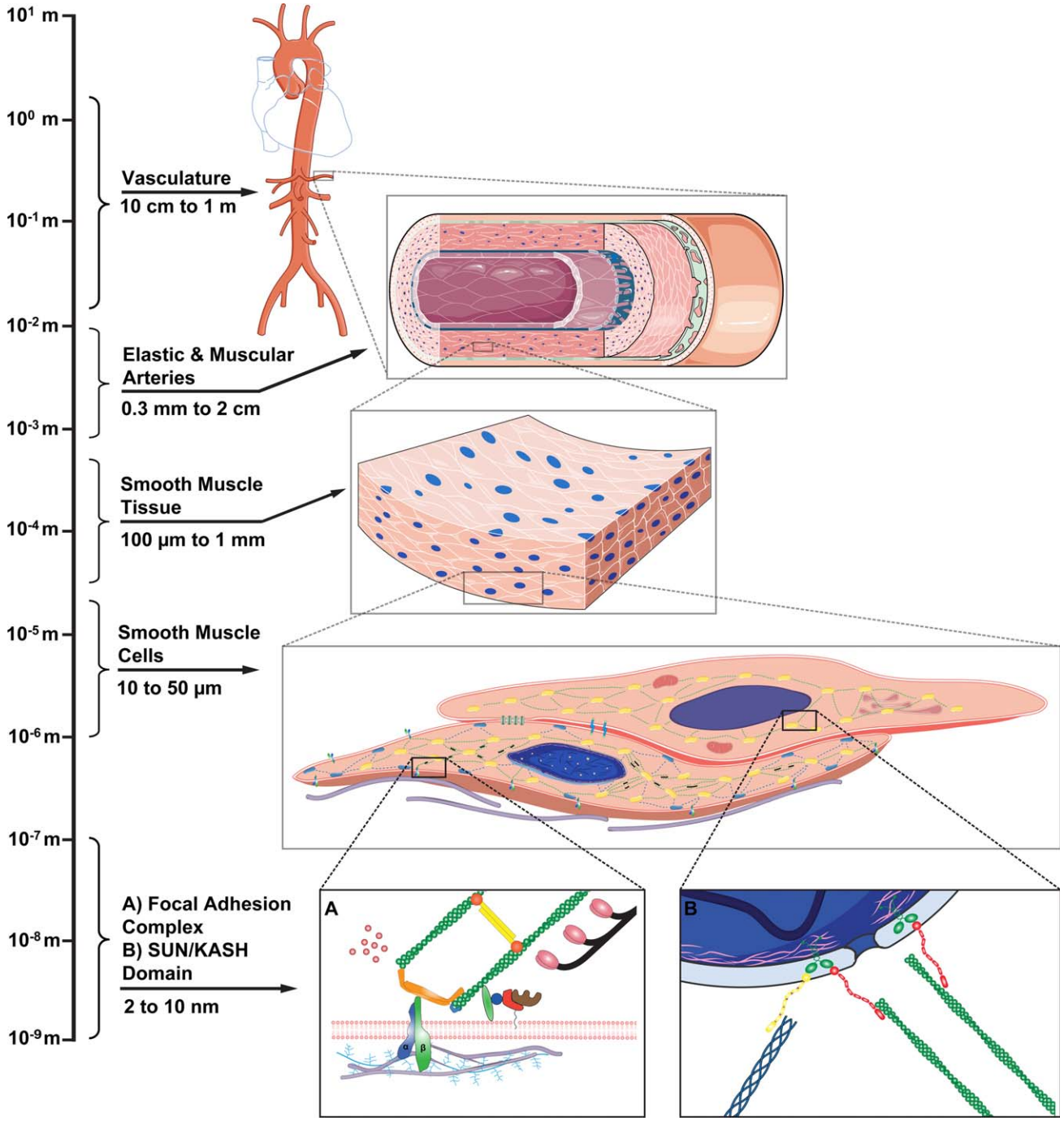

Fig. 1. Hierarchical organization of vascular tissue spans multiple spatial scales from nanometers to meters. Vascular smooth muscle cells assemble into muscle tissue that forms the media layer of the elastic and muscular arteries. The spindle-shaped cells contain nanometer-scale protein complexes that allow it to respond to mechanical cues in the cellular microenvironment. ADAPTED in part from Servier Medical Art (reproduction permitted: http://creativecommons.org/licenses/by/3.0/).

Ingber, 2006). In cardiomyocytes, mechanosensitive proteins embedded in the cytoskeletal network adapt their polymerization states and distributions in response to mechanical cues, which eventually translates into functional changes (McCain and Parker, 2011; Sheehy et al., 2012). In both striated and SM cells, cytoskeletal organization gives rise to cellular architecture; and, redistribution of the cytoskeletal network as a result of mechanotransduction leads to changes in cellular and tissue structure. Hence, understanding the interaction of the mechanotransductive machinery may provide new insights into health and disease.

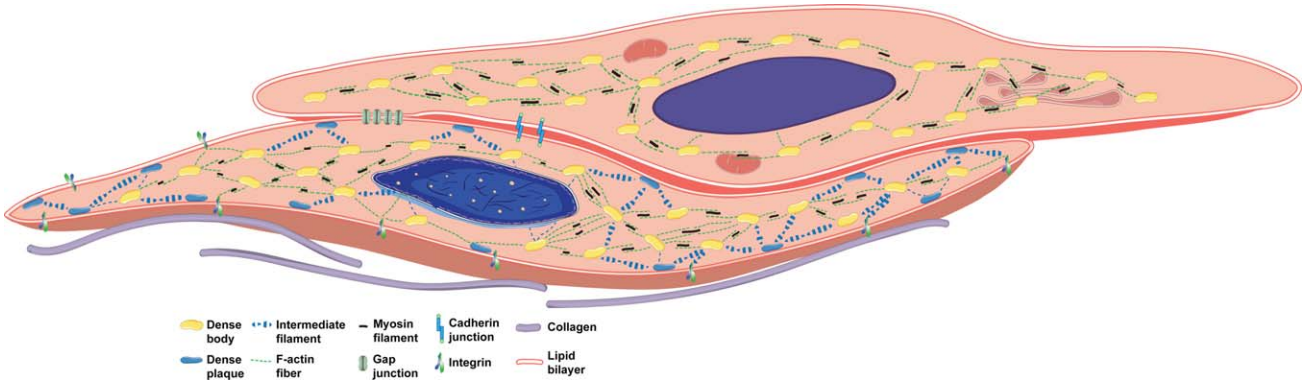

Fig. 2. Mechanotransductive cytoskeletal proteins in vascular smooth muscle cells. Integrin links extracellular matrix proteins such as collagen to actin fibers, allowing extracellular mechanical signals to be directly transmitted into the cell. Actin responds to mechanical input to the cells by rapidly changing the F- to G-actin ratios and also acts as an intracellular sensor. Cadherin junctions provide mechanical links between adjacent cells, allowing forces to be transmitted between cells.

In this review, we examine the contribution of mechanotransduction on VSM cytoskeletal organization and contractile function. We first discuss the collective network of mechanosensitive cytoskeletal proteins in the VSM extracellular, intercellular, and intracellular domains that enable translation and integration of mechanical stimuli into structural or biochemical changes. We then draw on evidence found from *in vitro* studies to show the responses of the VSM cytoskeleton to external mechanical cues and how this lead changes in VSM contractile function.

MECHANOSENSITIVE CONTRACTILE CYTOSKELETON IN VASCULAR SMOOTH MUSCLE CELLS

VSM experiences a wide range of mechanical stimuli throughout the cardiovascular system such as transmural pressure, pulsatile pressure, and shear stress (Osol, 1995). These mechanical signals can propagate within and between cells (Fig. 2). For example, integrin proteins directly connect the extracellular matrix to actin filaments within the cell, allowing forces to be transmitted from outside to inside the cells (Wiesner et al., 2005). Cadherin junctions directly couple adjacent VSM cells (VSMCs) together and propagate mechanical signals from one cell to the next (Philippova et al., 1998). Actin, intermediate filaments, and microtubules propagate mechanical signals through common hubs named dense plaques that are distributed throughout the VSMC cytoplasm (Gimbrone Jr. and Cotran, 1975). In the following sections, we will briefly review the VSMC cytoskeletal components in extracellular, intercellular, and intracellular domains that contribute to mechanotransduction and modulate contractile functions.

Mechanical Signaling through the Integrin-Extracellular Matrix Interface

Integrin proteins are transmembrane, heterodimeric receptors comprising α- and β-subunits. They connect the extracellular matrix (ECM) to the internal cytoskeletons typically clustered at the focal adhesion complex *via* the short cytoplasmic tail of the β-subunit (Fig. 3). Functionally, integrins transduce both "outside-in" and "inside-out" mechanical signals in many different cell types including VSMCs (Baker and Zaman, 2010). To date, 24 integrins have been described and among them, 13 out of 24 are found in VSMCs ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_6\beta_v$) (Glukhova et al., 1991; Moiseeva, 2001). Herein, we discuss how integrin subtypes are sensitive to ECM composition for transducing mechanical stimuli and perform contractile functions requisite for maintenance of proper vascular tone *in vivo*.

In vitro studies using isolated arterioles and VSMCs strongly suggest that integrins are crucial mechanotransductive elements for VSMCs. Wilson and coworkers (1995) demonstrated that the mitogenic response of the VSM to strain was dependent on the composition of the extracellular matrix to which it was adhered. Specifically, culturing VSMCs on fibronectin elicited the most significant mitogenic response to strain which corresponded with increased integrin binding. Further, soluble fibronectin, integrin binding peptide GRGDTP, and antibodies to β3 or αvβ5 integrins all independently blocked the mitogenic response of newborn rat VSMCs normally induced by mechanical strain, while soluble laminin, the inactive peptide GRGESP, and the antibody to the β1 integrin did not alter the mitogenic response to strain. Hence, specific integrin subunits sense and transduce the mechanical strain requisite for induction of the myogenic response in VSM (Wilson et al., 1995). Other studies subsequently showed that an integrin-recognizing synthetic RGD peptide can cause sustained vasodilation (Mogford et al., 1996) and decreased intracellular Ca^{2+} level in rat VSMCs (D'Angelo et al., 1997). These early studies demonstrated that integrins play an important role in transducing mechanical cues to intracellular signals that produce functional adaptive responses. More recently, studies on isolated rat arteriole tissue and VSMCs showed that antibody blocking of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins significantly inhibits myogenic constriction (Martinez-Lemus et al., 2005; Sun et al., 2008). However, pulling on fibronectin and

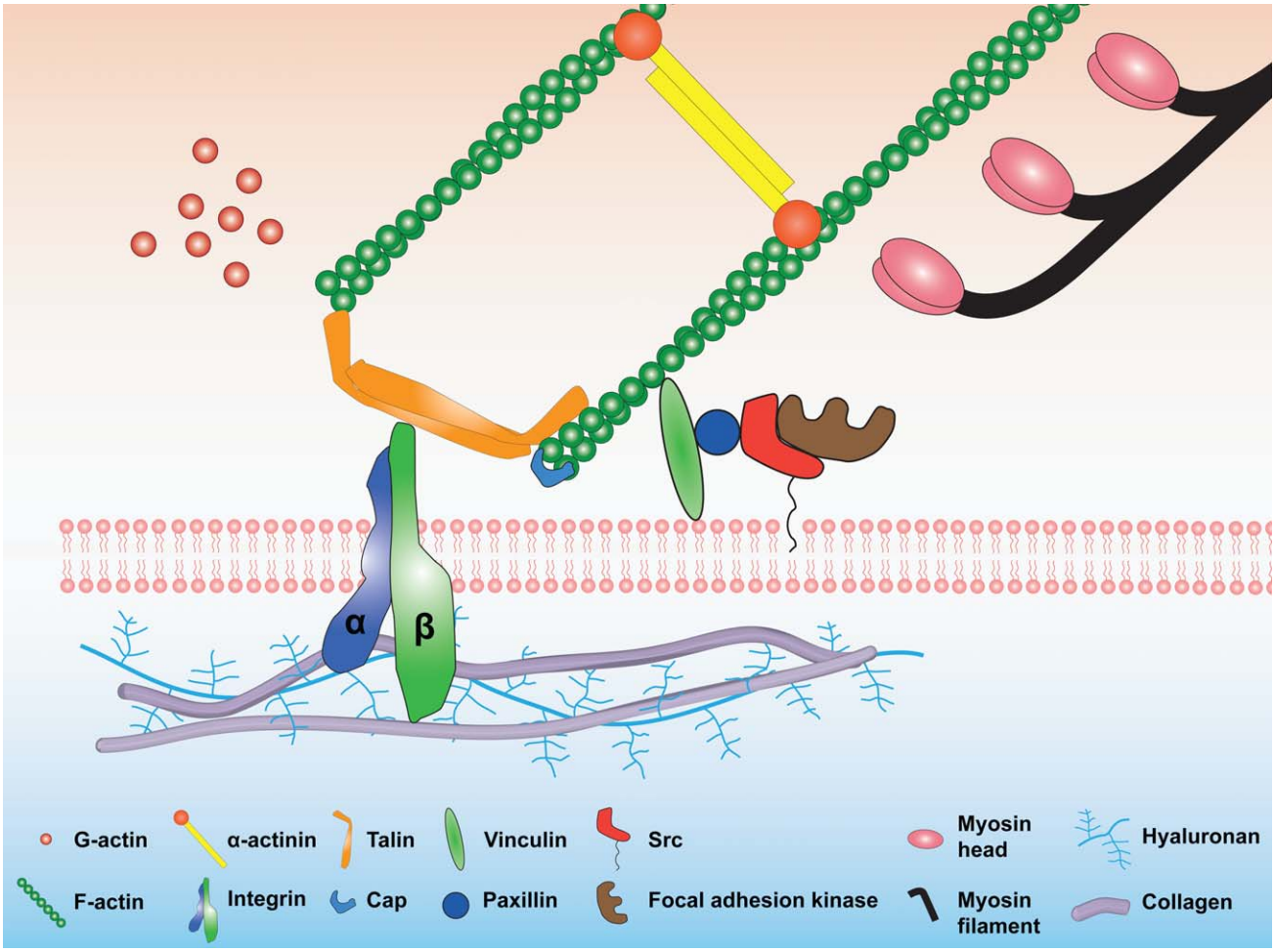

Fig. 3. Mechanotransductive proteins in the focal adhesion complex. The transmembrane protein integrin physically links extracellular matrix proteins such as collagen in the extracellular domain to intracellular structural proteins such as F-actin. This allows mechanical inputs to be transmitted bi-directionally, enabling both "outside-in" and "inside-out" signaling. Cellular components are not to scale.

β_1-integrin antibody-coated magnetic beads on isolated renal VSMCs elicits an increased cellular traction force and sustained traction, analogous to the sustained increase of vascular tone in pressure-induced myogenic response (Balasubramanian et al., 2013). The integrin mechanotransduction mechanism has also been linked to BK_{Ca} ion channel activities and *Src*-dependent pathways (Wu et al., 2008; Min et al., 2012). Collectively, these studies demonstrated that integrins are critical to mechanotransduction and inhibition of integrin function can reduce VSMC contraction.

Mechanical Signaling through Cadherin Intercellular Junctions

In addition to cell–ECM connections, VSMCs in the vascular wall contain a variety of cell-cell adherent junctions, including cadherin and gap junctions (Hill et al., 2009). The cadherin family of calcium-dependent transmembrane receptors is mechanically important: they bind adjacent VSMCs and link them intracellularly to actin filaments *via* catenins, allowing direct force transmission between neighboring cells during cellular contraction (Ganz et al., 2006; Desai et al., 2009; Liu et al., 2010).

VSMCs express multiple cadherins, including N-cadherin, T-cadherin, R-cadherin, cadherin-6b, and E-cadherin (in the case of atherosclerotic lesions) (Moiseeva, 2001). The predominant cadherin, N-cadherin, is expressed at a higher level in human venous smooth muscle cells (SMCs) than in arterial SMCs (Uglow et al., 2000). While N-cadherin has been investigated in the context of VSMC migration (Sabatini et al., 2008), proliferation (Jones et al., 2002), and survival (Koutsouki et al., 2005), Jackson et al. showed that selective blockage of N-cadherin or a cadherin inhibitory peptide in rat cremaster arterioles inhibits myogenic response to pressure changes independent of $[Ca^{2+}]_i$ (Jackson et al., 2010), implicating N-cadherin in mechanical load sensing and arteriolar contraction regulation. T-cadherin was originally identified in a membrane fraction of aortic SMCs (Tkachuk et al., 1998). Unlike classical cadherin family members, T-cadherin does not have transmembrane and cytosolic domains but instead is anchored to

membranes by means of glycosylphosphatidylinositol (GPI). An analysis of Triton-X fractionized human and rat VSMCs revealed that T-cadherin co-localizes with mechanotransducing signaling molecules such as Gαs protein and *Src*-family kinases in caveolin-rich membrane domains (Philippova et al., 1998), suggesting that T-cadherin may function as a local signal-transducing protein as well as an adhesion molecule.

Actins, Intermediate Filaments, and Microtubules in Intercellular Mechanical Signaling

Actin is the most abundant cytoskeletal protein in contractile VSMCs, contributing ~20% of total protein content (Kim et al., 2008). Four of the six vertebrate isoforms of actin are found in VSMCs: α-smooth muscle actin (SMA), β-non-muscle actin, γ-SMA, and γ-cytoplasmic actin. VSMCs in large arteries typically contain about 60% α-SMA, 20% β-non-muscle actin, and about 20% combined γ SM and γ non-muscle actin (Fatigati and Murphy, 1984). Both α- and γ-SMA are commonly referred to as contractile actin because of their association with myosin filaments in generating tension and cell shortening. The two remaining actin isoforms are referred to as cytoplasmic actin and are localized to the cell cortex (Gallant et al., 2011).

Although the precise role of cytoplasmic actin in arteriolar myogenic behavior remains uncertain, growing evidence supports the hypothesis that this subpopulation of actin contributes to VSMC mechanotransduction (Gunst and Zhang, 2008). Earlier studies using pharmacological agents demonstrated that a short exposure period to an actin depolymerizing agent cytochalasin D profoundly suppressed VSM tension development (Adler et al., 1983; Wright and Hurn, 1994; Saito et al., 1996; Cipolla and Osol, 1998), while exposure to an actin stabilizer enhanced myogenic tone (Cipolla et al., 2002), highlighting the critical role of actin polymerization in VSMC contraction and tension development. Independent studies using different techniques have demonstrated that actin polymerization is attributed to a small portion of G- to F-actin transition (Bárány et al., 2001; Cipolla et al., 2002; Flavahan et al., 2005; Srinivasan et al., 2008) that is associated with a redistribution of actin from the cell periphery (cortical region) to the cell interior (Flavahan et al., 2005). More recently, Kim et al. using labeled G-actin monomers, directly observed actin incorporation into cortical filaments upon agonist treatment (Kim et al., 2010) and that the nonmuscle cytoplasmic actin is primarily responsible for the agonist-induced actin polymerization (Kim et al., 2008). Given the known link between F-actin and putative mechanotransductive components such as integrins (Calderwood et al., 2000), cadherins (Yamada et al., 2005), and ion channels (Sharif-Naeini et al., 2009), these results suggest that the cortical non-muscle actin isoforms compose a dynamic subpopulation of actin that allows it to function as an intracellular sensor that actively remodels its polymerization state in response to the level of mechanical force applied to the cells.

In addition to actin fibers, intermediate filaments also function in providing structure and transducing mechanical signals. Intermediate filaments form bundles and associate with dense bodies to provide three-dimensional (3D) integrity to VSMCs (Berner et al., 1981). Two intermediate filament proteins are found in VSMCs, vimentin, and desmin (Berner et al., 1981). Vimentin production is high in VSMCs of large arteries. In human arteries, vimentin localization decreases gradually from proximal to distal, while desmin localization gradually increases (Frank and Warren, 1981; Gabbiani et al., 1981; Johansson et al., 1997). Vimentin- (Schiffers et al., 2000), and desmin-deficient mice (Loufrani et al., 2002) with normal myogenic responses display alterations in vasomotor properties such as agonist sensitivity and impaired flow-dependent dilation, suggesting that vimentin and desmin may be required for sensing mechanical cues in the local microenvironment. A similar dependence on vimentin occurs in airway SMCs. Wang et al. (2006) reported that downregulation of vimentin in canine airway SM attenuates force generation), while Tang et al. showed that airway SM stimulated with contractile agent 5-HT undergoes spatial rearrangement (Tang et al., 2005; Tang, 2008). Collectively, these results suggest that intermediate filaments of vascular and airway SMCs are important for adaptive remodeling to mechanical cues.

Lastly, microtubules are the cytoskeletal proteins that provide resistive forces in many cell types and are considered the compression bearing elements (Wang et al., 1993a). Since the ability to adequately stain and detect polymerized microtubules in dense contractile tissue depends on the tissue type and staining method (Yamin and Morgan, 2012), it is not surprising that contradicting findings on the role of microtubules in mechanotransduction have been reported for VSMCs. For example, one study showed that depolymerization of microtubules causes vasoconstriction in rat cremaster arterioles when pressurized intravascularly. Furthermore, this response involves Rho-A dependent Ca^{2+} sensitization without an overt increase in $[Ca^{2+}{}_i]$ (Platts et al., 2002), suggesting that regulation of microtubule dynamics may be directly linked with VSMC contraction and reactivity. However, in another study where porcine coronary arteries were used, a higher level of isometric force was associated with an increased level of intracellular calcium in porcine coronary VSMCs when treated with microtubule depolymerizing agent (Paul et al., 2000), suggesting that microtubules may modulate Ca^{2+} signal transduction. These studies suggest that microtubules play a role in regulating both calcium-independent and calcium-dependent contraction in SM.

Mechanical Functions of the Nucleus

There is emerging evidence that suggests the nucleus of SMCs can also respond to mechanical signals and participate in contractile activities. Unlike skeletal muscle, SMCs have a single, centrally located nucleus that typically takes on an elongated "cigar shape." The nucleus was recently found to interact directly with the cytoskeleton *via* nuclear membrane proteins such as the SUN/KASH domain proteins (Wilhelmsen et al., 2005; King et al., 2008; Xiong et al., 2008) (Fig. 4). This physical linkage allows mechanical forces exerted on the surface adhesion receptors to be transmitted along the cytoskeletons to the protein complexes in the cytoplasm and nucleus (Wang et al., 2009). Further, Kuo and Seow (2004) utilized electron microscopy to show that

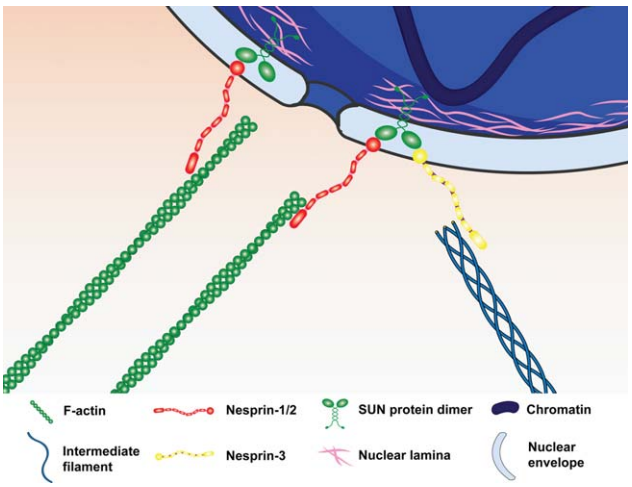

Fig. 4. Force transmissions via cytoskeleton to the nucleus. F-actin stress fibers and intermediate filaments are connected to the SUN protein dimers via the Nesprin-1/2 and Nesprin-3 protein complexes. SUN proteins bind to nuclear lamina and other nuclear envelope proteins, which are connected to DNA and chromatin inside the nucleus. These proteins couple the cytoskeleton mechanically to the nucleus, allowing mechanical signals to directly influence chromatin remodeling and cellular contraction.

contractile filaments of airway SM are arranged parallel to the longitudinal axis of the cell and centrally attach to the nuclear envelope, effectively making the nucleus a force-transmitting structure. Similar findings were observed by Nagayama et al. (2011) in aortic SMCs: stress fibers stabilize the position of intranuclear chromatin through mechanical connections with the nucleus, which in turn modulates gene and protein expression in VSMCs and alters functional behavior. Taken together, these studies suggest that the nucleus may play a role in SM mechanotransduction and force transmission during SMC contraction.

Mechanotransduction Disruption in Disease

The process of mechanotransduction can be disrupted by dysfunction of each of these mechanosensors discussed and ultimately result in disease. One example of a clinical manifestation that results due to an abnormality in one of the proteins in the mechanotransductive pathway of vascular SM is thoracic aortic aneurysm and dissection (TAAD). In TAAD, the longitudinally oriented VSM layer degenerates leading to a loss of regulation of blood flow and pressure (Milewicz et al., 2008). Diseases of the extracellular matrix such as Marfan syndrome (Milewicz et al., 2008; Tadros et al., 2009) and Ehlers-Danlos (Pepin et al., 2000) have long been known for the clinical manifestation of TAAD. In these syndromes as well as other cases of TAAD, a switch from a contractile phenotype to a synthetic phenotype in VSMCs is observed leading to subsequent dilation of the aorta (Lesauskaite et al., 2001; Huang et al., 2010). More recently, genetic mapping studies have found mutations in myosin heavy chain 11 (Zhu et al., 2006; Pannu et al., 2007) and SM α-actin (Guo et al., 2007) also lead to TAAD. These mutations resulted in decreased contractile function and loss of regulation of blood pressure (Schildmeyer et al., 2000). These genetic diseases demonstrate the concerted action of the ECM and cytoskeletal proteins is required for VSM to properly maintain vascular tone.

In summary, the extracellular, intercellular, and intracellular components of the VSMC cytoskeleton are embedded with proteins and filaments that are able to detect mechanical stimuli from the ECM, adjacent cells, or within the cytoplasm. Sensing these stimuli allows the cell to activate signaling pathways that promote structural remodeling of its cytoskeleton to offset or adapt to mechanical loading, forming a mechanotransduction feedback loop. When an abnormality exists within these mechanosensing proteins, cytoskeletal organization and function may undergo maladaptive remodeling resulting in disease.

MECHANOTRANSDUCTION FEEDBACK REGULATES CONTRACTILE CYTOSKELETON ARCHITECTURE

VSMCs are exposed to a wide range of mechanical signals from its extracellular microenvironment in physiological and pathological settings including cell shape deformations, pulsatile stretching, ECM rigidity, and subcellular surface topography. Due to the aforementioned mechanotransductive nature of the contractile cytoskeletal proteins, mechanical stimuli can regulate both cytoskeletal architecture and contraction. As a result, cytoskeletal proteins are tightly regulated spatiotemporally to ensure proper VSMC structure and function in normal physiological settings. To recapitulate desired VSMC structure and function, investigators exploit *in vitro* models to control mechanical parameters in the extracellular microenvironment. Here, we will summarize the influence of different mechanical cues on the VSMC cytoskeletal architecture.

Smooth Muscle Cell Shape

The shape of SMCs was observed to be dynamic during physiological and pathological developments; and, changes in SMC shape are closely associated with functional modulation. For example, irregular and more rounded VSM was found in muscular arteries of patients affected with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a hereditary vascular dementia characterized by a cerebral nonatherosclerotic, nonamyloid angiopathy that mainly affects the small arteries penetrating the white matter (Joutel et al., 1996). Recent advances in cellular engineering have enabled reproducible and precise studies of the role of cell shape in mechanotransduction (Borenstein et al., 2002; Park and Shuler, 2003; Parker et al., 2008). Our group has utilized microcontact printing (μCP) to micropattern ECM proteins on substrates to create user-defined cell-adhesive patterns that produce cells with various shapes (Kuo et al., 2012; McCain et al., 2012; Agarwal et al., 2013; McCain et al., 2013). More recently, our group engineered VSM tissues of varying widths by constraining the line width of micropatterned fibronectin and laminin proteins (Alford et al., 2011b). We found that, while the alignment of F-actin stress fibers is similar, the nuclear eccentricities of constituent VSMCs

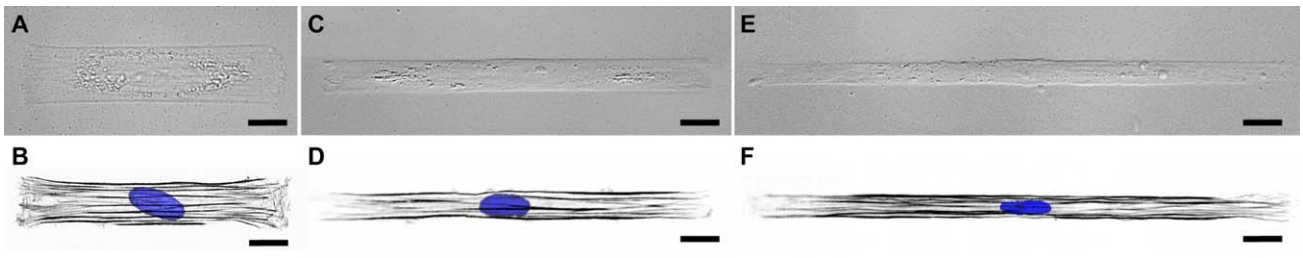

Fig. 5. Cellular shape directs cytoskeletal architecture. Engineered vascular smooth muscle cells with length-to-width aspect ratios of 5:1 (**A-B**), 10:1 (**C-D**), and 20:1 (**E-F**) self-assemble their cytoskeletons based on the boundaries of the micropatterned fibronectin. (A, C, E) are differential interference contrast images of engineered cells. (B, D, F) are stained with phalloidin for F-actin (black) and DAPI for nuclei (blue). Actin fibers and nuclei became increasingly aligned with the principle axis as cellular aspect ratio increased. Projected nuclear area also decreased as cell became more elongated. The remodeling of cytoskeleton as a result of cell shape re-distributes its mechanotransductive components and can lead to different contractile functions during healthy and diseased environment. (A–F) scale bars = 20 μm.

significantly correlates with cell shape with length-to-width aspect ratios (ARs) between 20:1 and 50:1 (Alford et al., 2011b). To investigate the shape-contractility relationship more rigorously, we recently engineered single VSMCs on fibronectin islands with ARs from 5:1 to 20:1 and quantified their F-actin alignment by measuring the orientational order parameter (OOP) and nuclear eccentricity (Fig. 5). In contrast to VSM tissues, we found that isolated VSMCs with higher ARs had increased OOP and nuclear eccentricity, suggesting elongated cell shape leads to more aligned stress fibers and elongated nuclei (Ye et al., 2014). Thakar et al. showed that bovine VSMCs cultured on micropatterned collagen strips with elongated cell shape have decreased expression of actin stress fibers and α-actin on narrower strips (Thakar et al., 2003). They also reported that elongated cell shape lowers the nuclear shape index of isolated VSMCs while reduced spreading area significantly reduces nuclear volume (Thakar et al., 2009). When isolated rat VSMCs were cultured on user-defined cell adhesive patterns fabricated by plasma lithography, Goessl and colleagues observed cell shape-dependent actin formation and nuclear shape change (Goessl et al., 2001). When rat VSMC volume was changed in 3D through hyperosmotic shrinkage or hyposmotic swelling, a dramatic elevation of F- to G-actin ratio was observed (Koltsova et al., 2008), suggesting that actin polymerization occurs in response to cell shape changes in 3D. Thus, these reports demonstrate that cellular shape and cytoskeletal architecture direct the location and organization of mechanosensitive components including stress fibers and nucleus, suggesting one potential mechanistic pathway in which cell shape changes in two dimension (2D) and 3D are translated to functional differences in VSMCs.

Pulsatile Stretching

The pulsatile nature of the vasculature exposes VSMCs to cyclic stretching in their native environment. Using ultrasonography and other methods, direct observation of the vasculature *in vivo* demonstrated that each cardiac cycle can radially strain human arteries, arterioles, and veins between 6 and 22%, with more distention experienced by larger, proximal arteries (Lyon et al., 1987; Wijnen et al., 1990; Laurent et al., 1992; Alfonso et al., 1994). These observations generated interest in the effect of stretching on VSMC behavior *in vitro*. Cyclic stretching on rat VSMCs *in vitro* produces rapid reorganization of stress fibers perpendicular to the stretching direction (Hayakawa et al., 2001). Longitudinal stretching of the vascular wall induces actin polymerization (Albinsson et al., 2004). In addition, cyclic stretching in rat VSMCs leads to increased expression level of insoluble focal adhesion contact components (Cunningham et al., 2002), paxillin, and vinculin (Na et al., 2008), suggesting that cyclic stretching may strengthen the number and size of focal adhesion complexes. These findings indicate that mechanical stimulation in the form of cyclic stretching can remodel the state and organization of actin stress fibers and focal adhesions, which may subsequently feed back to VSMC functional changes.

Extracellular Matrix Interactions

ECM components influence VSMC phenotype and functions like migration, proliferation, and contraction *in vitro*. Concomitantly, significant changes in cytoskeletal organization and expression have also been reported. One early study reported that isolated rat VSMCs cultured on laminin develop significantly fewer focal adhesions than cells cultured on fibronectin (Hedin et al., 1997). Another found different amounts of myofilament expression in rabbit VSMCs cultured on interstitial matrix (collagen I and fibronectin), basal lamina protein (collagen IV and laminin), and the serum adhesion protein vitronectin (Hayward et al., 1995). In addition, immunofluorescent staining of stress fibers with antibodies against α-actin, myosin heavy chain isoform SM2, and vimentin, revealed that stress fiber expression of VSMC cultured on fibronectin coated substrate over a 5-day culture period gradually reduced with time (Qin et al., 2000), suggesting that ECM can mediate active remodeling of cytoskeleton. More recently, distinct morphologies of actin organization and focal adhesion formation were found on VSMCs cultured on different ECM components (Lim et al., 2010). Specifically, for VSMCs cultured on fibronectin and collagen IV, cytoskeletal stress fibers organize along the long axis of the cell and tight bundles occur along the periphery; whereas this stress fiber organization is less typical for cells cultured on collagen I and laminin. In addition, rounded focal adhesions are induced by fibronectin, while elongated

morphology is more common for collagens. Furthermore, a significant decrease in both F-actin and vinculin area occurs only for cells on fibronectin matrix. These studies demonstrated that ECM regulates the assembly and organization of cytoskeleton in VSMCs.

Microenvironmental Stiffness

In a large number of cardiovascular diseases involving VSMCs, such as hypertension and atherosclerosis, the stiffness of the diseased blood vessels is dramatically altered (Niklason et al., 1999). Changes in substrate stiffness in 2D and 3D culture systems lead to VSMC cytoskeletal remodeling. Peyton and coworkers have shown that human VSMCs cultured on 2D polyacrylamide gels with a range of stiffness from 1.0 to 308 kPa display more visible F-actin bundles and punctate focal adhesion sites on a rigid substrate compared to cells cultured on soft substrates (Peyton and Putnam, 2005). In the same study, they also demonstrated that an intermediate stiffness produces an intermediate amount of fibers and focal adhesions. Extending those findings using a poly(ethylene glycol)-conjugated fibrinogen-based 3D culture system with compressive modulus between 448 and 5804 Pa, the group observed a higher level of F-actin bundling on VSMCs on stiff matrices after 14 days in culture (Peyton et al., 2008). These results suggest that VSMCs actively adapt to stiffness in the microenvironment by remodeling stress fiber and focal adhesion organization. This may provide insight into the mechanism of increased rates of hypertension associated with vascular stiffening in aging patients.

Extracellular Surface Interactions

VSMCs in their native environment in the vessel wall also interact with micro- and nanoscaled features such as pores, fibers, and ridges on the basement membrane (Abrams et al., 2000). Studies that mimic these micro- and nanoscale topographies *in vitro* have reported active remodeling of cellular cytoskeleton. VSMCs seeded on nanopatterned gratings of poly(methyl methacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS) assume elongated cell and nuclei shapes (Yim et al., 2005). VSMCs cultured in microchannels with channel widths of 20, 30, 40, 50, and 60 μm display highly aligned actin filaments and elongated nuclei on narrower microchannels (Glawe et al., 2005). More recently, Taneja and coworkers evaluated the effect of 13 μm 316L stainless steel microgrooved surface on VSMC phenotypic changes to understand how topography of endovascular stent contributes to restenosis (Taneja et al., 2011). They found that microgrooved surfaces induce significant cell elongation in addition to significantly higher levels of α-actin expression (Taneja et al., 2011). These studies suggest that micro- and nanoscaled topographical features can significantly alter the shape of both cell and nucleus and lead to cytoskeletal remodeling.

In Vivo Relevance

When stimuli deviate from the normal range experienced in health, maladaptive remodeling occurs in the cytoskeletal architecture and leads to diseased function in VSMCs. For example, in the case of vascular aging, wear and tear from cardiac cycling causes fatigue and fracture in the elastic fibers, promoting degeneration of the media layer and vessel stiffening (Lee and Oh, 2010). These changes in the ECM composition and substrate stiffness increase VSMC stiffness by increasing their adhesion molecule expression (Intengan and Schiffrin, 2000; Qiu et al., 2010) and drive the system away from healthy conditions and toward cardiovascular diseases.

Aberrant mechanical stimuli can also be modeled *in vitro* to more rigorously study the maladaptive remodeling that occurs in disease (Brown, 2000; Balachandran et al., 2011; Hemphill et al., 2011; Huh et al., 2012; McCain et al., 2013). Alford et al. modeled the cerebral vasospasm that occurs in some instances of traumatic brain injury (Alford et al., 2011a). In this *in vitro* model, human VSM was engineered on a flexible membrane and was subsequently subjected to acute tensile strain prior to performing studies of protein expression, structure, and contractile function. In this study, 10% strain was shown to induce hypercontraction in response to endothelin-1 1 hr after blast relative to the control; but, 24 hr after the blast, the engineered tissue was less contractile compared to the control. When the protein expression of smoothelin and SM myosin heavy chain were compared at the 1 and 24 hr time points, a decreased in expression of these cytoskeletal proteins was observed indicating remodeling in response to a mechanical stimuli, modeling both the acute hypercontraction as well as the chronic dysfunction seen in blast-induced cerebral vasospasm.

In summary, *in vitro* studies have enabled researchers to understand the effect of individual mechanical cues on VSMC cytoskeletal organization. These studies suggested that VSMC cytoskeleton is a dynamic network that constantly integrates mechanical cues and adapts its architecture accordingly to achieve homeostasis. These processes are also linked to many regulatory proteins within the cell, indicating that they are responsible for regulating a wide range of cellular functions.

SUMMARY

In summary, cytoskeleton proteins embedded in the extracellular, intracellular, and intercellular domains equip VSMCs with mechanosensing and mechanostransducing capabilities. This allows VSMCs to detect changes in the extracellular mechanical stimuli including tensile stress, cellular boundary, substrate stiffness and topography, in the forms of "outside-in" signaling. In response to these changes, VSMC cytoskeleton remodels by changing the rate of polymerization, distribution, and protein associations to adapt to the extracellular boundaries and external mechanical loads. This ultimately leads to differential activation of signaling pathways that mediates changes in VSMC functions such as proliferation, migration, contraction and gene expression. When the signaling pathways become disrupted under nonphysiological settings, maladaptive remodeling in cytoskeleton occurs and diseased function manifests.

Extensive studies with focuses on pharmacological perturbations on VSMCs *in vitro* and *ex vivo* provided a wealth of information on the functional outcomes of these inputs including protein expression profile, contractility and proliferation. However, little is known on the effect of these perturbations on cellular architecture and organization. While functional findings are

informative for treating vascular diseases, understanding mechanistically the role cytoskeleton played in sensing and transducing these changes allows investigators to directly target and correct maladaptive responses in the cytoskeleton to achieve the desired functions. From a vascular tissue engineering perspective, knowing the relationship between VSMC cytoskeleton and function equip investigators with new tools to design and build not only mechanically stable, but also functionally active vascular graft. This is particularly relevant for engineering small-diameter vascular grafts, where functional VSM tissue may improve graft patency and long-term survival. To achieve this, future studies focusing on understanding the mechanism between VSMC cytoskeleton and function interplay will be required.

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