SECTION B

Impact of Nuclear Mechanics on Function

CHAPTER 8

Mechanical Induction of Gene Expression in Connective Tissue Cells

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Abstract

The extracellular matrices of mammals undergo coordinated synthesis and degradation, dynamic remodeling processes that enable tissue adaptations to a broad range of environmental factors, including applied mechanical forces. The soft and mineralized connective tissues of mammals also exhibit a wide repertoire of mechanical properties, which enable their tissue-specific functions and modulate cellular responses to forces. The expression of genes in response to applied forces are important for maintaining the support, attachment, and function of various organs including kidney, heart, liver, lung, joint, and periodontium. Several high-prevalence diseases of extracellular matrices including arthritis, heart failure, and periodontal diseases involve pathological levels of mechanical forces that impact the gene expression repertoires and function of bone, cartilage, and soft connective tissues. Recent work on the application of mechanical forces to cultured connective tissue cells and various *in vivo* force models have enabled study of the regulatory networks that control mechanically induced gene expression in connective tissue cells. In addition to the influence of mechanical forces on the expression of type 1 collagen, which is the most abundant protein of mammals, new work has shown that the expression of a wide range of matrix, signaling, and cytoskeletal proteins are regulated by exogenous mechanical forces and by the forces generated by cells themselves. In this chapter, we first discuss the fundamental nature of the extracellular matrix in health and the impact of mechanical forces. Next we consider the utilization of several, widely employed model systems for mechanical stimulation of cells. Finally, we consider in detail how application of tensile forces to cultured cardiac fibroblasts can be used for the characterization of the signaling systems by which mechanical forces regulate myofibroblast differentiation that is seen in cardiac pressure overload.

I. Introduction

In mammals, connective tissues surround and are distributed throughout organs including liver, heart, lung, and kidney. Connective tissue cells synthesize and maintain extracellular matrices and provide mechanical support and attachment for contiguous tissues and organs. The cells embedded in soft or mineralized connective tissues live in specialized environments in which they experience tissue-specific chemical signals. They may also be subjected to various mechanical forces. In solid tissues, because of their surrounding extracellular matrices, cells likely can sense and respond to mechanical forces in ways that are quite different than the forces that impact, for example, blood cells flowing past endothelial cells (Tzima *et al.*, 2005).

In general, cells of connective tissues are mechanically adapted to the rheological properties of the extracellular matrix and their responses to mechanical stimuli are strongly affected by the matrix proteins that surround them. In this chapter, which focuses on mechanical signaling to mediate gene expression, connective tissues

provide critical physical and biological elements for transmission of gravitational and muscular forces. For example, connective tissues anatomically and functionally join muscles to bone, thereby enabling force transfer between these two tissues. Another important aspect of the extracellular matrix is its contribution to stabilization of tissues that are subjected to physical forces. This general property enables organs and tissues to preserve their shape and helps to prevent cellular damage induced by mechanical forces.

II. Extracellular Matrix Environment

The extracellular matrices of connective tissues are comprised of collagen fibers and a large gruop of other fibrillar and globular proteins, which may include fibronectins, laminins, glycosaminoglycans, tenascins, and several other glycoproteins. In mineralized connective tissues, highly ordered arrays of hydroxyapatite crystals that are distributed throughout a soft connective tissue matrix, can display a wide range of stiffness values. For example, cortical bone is much stiffer than cartilage. For all connective tissues, collagen is the principal molecular building block and indeed fibrillar collagen is the most abundant protein in mammals (Perez-Tamayo, 1978). Further, in soft connective tissues, collagen fibrils can transmit tensile forces (Provenzano and Vanderby, 2006) to fibroblasts and many other cell types in which there is appropriate expression of collagen receptors. Depending on the structure and makeup of the proteins in connective tissues, extracellular matrices are well adapted to transmit forces and to protect cells against a wide variety of mechanical loads. These loads could include tensile, compressive, and/or shearing forces (Warden *et al.*, 2005).

When connective tissues are subjected to increased loading, there is enhanced remodeling of connective tissue matrices (Ozaki *et al.*, 2005) and increased proliferation of fibroblasts and osteoblasts. These cells are the principal mesenchymal cell type of soft and mineralized connective tissues, respectively. Their sensitivity to mechanical forces facilitates force-induced remodeling of extracellular matrices. It is evident from many *in vivo* studies that mechanical forces do indeed regulate gene expression in connective tissue cells. Further, the mechanical properties of the extracellular matrix itself have an important influence on the morphology and function of osteoblasts and fibroblasts (Hinz and Gabbiani, 2003). For example, when collagen lattices are subjected to mechanical forces, a “synthetic” fibroblast phenotype emerges, which is characterized by increased expression of connective tissue matrix proteins and inhibition of matrix protein degradation (Kessler *et al.*, 2001). In experiments that employ three-dimensional collagen gels, mechanical loading influences matrix remodeling (Mudera *et al.*, 2000). Accordingly, while the extracellular matrix can transmit mechanical forces, its structure and protein composition is affected by the cellular responses to the forces that are applied or that are generated by the cells themselves.

III. Mechanical Signaling

A. Systems

Much is known of how soluble chemical ligands, after binding to cognate receptors, activate cellular signaling pathways. In contrast, there is less definitive knowledge on transduction of mechanical signals although it is well recognized that physical forces impact the metabolic responses of many tissues including the stimulation of bone formation (Martin, 2007; Skerry and Suva, 2003; Turner and Robling, 2004), the remodeling of the periodontium during orthodontic treatment and dental occlusal trauma (Krishnan and Davidovitch, 2009; Rygh, 1973), the induction of cardiac hypertrophy by volume or pressure overload (Catalucci *et al.*, 2008; Tarone and Lembo, 2003), the generation of ventilator-induced injury in lung (Lionetti *et al.*, 2005; Stenqvist *et al.*, 2008), and the sensations of pain (Lewin and Moshourab, 2004; Tsunozaki and Bautista, 2009) and hearing. Currently, the molecular identity of specific mechanotransducers has not been defined but analysis of genetic models of mechanotransduction in *Caenorhabditis elegans* (Syntichaki and Tavernarakis, 2004) and a large number of mammalian cell models (Tsunozaki and Bautista, 2009) have suggested several possible mechanisms. One possible mechanism of mechanotransduction invokes force–activation of mechanosensitive plasma membrane channels (Kiselyov and Patterson, 2009; Martinac, 2004), thereby allowing inflow of Ca^{2+} that can act as a second messenger to regulate gene expression. Recent evidence indicates that one family of channels, the transient receptor potential channels, play central roles as specific mechanosensitive channels in hearing and mechanosensation (Corey, 2003; Yin and Kuebler, 2010), but how mechanically gated channels are regulated by forces acting on the cell surface and how these signals are translated into biological outcomes is not defined. The potential therapeutic importance of mechanically gated ion channels is underlined by their identification as possible drug targets for a variety of diseases involving dysregulated mechanotransduction (Cortright and Szallasi, 2009;Gottlieb *et al.*, 2004).

B. Cell Contacts

In connective tissues, direct transfer of forces to cells may involve cell-to-cell and/or cell-to-matrix contacts (Chen *et al.*, 2004). One of the cell surface receptors that bind to matrix molecules, the integrins, are of particular importance because they functionally integrate cell adhesion and cell signaling processes, and because they may be able to transfer forces from the extracellular matrix to the cytoskeleton (Chiquet *et al.*, 2009; Katsumi *et al.*, 2004). While specific macromolecular platforms may provide cells with the ability to respond specifically to mechanical stimuli (Helmke and Schwartz, 2004), the proteins which comprise the force sensor and effector systems are not defined. This is important since tissue and organ dysfunction is mediated by high-amplitude/high-frequency applied forces to cell surface receptors, including the integrins (Ingber, 2003a; Thodeti *et al.*, 2009). Among the cellular elements that are thought to

contribute to mechanotransduction, the cytoskeleton is of particular interest because it can transmit cellular forces, contributes to the information processing of mechanically induced signals and may protect cells against damage induced by excessive force levels (Janmey and Weitz, 2004).

Cells in connective tissues adhere to extracellular matrices by a wide variety of matrix receptors. Under certain circumstances these receptors can become clustered into aggregates, which, in cultured cells, are termed focal adhesions or focal complexes. These protein complexes are potential sites for transfer of contractile forces to the cytoskeleton in cultured cells and possibly for cells in tissues (Ingber, 2003b). Because of the ease of culturing fibroblasts and for then applying a wide variety of exogenous forces to these cells, cultured fibroblasts are now widely used models for exploring mechanosensing and force response mechanisms in solid tissues. In fibroblasts, force transmission is critically dependent on the attachment of cells to matrix molecules such as fibrillar collagen or fibronectin applied to either the culture substrate (Hinz, 2006) or beads. With this methodology, mechanical induction of gene expression is experimentally testable: tensile forces applied through matrix proteins like collagen (but not poly-L-lysine-coated beads) promote increased expression of the actin-binding protein filamin A (D'Addario *et al.*, 2002).

Trans-membrane proteins can activate intracellular biochemical signaling pathways either by binding an extracellular ligand (chemical signaling) or when they are unfolded or otherwise deformed by force (mechanical signaling). Thus, the adhesive functions of attachment molecules such as integrins and cadherins are key elements in mechanosensing and mechanotransduction. Integrins are enriched in the extensively studied focal adhesions described above. Focal adhesions are multimolecular complexes consisting of more than 50 different proteins that link extracellular matrix-attached integrins to the actin cytoskeleton (Geiger and Bershadsky, 2002). The assembly and maintenance of focal adhesions depend in part on local mechanical forces. These forces may be generated by myosin II-driven contraction of the actin cytoskeleton or by stretching forces originating from the extracellular matrix. Force-induced assembly of focal adhesions leads to activation of a variety of signaling pathways that control cell proliferation, differentiation, the organization of the cytoskeleton, and the expression of specific genes.

While attachment of connective tissue cells to the extracellular matrix is generally reliant on the formation and remodeling of integrin-mediated adhesions, connective cells can also adhere to each other by intercellular adhesive molecules (e.g., cadherins) that may also act as force sensors (Ko *et al.*, 2001) and possibly be able to regulate gene expression. As *N*-cadherin-mediated adherens junctions are influenced by integrin biology, fibroblasts may be able to integrate mechanical signals from both adhesion systems to coordinate gene responses relevant to differentiation, organogenesis, and wound healing (Linask *et al.*, 2005). Notably, several reports have described mechanical signaling through both cadherin and integrins (Ko *et al.*, 2001; Potard *et al.*, 1997). Consequently, mechanotransduction, may not be a single, restricted process but may instead be a chain of interrelated processes that require the recruitment of a large variety of attachment, cytoskeletal, and signaling proteins. These proteins may then be

able to form docking and signaling complexes that are appropriately oriented in time and space to optimize transmission and processing of mechanical signals.

IV. Overview of Methods for Mechanical Cell Stimulation

Here, we provide an overview of different culture methods that are currently used to study mechanotransduction at the cellular and subcellular levels. More detailed technical descriptions are covered in excellent recent reviews (Brown, 2000; Jonas *et al.*, 2008; Kim *et al.*, 2009; Lele *et al.*, 2007). As introduced above, mechanical cues determine cell fate, phenotype, and behavior (Bao and Suresh, 2003; Chen, 2008; Discher *et al.*, 2005; Hoffman and Crocker, 2009; Ingber *et al.*, 2006; Janmey and McCulloch, 2007; Janmey and Weitz, 2004; Orr *et al.*, 2006; Vogel and Sheetz, 2006; Wang and Thampatty, 2008). When studying cell "mechanoperception" it is important to consider that different cells types are exposed to different qualities and quantities of mechanical load *in vivo*. As mentioned above, circulating blood cells and endothelial cells of the vascular wall experience fluid flow shear stress (Makino *et al.*, 2007), which is very different than the compressive and tensile forces that are sensed by cells in solid connective tissues. Thus while cells residing in bone and cartilage are under compressive load (Adams, 2006; Turner, 2006), a large number of different cell types are subjected to stretching forces. For example, cyclic stretch and compression are characteristic mechanical stimuli for cardiomyocytes (Lammerding *et al.*, 2004) and endothelial and smooth muscle cells in the vessel wall (Halka *et al.*, 2008; Reinhart-King *et al.*, 2008; White and Frangos, 2007), of the intestine (Jones and Bratten, 2008), and of the airways (Choe *et al.*, 2006; Hasaneen *et al.*, 2005; Pugin, 2003). Skeletal muscle cells, connective tissue fibroblasts, and epidermal keratinocytes are subject to rather more gradual stretching of various degrees and rates (Chiquet *et al.*, 2003; Hinz, 2010; Reichelt, 2007). We will describe below several different systems to manipulate cells mechanically, consider their potential pitfalls and provide simple guidelines on how to select the appropriate instruments from this mechanical toolbox.

A. Shear Forces

Fluid flow is commonly applied to expose cultured cells to shear forces. The physiological relevance of fluid flow shear force is most obvious for hematopoietic cells transported in the bloodstream and for cells lining the inner surfaces of fluid-filled cavities, including endothelial cells of blood and lymphatic vessels but also epithelial cells in the respiratory and gastrointestinal tract. Two major construction principles exist for two-dimensional culture systems: (1) the cone-and-plate system where rotation of a cone-shaped body over a flat culture plate sets the cell culture medium in turbulent motion (Chung *et al.*, 2005; Dewey, 1984) and (2) parallel plate flow chambers in which cells are grown in flow channels of defined dimensions (Bacabac *et al.*, 2005; Usami *et al.*, 1993). Variations of the second device are more widely used, allowing precise control over flow and corresponding shear rates.

Flow-induced shear stress also acts on cells populating three-dimensional tissue environments, such as chondrocytes, osteoblasts, and osteocytes. High levels of shear are generated in interstitial fluid with these models by the compression of the porous tissue structure (Chen *et al.*, 2010; Fritton and Weinbaum, 2009). This can be recapitulated in culture by perfusing cell-containing three-dimensional porous scaffolds, which are produced from various materials infused with medium (Brown, 2000; Datta *et al.*, 2006; Griffith and Swartz, 2006; Stiehler *et al.*, 2009). Notably, even the very small interstitial flow rates that occur during tissue swelling, microvascular permeability, and lymphatic drainage, have profound influences on the behavior of fibroblasts, tumor cells, and inflammatory cells (Rutkowski and Swartz, 2007). It is unlikely that such low flow rates produce shear forces that are sensed by the cells. Instead, cellular responses are generally explained by directed solute transport (Fleury *et al.*, 2006). Indeed, transport processes always have to be considered as "contaminating" parameters in flow experiments. Further, it is important to consider that shear forces are calculated on the basis of the applied flow rates and the dimensions of the fluid-filled channels, which define the pressure differential between the inlet and the outlet. This is an accurate approximation for two-dimensional flow chambers and for cells grown in flat monolayers but requires extensive mathematical modeling for more complex three-dimensional materials (Anderson and Knothe Tate, 2008; Porter *et al.*, 2005).

B. Compression

Mechanically loading of cells in three-dimensional environments not only produces fluid flow and shear stress but also produces a compressive component. Compression devices are principally used to study the mechanobiology of chondrocytes, osteocytes, and osteoblasts. Physiological challenge of cartilage and bone is estimated to generate ~40 times higher hydrostatic pressure in the porous structure than values measured in the vasculature (Chen *et al.*, 2010; Zhang *et al.*, 1998). Positive and negative hydrostatic pressures can be generated using gas pressure incubators (Brown, 2000; Yousefian *et al.*, 1995). Hence, no direct contact is needed between the pressure-imposing device and the cells. In addition, the cells do not need to adhere to a substrate. On the other hand, high pO_2 and pCO_2 conditions may alter the culture medium chemistry, which requires appropriate countermeasures (Ozawa *et al.*, 1990). Solid specimens, such as cartilage, bone, or biomimetic scaffolds can be subjected to pressure by a direct platen abutment. Loading of the sample can be unconfined so that the lateral edges are free to move under compression (Burton-Wurster *et al.*, 1993; Torzilli *et al.*, 1997), or the lateral edges can be confined (Freeman *et al.*, 1994). With recent advances in microtechnological devices and their application to biological problems, miniaturized versions of compression devices are now available for multiple sample analysis (Moraes *et al.*, 2010b).

C. Stretch

Stretchable substrates are probably the most frequently used tools to study the mechanisms of cell mechanosensing, the consequences of mechanical protein

deformation, and gene expression (Jonas *et al.*, 2008; Little *et al.*, 2008; Sawada and Sheetz, 2002; Wipff *et al.*, 2007; Zhong *et al.*, 1998). Most two-dimensional culture stretch systems are based on transparent silicone-based culture membranes that require surface activation for cell adhesion (Lateef *et al.*, 2002; Wipff *et al.*, 2009). Standard models have used polydimethyl siloxane to allow linear extensions of up to 25%, which is in the range of physiologically relevant strain (e.g., smooth muscle cells in the arterial wall experience strain rates of 3–10% in normal conditions). Novel biocompatible silicones have been developed to reach much larger culture surface expansions (Majd *et al.*, 2009), which allow generation of even higher strain of $> 30\%$ (e.g., those that might occur in hypertensive vessels) (Califano and Reinhart-King, 2010). Micromachined stretch systems have recently been introduced to strain cells with a high throughput (Moraes *et al.*, 2010a). Alternatively, for growth on two-dimensional stretched membranes, cells can be embedded in three-dimensional substrates such as collagen, fibrin, hydrogels, and these mixtures are then clamped to strain devices (Brown *et al.*, 1998; Lee *et al.*, 2008; Raeber *et al.*, 2008). For cells growing in three-dimensional tissue environments, these systems more closely approximate *in vivo* conditions but are prone to cell-mediated alterations. Cell remodeling processes can lead to matrix anisotropy and local strain distributions that are difficult to predict (Balestrini and Billiar, 2009).

The process of selecting the appropriate stretch protocol should be guided by the physiologically relevant conditions for a particular cell type and biological condition. Depending on the design of the stretch device, cells can be stretched uniaxially (substrate and cells strained in one direction) or biaxially (strain in multiple directions) (Banes *et al.*, 1985; Brown, 2000; Jungbauer *et al.*, 2008; Lee *et al.*, 1996). In equibiaxial stretch systems, cells experience the same strain in all directions in contrast to nonequibiaxial apparatus, where different strain magnitudes act in different directions. In addition to the direction and magnitude of strain, automated stretch devices control whether cells are gradually strained or subjected to cyclic stretches of various frequencies.

Some limitations of silicone-based strain devices have to be taken into consideration: (1) the magnitude of the imposed stretch is in most cases higher than the actual stretch experienced at the cellular level. In two-dimensional systems this can be due to friction between the silicone membrane and the stretch device, loss of cell adhesion to the membrane coating, or slipping of the coating membrane with respect to the membrane (Wipff *et al.*, 2007). In three-dimensional cultures, the protein architecture of extracellular matrix will function as a stress buffer according to the level of organization. (2) In uniaxial systems cells are compressed perpendicular to the axis of stretch if the free edges of the clamped membrane are not confined. This effect is prevented in strip-biaxial systems where the substrate is held fixed in the nonstretched axis (Lee *et al.*, 2008). (3) Cells will react to cyclic uniaxial strains by typically aligning along or perpendicular to the axis of stretch. Once aligned, cells will perceive different mechanical inputs compared with their random initial orientation (Mata *et al.*, 2002; Syedain *et al.*, 2008). Cell alignment does usually not occur upon equibiaxial stretch due to the lack of a major axis of strain. It may appear trivial but morphological study and verification of the proper force application to the cells under stretch is an essential component of these types of experiments.

D. Static Mechanical Stimuli—Substrate Stiffness Matters!!

In addition to dynamic mechanical stimuli, static mechanical conditions such as the rigidity of the microenvironment profoundly influence cell behavior (Engler *et al.*, 2007; Janmey *et al.*, 2009; Tenney and Discher, 2009). There is an expanding list of different cell types that respond to substrate stiffness including cancer cells (Paszek *et al.*, 2005), mesenchymal stem cells (Engler *et al.*, 2006; Winer *et al.*, 2009), neurons (Georges *et al.*, 2006), epithelial cells (Pelham and Wang, 1997), myotubes (Engler *et al.*, 2004), cardiomyocytes (Engler *et al.*, 2008), and fibroblasts (Goffin *et al.*, 2006; Klein *et al.*, 2009). To replicate known levels of tissue stiffness in culture, different two-dimensional polymer coatings are frequently applied. Biopolymer substrates produced from purified collagen, fibrin, and complex protein mixtures like Matrigel usually bracket the lower end of the physiological stiffness spectrum of tissues (Grinnell, 2009; Velegol and Lanni, 2001). However, mechanical signals are difficult to uncouple from chemical influences in biopolymer substrates. Moreover, biopolymers are remodeled by cells, which better matches the *in vivo* situation (Storm *et al.*, 2005) but compromises the reproducibility of culture experiments due to the resulting anisotropic stiffness and stress distributions. In this context, the development of synthetic polymer substrates with tunable elastic modulus and almost ideal elastic properties represent a major advance.

Two-dimensional compliant substrates are mainly produced from polyacrylamide-, polyvinyl alcohol-, or silicone-based elastomers. They provide tissue-like stiffness and excellent optical properties (Brown *et al.*, 2005; Harris *et al.*, 1980; Kandow *et al.*, 2007; Pelham and Wang, 1997; Zajaczkowski *et al.*, 2003). Nevertheless, two-dimensional compliant culture substrates have limitations that should be considered: (1) they cannot reproduce three-dimensional tissue environments, which is a major challenge for biomaterials and tissue engineering (Lutolf and Hubbell, 2005; Place *et al.*, 2009). (2) The mechanical qualities of elastic synthetic polymers and of viscoelastic biopolymers are very different at the cell "perception" level (Storm *et al.*, 2005). (3) Cells may circumvent the direct mechanical input from the compliant substrate surface by secreting their own extracellular matrix proteins, which may become stiffer than the underlying polymer. (4) The mechanical influence of intercellular adherens and tight junctions with neighboring cells in confluent cultures may override the significance of the substrate (Yeung *et al.*, 2005).

E. Subcellular Mechanical Stimulation

There are alternatives to stimulating whole cells and cell populations. A variety of methods are now available for application of local forces at different levels of force and spatial resolution. Several currently used methods to apply stress to cells locally involve microparticles that are coated with cell adhesive proteins and couple to cytoskeleton-linked receptors in the dorsal plasma membrane (see the section below for a detailed description of one system). These particles have a typical diameter of 1–10 μm and can be actuated in two principle ways, using magnetic forces or optical forces. In magnetic twisting cytometry, ferromagnetic beads are manipulated

by transiently applying a strong magnetic field that orients the magnetic dipoles of the beads horizontally. Subsequent application of a weak but sustained magnetic field in an orthogonal direction induces bead rotation and thereby "twists" the membrane (Gosse and Croquette, 2002; Lele *et al.*, 2007; Wang *et al.*, 1993). Magnetically induced forces in magnetic twisting cytometry can range from pN to several nN and are applicable to whole cell populations; however, the inconvenience of magnetic twisting cytometry is its relatively poor spatial resolution. This limitation has been overcome with the development of magnetic pulling cytometry (or "magnetic tweezers") using magnetic microneedles to apply forces to superparamagnetic particles (Lele *et al.*, 2007). Even higher spatial resolution is gained with optical tweezers; however, the forces produced by these optical traps are limited to the hundreds of pN range (Bar-Ziv *et al.*, 1998; Dai and Sheetz, 1995; Grier, 2003; Nieminen *et al.*, 2007; Svoboda and Block, 1994). One potential danger of all microparticle-based technologies is bead internalization by phagocytosis; since small beads can become engulfed within minutes the time for experiments is limited. This is prevented by using atomic force microscopy cantilever as micro- and nanostimulators (Charras *et al.*, 2001, 2002; Shroff *et al.*, 1995). An elegant variation of this theme is the combination of the magnetic bead technology and microstructured elastic substrates. By growing cells on elastic micropillars with integrated cobalt nanowires, applied magnetic field lead to pillar deflections and locally stimulate the ventral cell surface (Sniadecki *et al.*, 2007).

V. Cardiac Fibrosis and Mechanical Induction of Gene Expression

The application of supraphysiological forces *in vivo* can lead to tissue damage, which is frequently manifest as fibrosis, a process which involves the formation of poorly organized and dysfunctional connective tissues. Impaired collagen turnover in fibrotic lesions is thought to contribute to collagen accumulation, thereby leading to loss of appropriate connective tissue function. Fibrosis disturbs the protective features of extracellular matrices by disrupting the stress-shielding, cross-linked architecture of these tissues (Hinz *et al.*, 2007). In heart failure, the activation of cardiac fibroblasts, which involves their differentiation into myofibroblasts and the excessive accumulation of extracellular matrix proteins, is an example of a medically important fibrotic response and which is strongly associated with abnormal cardiac diastolic function (MacKenna *et al.*, 2000). This process is mediated in part by mechanical signals and leads to the *de novo* expression of α-smooth muscle actin by cardiac fibroblasts. α-Smooth muscle actin is a hallmark gene expressed by myofibroblasts (Tomasek *et al.*, 2002). Below we will consider in detail how inappropriate remodeling of the extracellular matrix of the cardiac interstitium by myofibroblasts can be modeled by mechanical force induction of α-smooth muscle actin. We use this system as a model to study mechanical induction of gene expression in cultured fibroblasts.

A. Cardiac Interstitium

The cardiac interstitium is composed of nonmyocyte cells and a structural protein network that plays a dominant role in governing the architecture and mechanical behavior of the myocardium (Brilla *et al.*, 1992, 1995; Weber *et al.*, 1994). The cardiac extracellular matrix is composed predominantly of collagen fibers and a variety of other extracellular matrix proteins including fibronectin, laminin and tenascin. Cardiac muscle contains about sixfold more collagen than skeletal muscle. Accordingly, differences in the resting tension relationships in cardiac and skeletal muscle may result largely from differences in the connective tissue matrix (Covell, 1990). The fibrillar elements form a stress-tolerant network that facilitates the distribution of forces generated in the heart and provide for appropriate alignment of cardiac myocytes (Carver *et al.*, 1991). The long-term performance of cardiac muscle is regulated through a complex but poorly understood group of feedback mechanisms in which mechanical loading controls the organization of myofibrils, the size of muscle fibers, the expression of muscle-specific genes, and the synthesis and secretion of a wide variety of extracellular matrix products and trophic factors (Yamazaki *et al.*, 1998). This phenomenon is tightly regulated during growth or adaptive responses. Increases of muscle mass occur because of hypertrophic enlargement of terminally differentiated cardiomyocytes, increased numbers of fibroblasts, and increased volume of the extracellular matrix (Olson and Srivastava, 1996). When after-load is increased, the adult heart adapts by hypertrophy. This compensatory response in adult hearts is associated with up to a sixfold increase of type I and III collagens (Butt *et al.*, 1995; Sun and Weber, 1996) and an increase of the ratio of type III/I collagens (Carver *et al.*, 1991).

Contemporaneous with the increased collagen synthesis is a reduction of collagen degradation, possibly mediated by reduced collagenolytic activity (Gonzalez *et al.*, 2009). The net increase of collagen in the interstitium is an important determinant of pathological hypertrophy since it may account for abnormal myocardial stiffness (Wilke *et al.*, 1996). Over the long term, this adaptive response can contribute to impairment of cardiac function and heart failure (Keating and Sanguinetti, 1996). Thus the regulatory mechanisms that are related to the fibrous tissue response in various cardiovascular diseases (e.g., hypertensive heart disease, dilated cardiomyopathy, postmyocardial infarction) are of primary clinical interest (Brilla *et al.*, 1995).

B. Mechanical Induction of Myofibroblasts

While cardiac myocytes comprise the largest volume fraction of the adult heart, they represent <25% of cell number (Grove *et al.*, 1969). By far the most abundant nonmyocytic cell in the myocardium is the fibroblast (Eghbali, 1992) (30–50% of cell number), which, along with endothelial and smooth muscle cells, pericytes, neurons, and blood-borne cells make up the remaining 75%. The fibroblast is the principal cell involved in the synthesis and remodeling of the extracellular matrix and therefore plays a central role in the hypertrophic response (Khan and Sheppard, 2006). The conversion of the fibroblast to the myofibroblast is a critical step in

cardiac pathology since in contrast to fibroblasts, myofibroblasts elaborate a poorly organized collagen matrix that impairs diastolic function (Kuwahara *et al.*, 2002).

Currently, the mechanisms that regulate conversion of fibroblasts to myofibroblasts and increased collagen deposition in hypertrophic and fibrotic hearts are not defined. But almost certainly the application of increased forces to cardiac cells is required (Chien, 1999; Komuro *et al.*, 1996; Sadoshima and Izumo, 1997)). At least three effector responses involved in mechanotransduction have been identified in cardiac fibroblasts:

1. Increased mechanical load by itself stimulates fibroblast proliferation, reduces collagenolytic activity and increases collagen production (Husse *et al.*, 2007).
2. The release of autocrine and paracrine growth factors is stimulated by mechanical loading (Kaye *et al.*, 1996; van Wamel *et al.*, 2000).
3. Mechanical load may upregulate vessel wall permeability and thereby increase the availability of systemic factors that can activate fibroblasts (Nicoletti and Michel, 1999).

All of these responses likely involve increased mechanical loading of cardiac fibroblasts, but how are these mechanical forces applied to the cells? In brief, and as described above, force transmission can be mediated by cell-to-cell interactions, cell–matrix interactions, and perhaps by cytoskeletal transmission of force directly to the nucleus (Ingber, 1997, 2003a). As physical forces *in vivo* can be transmitted from extracellular matrix molecules to integrins and to cytoskeletal proteins (Juliano and Haskill, 1993), the integration of the extracellular matrix, integrins, cytoskeleton, and ion channels in a connected and dynamic network provides an attractive scheme for sensing force in cardiac fibroblasts (Kiseleva *et al.*, 1996).

C. Regulation of Gene Expression in Mechanically Loaded Cardiac Cells

α-Smooth muscle actin is not expressed in fibroblasts of normal hearts but is a marker for cardiac myofibroblasts in hypertrophic and fibrotic hearts (Campbell and Katwa, 1997; Leslie *et al.*, 1991) and in heart failure in humans (Suurmeijer *et al.*, 2003). The abundance of myofibroblasts is also locally increased at sites of myocardial infarction (Campbell *et al.*, 1995; Katwa *et al.*, 1997). Further, the expression of α-smooth muscle actin is reactivated during cardiac hypertrophy (Black *et al.*, 1991). In cultured fibroblasts, expression of α-smooth muscle actin is required for integrin-mediated collagen gel remodeling (Leslie *et al.*, 1991; Tomasek *et al.*, 2002).

D. Regulation of the α-Smooth Muscle Actin Promoter

Serum response factor is a MADS-box transcription factor that regulates genes involved in cell proliferation, migration, cytoskeletal dynamics, and myogenesis by binding a conserved DNA sequence [$CC(A/T)_6GG$], known as a CArG box or serum response element (Kuwahara *et al.*, 2005). The ability of serum response factor to distinguish between different target genes depends on the presence of binding sites for

other transcription factors, the number of CArG boxes, and a large number of cofactors. Myocardin and the myocardin-related transcription factors A and B are expressed in a broad range of cell types (Wang *et al.*, 2002a) and comprise a family of coactivators that mediates the transcriptional regulating activity of serum response factor (Wang *et al.*, 2001) (Fig. 1). The CArG box serves as a docking site for myocardin and myocardin-related transcription factors. These proteins enhance the transcriptional activity of serum response factor by forming a ternary complex with serum response factor on DNA (Wang *et al.*, 2002a). MRTF-A and -B also convey stimulatory signals from the Rho GTPase and the actin cytoskeleton to serum response factor via their regulated translocation into the nucleus (Miralles *et al.*, 2003).

The α-smooth muscle actin promoter contains several conserved *cis* elements (Blank *et al.*, 1992; Tomasek *et al.*, 2005) that both positively and negatively affect transcription. Within the proximal 400 bases of the α-smooth muscle actin promoter, in addition to CArG elements are Transforming growth factor (TGF)-β control elements (Hautmann *et al.*, 1997) as well as E-box elements that can regulate α-smooth muscle actin promoter activity (Kumar *et al.*, 2003). We have shown that in fibroblastic cells with low basal levels of actin filaments, stretch-induced activation of α-smooth muscle actin relies on the CArG B box in the α-smooth muscle actin promoter (Wang *et al.*, 2002b). When we first attempted these experiments in myofibroblasts that expressed high levels of α-smooth muscle actin, we did not see additional stretch-induced increases of α-smooth muscle actin protein. Instead, force reduced α-smooth muscle actin protein (Wang *et al.*, 2000) and inhibited skeletal α actin promoter activity (Lew *et al.*, 1999). Subsequently, we have discovered that the basal, unstimulated level of actin assembly in cultured cells is critical in these experiments since the abundance of actin filaments are now known to regulate serum response factor-dependent transcription (Yoshida *et al.*, 2003). To overcome this limitation, we used ROS 17/2.8 cells that develop low levels of actin filaments in vitro and are readily transfected (Wang *et al.*, 2002b). These experiments underline the importance of careful control of cell culture conditions when performing experiments on α-smooth muscle actin activation by force (Wang *et al.*, 2000, 2003).

E. Cell Culture Models for Mechanical Induction of α-smooth Muscle Actin Expression

To study cytoskeletal involvement in the regulation of the hypertrophic response in cardiac myocytes and fibroblasts, it is essential to use models that generate membrane strain as a result of direct manipulation of integrins. As described previously (Chan *et al.*, 2009; Glogauer and Ferrier, 1998; Glogauer *et al.*, 1995, 1997; Wang *et al.*, 2000, 2002b; Zhao *et al.*, 2007), we developed and tested a model, based on magnetically generated tensile forces applied to collagen-coated beads, which does indeed deliver measurable, physiologically meaningful forces through integrins to the actin cytoskeleton. Moreover, this model provides very useful correlates for examining the expression of α-smooth muscle actin by fibroblasts *in vivo* and what might be important upstream regulators and mechanosensors, such as the focal adhesion kinase (Chan *et al.*, 2009).

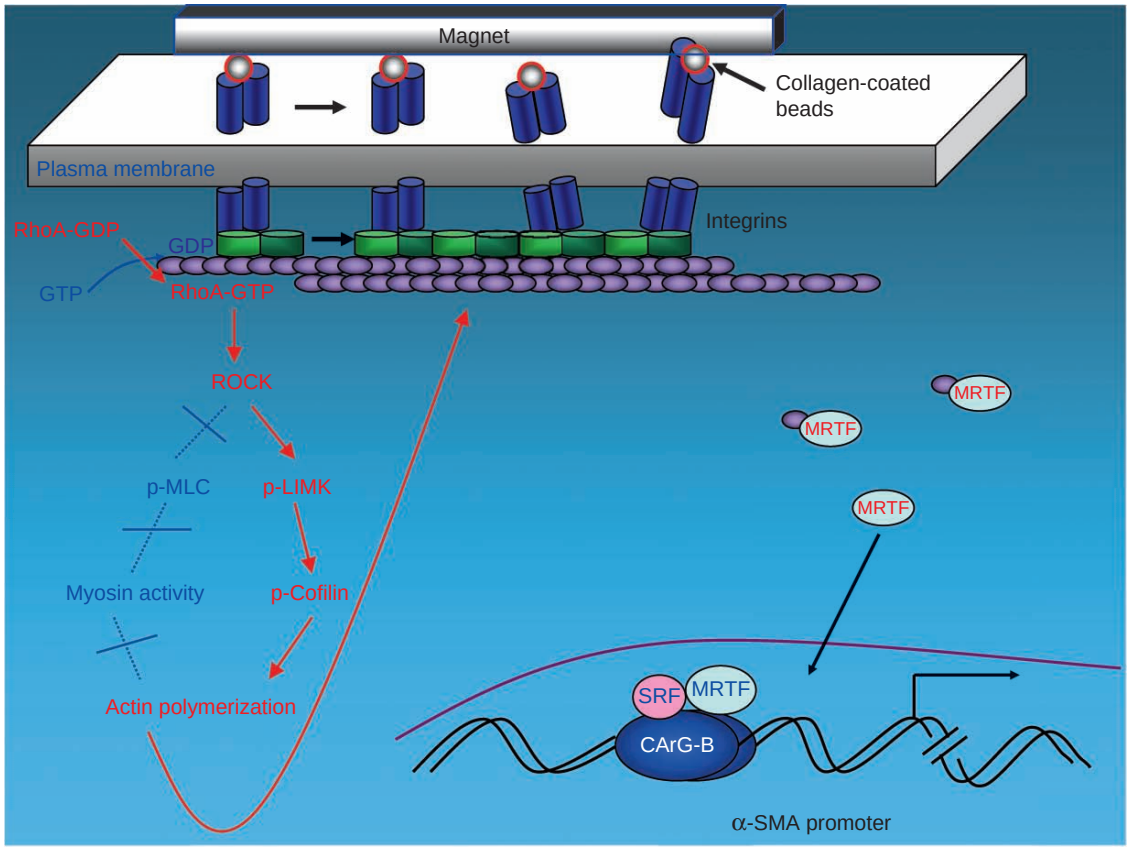

Fig. 1 Model to demonstrate application of tensile forces to cells using magnetically generated forces, collagen-coated beads, and the resultant activation of the Rho–LIM kinase–cofilin–actin filament signaling system that leads to enhanced expression of α-smooth actin.

We have developed magnetic devices that deliver precise tensile forces to fibrillar collagen-coated magnetite beads attached to integrins on cultured cells. Force levels are evenly distributed across the whole diameter of the culture dish, unlike other substrate stretch methods that may produce a nonlinear strain gradient (Fig. 2). The beads are attached to the ventral surfaces of cultured cells through $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins. The stretch system pulls the beads vertically, delivering tensile forces of 0.6 pN/μm^2 (force/cell area), which are within the range of physiological force levels that are expected to be encountered by cardiac fibroblasts *in vivo* (Hamrell and Dey, 1993; Wang *et al.*, 2000). Notably, to model the forces that might be expected in cardiac overload, the force levels can be increased by twofold (to 1.2 pN/μm^2 cell area), which is in the range of predicted increases of force that might be generated in hypertrophic conditions *in vivo* (Mann *et al.*, 1991). Thus it is possible to model "physiological" and "pathological" force levels, respectively. Further, compressive forces can be applied by placing the magnet below cells, and twisting forces can be applied by rotating the magnet placed above cells by 90° after half the total length of exposure time (Mak *et al.*, 2008).

The forces generated by magnetic fields on 5-μm beads have been estimated from Stokes' law and from direct measurements of bead velocities in viscous fluids (Glogauer and Ferrier, 1998). Magnetic fields producing one of two force levels at the cell surface will be used for all experiments. Since we have carefully mapped out the magnetic flux densities that are generated by the permanent and electric magnets, we have precise estimates of flux densities at various distances from the magnetic pole

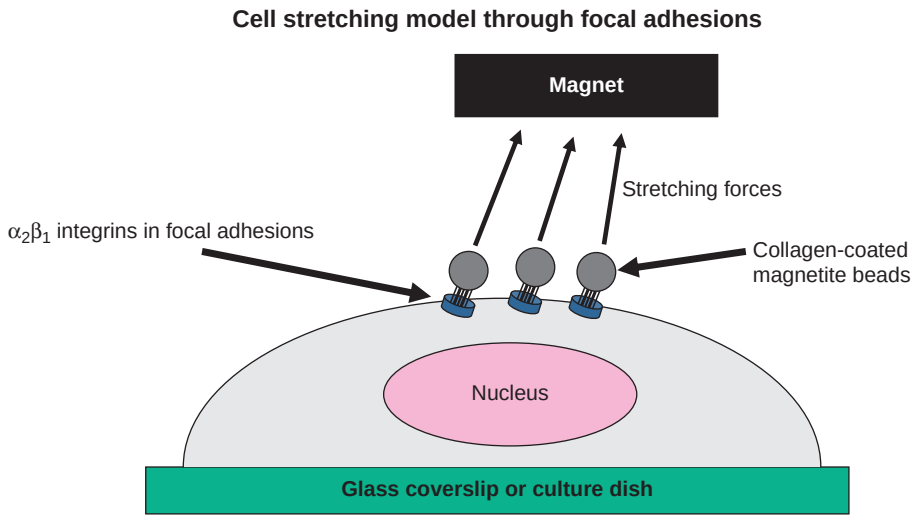

Fig. 2 Cartoon of experimental model system used for in vitro cell stretching. Collagen beads are attached to the dorsal surfaces of cells through β1 integrins. Stretching forces are applied (0.5–2 pN/mm^2 surface area of cell). The stretching forces can be adjusted in terms of amplitude, direction, and duration, to suit the desired experimental condition.

face. Another important variable in determining force application to the cells is to estimate the total cell surface area and the percent surface area covered by beads. This is determined by using an image analyzer as described (Glogauer *et al.*, 1995). From these data, precise estimates of the force applied per cell can be obtained.

F. Coating Methods for Beads

Based on the abundance of type I collagen in the myocardial extracellular matrix and its impact on the cardiac hypertrophic process (Pelouch *et al.*, 1993; Shirwany and Weber, 2006) and its apparent role in force transmission to fibroblasts (Provenzano and Vanderby, 2006), collagen seems to be a good candidate molecule for bead coatings. Further, previous data (Wang *et al.*, 2003) indicate that when force is applied through fibronectin-coated beads in rat cardiac fibroblasts, there is no activation of the p38 MAP kinase or increased expression of α-smooth muscle actin. In contrast, force applied to collagen-coated beads strongly upregulates p38 kinase activity and α-smooth muscle actin content. This finding is in agreement with *in vivo* findings of pressure overload-induced p38 activation in rats.

To test for nonspecific binding to the cell membrane and the specificity of the collagen effect, beads are coated with the nonintegrin-dependent adhesive poly-L-lysine or with bovine serum albumin (BSA) as described (Wang *et al.*, 2003). These different bead coatings permit comparisons of loading through integrins versus stretching through the cell membrane and other integral membrane proteins. They should also indicate which one of the collagen receptors is most important in the stretch response. Beads can be coated with antibodies to the specific α subunits that are expected as collagen receptors and determine which antibody is most effective in inducing alteration of α-smooth muscle actin levels in response to stretching (Wang *et al.*, 2000, 2003). As the antibodies do not exhibit multivalent interactions with receptors, it is possible to study clustering by confocal microscopy. Beads should be coated with equimolar concentrations of proteins. Bead size can be estimated by electronic particle counting (Glogauer *et al.*, 1997). Verification of collagen or antibody coating on beads is assessed with antibodies to type I collagen (rabbit antibovine, antiserum; Chemicon).

Beads are added to well-spread cells for 10 min and cells are washed 3 times to remove unbound beads. For longer duration experiments when force is applied (1–4 h), bead internalization and bead detachment is minimal under these conditions, but this must be carefully examined as mentioned in the section above, as phagocytosis of beads confounds experimental results. For experiments in which large numbers of cells are analyzed, phase-contrast microscopy is used to assess the equality of bead loading and the relatively even distribution of beads across the dish. Cultures in which bead loading does not conform to previously established protocols (Glogauer *et al.*, 1995) should not be used as this would indicate that the cells were not loaded equivalently. The force applied is proportional to the volume of the beads (Glogauer and Ferrier, 1998). From the distribution of bead diameters we have calculated a cross-sectional-area-weighted mean diameter of ~5 μm. The latter value is appropriate for calculating the force on a per unit area basis.

G. Cell Transfection and Promoter Methods

The tensile force model system described above is particularly well suited to study of force-induced gene expression. Consequently, the use of rapid readout cell systems is helpful. For promoter studies, a Rous sarcoma virus (RSV; −124 to +34)/luciferase construct and a similar Renilla luciferase construct are used as internal controls as they are not affected by cell stretching (Lew *et al.*, 1999). Subconfluent cardiac fibroblasts are transiently transfected with Qiagen-purified plasmid DNA using the transfection reagent Lipofectamine 2000 or Fugene (Roche) as described (Lew *et al.*, 1999; Sayegh *et al.*, 2005; Wang *et al.*, 2002a). Standardized amounts of plasmid (typically 10 μg/100 mm plate of cells) are used in quadruplicate replicate cultures for each promoter construct. Based on our experience with cardiac fibroblasts, we usually obtain reproducible and meaningful results with up to threefold increased α-smooth muscle actin promoter activity in response to stretch in cells with no detectable baseline α-smooth muscle actin expression. After 16 h incubation, the medium is replaced with serum-free medium. Collagen or poly-L-lysine or BSA-coated beads are added to the transfected cells for 15 min at 37°C and force applied. After washing in phosphate-buffered saline (PBS), cells are collected and processed for β-galactosidase activity as described (Lew *et al.*, 1999). The assessment of β-galactosidase or luciferase activity can be used to analyze the effect of force on promoter activity and of the various perturbations that are sought.

H. Identification of Adhesion-Associated Proteins

The system described above can be used also to study which proteins in the collagen bead adhesion complex may be regulated by the application of force. After addition of collagen- or BSA-coated magnetite beads, and then application of force, bead-associated proteins are isolated from cells as described (Wang *et al.*, 2006) (Fig. 3). Briefly, cells are washed with cold PBS to remove unbound beads, scraped into cold cytoskeleton extraction buffer, and sonicated for 10s. The beads are isolated from the lysate with a magnet, resuspended in cold cytoskeleton extraction buffer, homogenized, and reisolated magnetically. Bead-associated proteins are removed by boiling in Laemmli sample buffer. In control experiments, cell cultures are treated with swinholide A (50 nM, 25°C, 20 min) prior to bead binding to dissipate focal adhesion formation. The resulting lysates can then be analyzed for bead-associated proteins by immunoblotting (for known proteins of interest) or with the use of proteomic methods (e.g., tandem mass spectrometry and isotope-coded affinity tags; Pho *et al.*, 2008) for a wider screen of potentially interesting proteins that are regulated by force.

VI. Conclusions

The application of the methods described here should provide relatively facile approaches to identify gene responses in cultured mesenchymal cells to a variety of different mechanical perturbations. We have described in detail the utilization of one

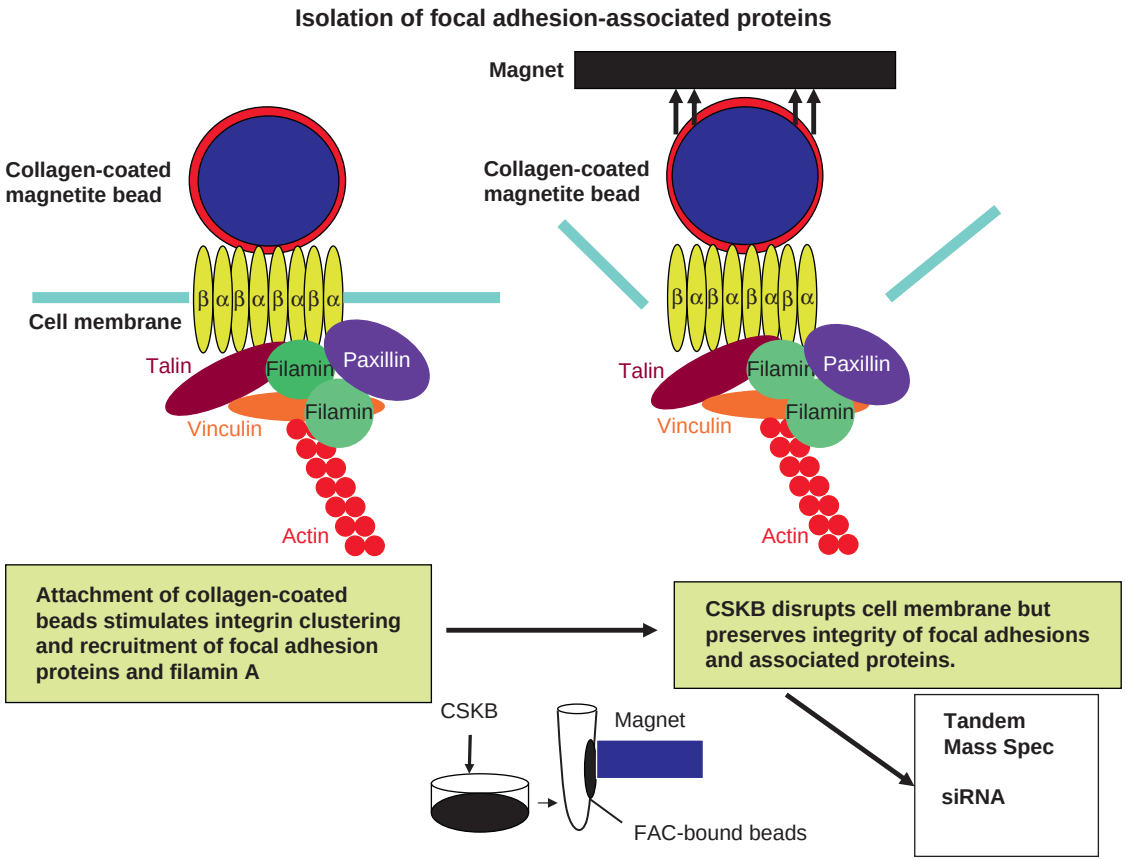

Fig. 3 Diagram to illustrate methods for purification of adhesion-associated proteins from collagen-coated beads attached to the dorsal surface of cells. Bead-associated proteins can be analyzed by immunoblotting or, for searching for novel proteins, by mass spectrometry.

tensile force model to the cardiac fibroblast situation and the specific focus on the regulation of the α-smooth muscle actin promoter, but this approach may be applicable to other genes in which short-term responses can be evaluated. Notably, there are limitations to all of these methods and we have described limitations and pitfalls, which must be considered for the appropriate interpretation and application to the *in vivo* situation. In particular, the use of controls and the need for reasonable force levels cannot be overstated.

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