Am J Physiol Heart Circ Physiol 309: H532–H542, 2015.
First published June 19, 2015; doi:10.1152/ajpheart.00299.2015.

Mechanoregulation of cardiac myofibroblast differentiation: implications for cardiac fibrosis and therapy

Kar Wey Yong,[1,2] YuHui Li,[1,3] GuoYou Huang,[1,3] Tian Jian Lu,[1] Wan Kamarul Zaman Wan Safwani,[2] Belinda Pingguan-Murphy,[2] and Feng Xu[1,3]

[1]*Bioinspired Engineering and Biomechanics Center, Xi'an Jiaotong University, Xi'an, People's Republic of China;* [2]*Department of Biomedical Engineering, Faculty of Engineering, University of Malaya, Kuala Lumpur, Malaysia; and* [3]*The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, People's Republic of China*

Submitted 27 April 2015; accepted in final form 19 June 2015

Yong KW, Li Y, Huang G, Lu TJ, Safwani WK, Pingguan-Murphy B, Xu F. Mechanoregulation of cardiac myofibroblast differentiation: implications for cardiac fibrosis and therapy. *Am J Physiol Heart Circ Physiol* 309: H532–H542, 2015. First published June 19, 2015; doi:10.1152/ajpheart.00299.2015.—Cardiac myofibroblast differentiation, as one of the most important cellular responses to heart injury, plays a critical role in cardiac remodeling and failure. While biochemical cues for this have been extensively investigated, the role of mechanical cues, e.g., extracellular matrix stiffness and mechanical strain, has also been found to mediate cardiac myofibroblast differentiation. Cardiac fibroblasts in vivo are typically subjected to a specific spatiotemporally changed mechanical microenvironment. When exposed to abnormal mechanical conditions (e.g., increased extracellular matrix stiffness or strain), cardiac fibroblasts can undergo myofibroblast differentiation. To date, the impact of mechanical cues on cardiac myofibroblast differentiation has been studied both in vitro and in vivo. Most of the related in vitro research into this has been mainly undertaken in two-dimensional cell culture systems, although a few three-dimensional studies that exist revealed an important role of dimensionality. However, despite remarkable advances, the comprehensive mechanisms for mechanoregulation of cardiac myofibroblast differentiation remain elusive. In this review, we introduce important parameters for evaluating cardiac myofibroblast differentiation and then discuss the development of both in vitro (two and three dimensional) and in vivo studies on mechanoregulation of cardiac myofibroblast differentiation. An understanding of the development of cardiac myofibroblast differentiation in response to changing mechanical microenvironment will underlie potential targets for future therapy of cardiac fibrosis and failure.

cardiac myofibroblast differentiation; mechanical cues; mechanical microenvironment; ECM stiffness; mechanical strain

HEART INJURY from many causes, e.g., ischemic heart diseases and hypertension, can end up with cardiac fibrosis. Cardiac fibrosis is an initial healing process essential for heart repair, but which if dysregulated is liable to cause adverse remodeling of cardiac tissues, leading to the development of congestive heart failure (108, 111). Cardiac fibrosis results from the excessive accumulation of fibrous connective tissues (components of the ECM, such as collagen) deposited by an increased number of cardiac fibroblasts and myofibroblasts around damaged heart tissues, resulting in permanent scarring and impaired cardiac functions (16, 101, 108). The origin of cardiac fibroblasts generated during fibrosis has been determined using various nonuniversal cardiac fibroblast markers (e.g., vimentin, discoidin domain receptor-2, and fibroblast-specific protein-1) and fate-mapping strategies (7, 110). These studies reveal that cardiac fibroblasts may be derived from endothelial cells [via endothelial mesenchymal transition (EndoMT)], bone marrow-derived precursors, or epicardial cells [via epithelial mesenchymal transition (EMT)] (32, 111, 113). Recently, it has been suggested that the origin of cardiac fibroblasts is dependent on the heart conditions (e.g., whether during postnatal development or injury). With the use of a robust cardiac fibroblast marker (collagen1a1-green fluorescent protein), it is possible to demonstrate that cardiac fibroblasts generated during fibrosis originate from the activation and proliferation of resident epicardial- and endothelial-derived fibroblasts (1, 7, 60, 61). On the other hand, cardiac fibroblasts during postnatal heart development are formed via EndoMT and EMT. Taken together, this requires further investigations to obtain a universal cardiac fibroblast marker to accurately identify the origin of cardiac fibroblasts, especially for those generated during cardiac fibrosis. Furthermore, while traditional dogma states that cardiac fibroblasts represent the most prevalent cell type in the mammalian heart (~70% of total cell numbers in heart) (71,

Address for reprint requests and other correspondence: F. Xu, School of Life Science and Technology, Xi'an Jiaotong Univ., No. 28 Xianning Xi Rd., Xi'an, 710049, P.R. China (e-mail: fengxu@mail.xjtu.edu.cn).

86), but a few recent reports show that the cardiac cellular makeup varies greatly between species of mammal. The adult mouse heart contains ~27% fibroblasts, whereas the human heart only ~10% fibroblasts, with the majority of heart cells being cardiomyocytes (5, 70). Such controversial issues regarding the total numbers of cardiac fibroblasts in the heart and the origin of cardiac fibroblasts remain to be debated.

In general, the differentiation of cardiac fibroblasts to more active myofibroblasts is the hallmark of cardiac fibrosis, as characterized by increased collagen production and expression of α-smooth muscle actin (α-SMA) (88, 91). Although cardiac myofibroblasts have been assumed to be solely derived from differentiation of resident fibroblasts, it is now apparent that they can be derived from various alternative cellular precursors. Upon heart injury, resident cardiac fibroblasts and a variety of other cell lineages (e.g., EndoMT, bone marrow-derived precursors, and EMT) are stimulated under various profibrotic cytokines and growth factors released by cardiac cells and immune cells (e.g., macrophages and lymphocytes) to differentiate into myofibroblasts, which are not present in the normal adult heart (44, 45). In the normal healing process, myofibroblasts undergo apoptosis and rapidly disappear. However, failure of the apoptotic mechanism (e.g., due to mechanical alteration) leads to persistent myofibroblast expansion, excessive ECM production, and pathological scar formation that leads in turn to cardiac fibrosis (46, 102). Therefore, it is of great importance to understand cardiac myofibroblast differentiation for pathophysiological studies and therapeutic purposes.

To date, significant effort has been made in the study of the differentiation of cardiac fibroblasts into myofibroblasts from a biochemical aspect. For instance, soluble factors [e.g., transforming growth factor-β (TGF-β), angiotensin II (ANG II), and endothelin-1 (ET-1)], cytokines (e.g., interleukin-6), matricellular proteins (e.g., connective tissue growth factor), and extracellular proteins [e.g., fibronectin extra-domain A (EDA)] are implicated in cardiac myofibroblast differentiation (14, 47). TGF-β is a major inducer of myofibroblast differentiation (55), which enhances the expression of α-SMA and synthesis of ECM molecules (e.g., fibronectin EDA), further mediating myofibroblast differentiation (80). On the other hand, ANG II, a neuroendocrine factor serving as upstream inducer of TGF-β signaling for myofibroblast differentiation (31), has been shown to increase the expression of ET-1 (a bioactive peptide produced during cardiac injury) in cardiac fibroblasts (11, 48). ET-1 appears to function synergistically and downstream of both TGF-β and ANG II to promote and maintain the myofibroblast phenotype (47). However, these studies focused solely on biochemical factors cannot explain the whole mechanism of cardiac myofibroblast differentiation.

Besides biochemical factors, mechanical cues (e.g., mechanical strain and ECM stiffness) also play an important role in regulating myofibroblast differentiation (35). Cardiac cells (including cardiac fibroblasts) reside in one of the most mechanically dynamic environments of the body, where the heart chamber filling and wall distention within diastole induces rapid changes in pressure and volume that are released by the wave of contraction produced to pump blood through the body (12). These pulsatile stimuli are experienced by cardiac cells as cyclic strain (relative deformation) and stresses (force per unit area). In certain conditions, such as during pathogenesis, these cells are placed under cyclic strain with magnitude and frequency that vary with heart rate and pressure load (29). Strain can be mimicked in vitro by the controlled stretching of a culture substrate containing cardiac cells with various magnitudes (64). The literature reviews show that cardiac fibroblasts respond differently in terms of myofibroblast differentiation after stimulation with various magnitudes of strain (13, 25). Furthermore, the mechanical properties of ECM (e.g., matrix stiffness, a measure of matrix resistance to mechanical deformation) also regulate myofibroblast differentiation (36). The relatively high stiffness of injured and fibrotic tissues may promote myofibroblast differentiation, which prolongs the existence of fibrosis via increases in the secretion of TGF-β (103). Stiff ECM-induced myofibroblast differentiation has been reported in fibroblasts isolated from heart tissue (27, 112). Both strain- and stiff ECM-induced myofibroblast differentiation is associated with the activation of TGF-β, a master regulator of mechanical stress-induced myofibroblast differentiation (25).

There are many existing reviews of the role of cardiac fibroblasts and myofibroblasts during cardiac wound healing and their interaction with ECM, which contributes to cardiac fibrosis (17, 71, 91, 102). However, there is still no comprehensive review of the interaction of cardiac fibroblasts and myofibroblasts with mechanical strain and stiffness of ECM. In this review, we focus on the important parameters for cardiac myofibroblast differentiation evaluation and the development of in vitro [2 and 3 dimensional (2-D and 3-D, respectively)] and in vivo studies on the use of mechanical cues to regulate cardiac myofibroblast differentiation. We first present parameters (e.g., expression of α-SMA, fibronectin EDA, collagen, and contractile activity) used to distinguish cardiac fibroblasts and myofibroblasts and also their interaction with mechanical cues. We then review the mechanisms of mechanical cues incorporated with biochemical cues in regulating in vitro and in vivo cardiac myofibroblast differentiation. Understanding of the mechanisms of cardiac myofibroblast differentiation in response to changing mechanical microenvironment is important for uncovering new targets for future cardiac fibrosis and failure therapy.

Important Parameters for Cardiac Myofibroblast Differentiation Evaluation

Cardiac myofibroblast differentiation occurs in two stages (89). In the first stage, cardiac fibroblasts develop into protomyofibroblasts, which are characterized by the assembly of cytoplasmic actin stress fibers and fibronectin EDA (not found in cardiac fibroblasts). Together with the small adhesion complexes such as β- and γ-actin microfilaments, these allow protomyofibroblasts to migrate into the wounded area (15, 37). The second stage is initiated in accord with high levels of cytokines (e.g., interleukin-6), TGF-β, fibronectin EDA, and mechanical stress that have accumulated within the wounded area, promoting differentiation of protomyofibroblasts into active myofibroblasts (89).

Morphologically, myofibroblasts are spindle-shaped with protruding dendrite-like processes and extensive areas of endoplasmic reticulum (22, 37). The defining marker of fully differentiated myofibroblasts in research and clinical diagnostics is the relatively high expression of α-SMA (not expressed

by protomyofibroblasts) that incorporates into a prominent stress fiber network underlying their contractile function (17). The expression of α-SMA can be determined using molecular-based (e.g., quantitative real-time polymerase chain reaction method) or protein-based (e.g., immunofluorescence staining or Western blot analysis) assays (25, 99, 100). Contractile activity of myofibroblasts can be detected by measuring their ability to contract a collagen gel (18). Myofibroblasts induce a fourfold greater contraction than is seen in collagen gel without cells, and cardiac fibroblasts induce lesser contraction. Furthermore, myofibroblasts actively secrete TGF-β and ECM proteins like periostin, fibrillar collagens (e.g., collagen I and collagen III), nonfibrillar collagen (e.g., collagen VI), fibronectin, and EDA splice variant of fibronectin (15, 91) (Fig. 1). Periostin promotes myofibroblast recruitment and collagen synthesis (83), whereas ECM proteins such as collagen I, collagen III, and fibronectin are secreted to replace the damage myocardium (55). Collagen I and collagen III (two major components of ECM in heart) could enhance the proliferation of cardiac fibroblasts; collagen VI (a minor component of ECM in heart) is a mediator of cardiac myofibroblast differentiation (63). EDA fibronectin is essential for connecting ECM to integrins and stress fibers, which allows myofibroblasts to exert mechanical traction on the ECM (78) to increase their contractility. This is important for structural integrity and supporting the new matrix to strengthen the scar (34).

Mechanical and biochemical cues are often interdependent in biological processes, and this includes cardiac myofibroblast differentiation (57). For instance, cell traction forces (tensile forces generated by the cells transmitted to the ECM via focal adhesions) are regulated by intracellular proteins (e.g., α-SMA) and soluble factors (e.g., TGF-β) or vice versa (4). These forces are essential for mechanical signal generation, cell-shape maintenance, and cell migration in biological processes. In general, TGF-β is usually stored in the ECM as part of a latent complex. Notably, the latency-associated peptide (one of the component of latent complex) binds directly to integrins (e.g., $\alpha_v\beta_5$) and controls the release of extracellular stores of TGF-β (103). Cells will exert traction on the latency-associated peptide connected to ECM through integrins, causing conformational changes and releasing active TGF-β (107). Under the traction applied by cells, soft ECM preferentially deforms, leaving the latent complex intact and inactivation of TGF-β, whereas stiff ECM resists deformation, resulting in distortion of the latent complex and the release of active TGF-β. Taken together, high ECM stiffness, cell traction forces, and TGF-β activation are required to increase the expression of α-SMA protein (104). Increased α-SMA proteins interact with myosin to contract and produce increased tractions, which is effectively a feed-forward loop incorporating both biochemical and mechanical signals to promote myofibroblast differentiation and maintain myofibroblast phenotype (107). In addition to cell traction force, other mechanical forces including stretch and interstitial fluid flow have also been implicated in paracrine release of TGF-β from cardiac fibroblasts (13, 25), which induces myofibroblast differentiation.

2-D In Vitro Mechanoregulation of Cardiac Myofibroblast Differentiation

Effect of ECM stiffness on cardiac myofibroblast differentiation in 2-D culture. METHODS OF ENGINEERING HYDROGEL SUBSTRATES WITH TUNABLE STIFFNESS. In general, fibroblasts from various organs (e.g., lungs, liver, heart, and aortic valve) have been studied for myofibroblast differentiation on 2-D substrates [e.g., polyacrylamide, polyethylene glycol (PEG), polydimethylsiloxane (PDMS), and methacrylated hyaluronic acid (MeHA)] with tunable stiffness (0.15–154 kPa) according to polymer fabrication methods. Notably, polyacrylamide gels coated with collagen I with stiffness gradients, fabricated by varying cross-linking density, have been particularly useful for assessing the effects of matrix stiffness on cellular responses, including myofibroblast differentiation (50, 65, 73, 105). PEG diacrylate gels have also been used to study myofibroblast

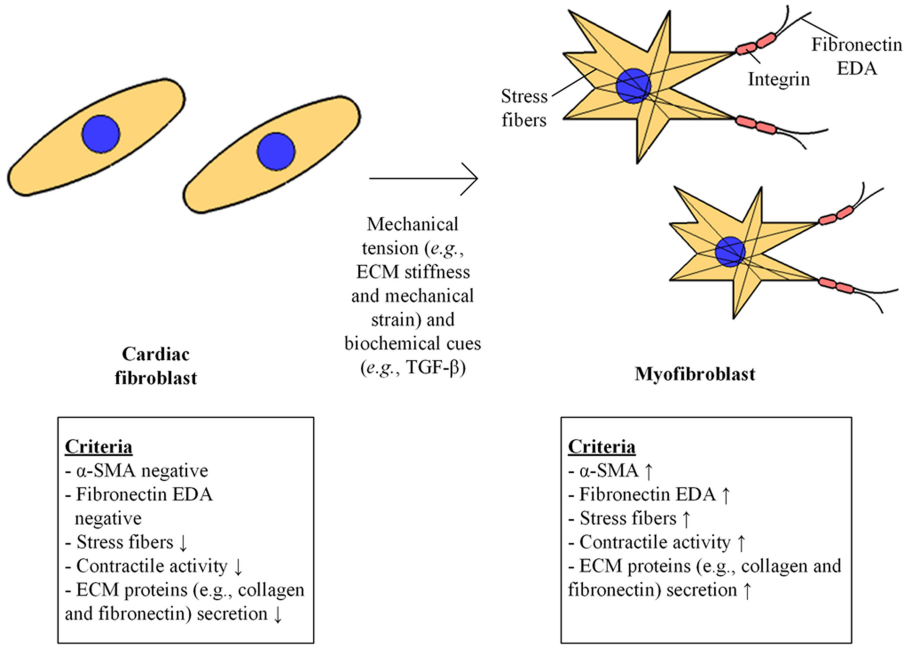

Fig. 1. The important parameters for evaluating myofibroblast differentiation upon mechanical tension, e.g., extracellular matrix (ECM) stiffness and mechanical strain. TGF-β, transforming growth factor-β; α-SMA, α-smooth muscle actin; EDA, extra domain A.

differentiation and are formed from free radical chain growth polymerization in the presence of ultraviolet (UV) light and a photoinitiator (92, 112). Interestingly, the stiffness of modified PEG (added with photodegradable cross-linkers) can be reduced (from 32 to 7 kPa) by exposure to UV light, enabling the reversion of myofibroblast differentiation in aortic valvular interstitial cells (92, 93). Besides that, the stiffness of PDMS can be controlled by concentration of cross-linking agent, temperature, and duration of baking (24, 82) for cell behavior studies, including myofibroblast differentiation. With a variation of the methacrylate consumption through Michael-type addition cross-linking and UV exposure time, MeHA with tunable stiffness (3–100 kPa) can be fabricated (58). For instance, soft MeHA (2 kPa) was fabricated using Michael-type addition alone, whereas stiff MeHA (24 kPa) was generated with the addition of secondary cross-linking with UV light to study myofibroblast differentiation in liver fibroblasts (30).

EFFECT OF ECM STIFFNESS ON CARDIAC MYOFIBROBLAST DIFFERENTIATION. PEG hydrogel-based ECM with patterned stiffness was created for constructing an in vitro fibrosis model to study cardiac myofibroblast differentiation. Fabrication of such mechanically patterned hydrogel substrate is demonstrated in Fig. 2*A*. With the use of this model, a patterned distribution of myofibroblasts-fibroblasts was observed on soft and stiff areas of PEG diacrylate, as indicated by α-SMA and fibronectin expression. This model revealed the migration of cardiac fibroblasts across the border from the soft area (Young's modulus of 10 kPa) to the stiff area (Young's

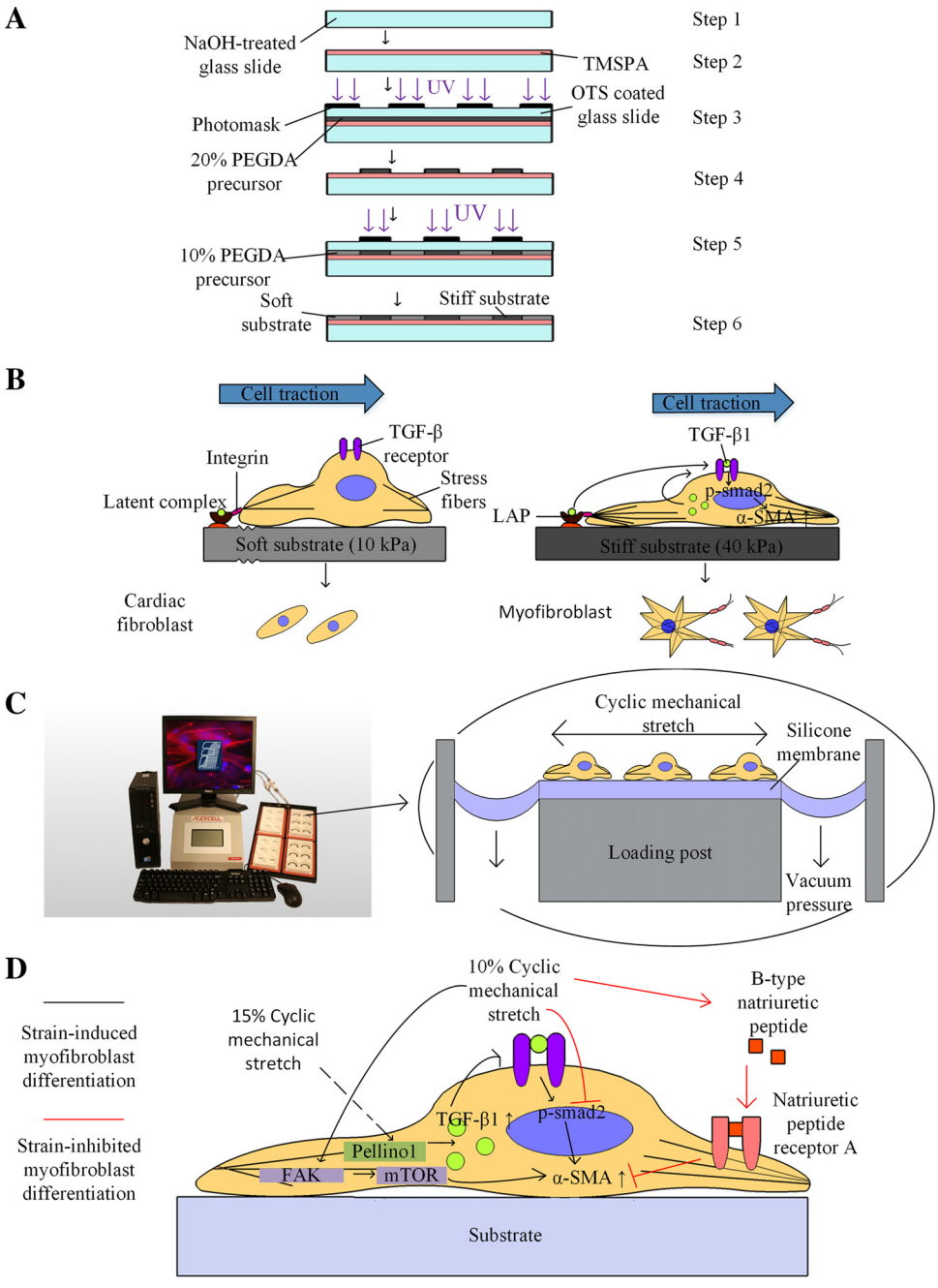

Fig. 2. Methods for engineering 2-dimensional cell mechanical microenvironment and the mechanisms proposed for cardiac myofibroblast differentiation. *A*: mechanically patterned stiffness of PEG diacrylate (PEGDA) was generated to study the stiffness-induced myofibroblast differentiation. Fabrication procedures are stated as follows: *Step 1*, treat glass slide with NaOH; *step 2*, functionalize glass slide with 3-(trimethoxysilyl)propylmethacrylate (TMSPA); *step 3*, expose 20% PEGDA to ultraviolet (UV) light; step 4, form-patterned stiff substrate; *step 5*, expose 10% PEGDA to UV; and *step 6*, form mechanically patterned substrate. *B*: mechanisms proposed for stiff ECM-induced cardiac myofibroblast differentiation. *C*: Flexcell tension system was used to apply stretch on cardiac fibroblasts via pneumatic deformation of the membrane using vacuum pressure to evaluate the effect of mechanical strain on myofibroblast differentiation. *D*: mechanisms proposed for mechanical strain in regulating cardiac myofibroblast differentiation. OTS, octadecyltrichlorosilane; LAP, latency-associated peptide; p-smad2, phosphorylated smad2; FAK, focal adhesion kinase; mTOR, mammalian target of rapamycin.

modulus of 40 kPa), which differentiated into myofibroblasts in the stiff areas (Fig. 2*B*) through the mechanism as mentioned earlier in *Important Parameters for Cardiac Myofibroblast Differentiation Evaluation*. Furthermore, upon treatment of a Rho-associated protein kinase inhibitor, a significant reduction of myofibroblasts was observed, indicating promising application of this model for exploring potential mechanisms for reversing myofibroblast differentiation (112).

Effect of mechanical strain on cardiac myofibroblast differentiation in 2-D culture. METHODS OF APPLYING MECHANICAL STRAIN ON CARDIAC FIBROBLASTS CULTURED IN 2-D. Cardiac fibroblasts have been subjected to mechanical strain with various parameters, such as stretching mode (e.g., static and cyclic), magnitude of strain, and frequency using various stretching devices. Initially, static stretching devices were used to study the changes of biological responses (e.g., collagen, TGF-β, and insulin growth factor type-1 secretion) in cardiac fibroblasts upon mechanical stimulation (28, 39, 49, 77). In fact, cardiac fibroblasts either in normal or pathological conditions are constantly subjected to dynamic mechanical changes. In accordance with the fact, self-designed, cyclic-stretching devices were developed for creating engineered, in vitro, mechanical microenvironment to determine the changes of biological responses (e.g., proliferation capacity, collagen, and insulin growth factor type-1 secretion) in cardiac fibroblasts in an accurate manner (3, 9, 39). However, these devices lack strain profile characterization, a factor that is often overlooked, thus making data comparison between studies difficult (75). To address this, many studies have used commercially available cyclic-stretching devices, such as Flexcell (8, 41, 42, 51, 67, 76, 90, 109), which provide relatively well-characterized strain profile and tunable magnitude of strain (1–33%) (75). Overall, these studies focused more on mechanical force regulation of alterations in ECM (8, 41, 67), activation of stress-induced cell-signaling pathways (42, 51), and secretion of peptides and growth factors (76, 109) in cardiac fibroblasts. However, these studies provide useful information for investigating the roles of mechanical cues in regulating cardiac myofibroblast differentiation. Notably, only studies within the last 5 years focused on the mechanism for mechanoregulation of cardiac myofibroblast differentiation (13, 85, 99, 100).

EFFECT OF MECHANICAL STRAIN ON CARDIAC MYOFIBROBLAST DIFFERENTIATION. The initial evaluation of mechanical strain on cardiac myofibroblast differentiation was carried out by applying static stretch on cardiac fibroblasts using collagen-coated magnetite beads. The results show that static stretch (0.65 $pN/\mu m^2$, 4 h) reduces myofibroblast differentiation by reducing the expression level of α-SMA in cardiac fibroblasts via a p38 kinase and eukaryotic initiation factor-2α pathway (94–96). However, as the well-characterized, cyclic-stretching device (Flexcell tension system) is developed, extensive studies on this were performed. Cardiac fibroblasts seeded on substrate coated with matrix proteins such as collagen or fibronectin were subjected to the various magnitudes, same frequency (1 Hz), and various durations of strain (13, 85, 99, 100) by using this device (Fig. 2*C*).

To explore the mechanisms involved in cardiac myofibroblast differentiation activated by mechanical stimulation, Dalla Costa et al. (13) investigated the role of an integrin, focal adhesion kinase (FAK), in differentiation of cardiac fibroblasts seeded on collagen type I in response to cyclic stretch (10%, 1 Hz, 4 h). After cyclic stretch, they observed that cardiac fibroblasts differentiated to myofibroblasts, as indicated by increased expression of α-SMA and collagen type I. Treatment with RGD peptide (FAK inhibitor) and FAK silencing significantly inhibited the stretch-induced myofibroblast differentiation. Furthermore, their findings demonstrate a critical role of the mammalian target of rapamycin complex, downstream from FAK, in mediating cardiac myofibroblast differentiation in response to mechanical stretch. Overall, it showed that mechanical stretch can induce cardiac myofibroblast differentiation through the activation of FAK and mammalian target of rapamycin complex (Fig. 2*D*).

Furthermore, Watson et al. (100) have investigated the combined effect of mechanical stretch and soluble factors [e.g., TGF-β1 and B-type natriuretic peptide (BNP)] on myofibroblast differentiation in cardiac fibroblasts seeded on fibronectin. They observed that cyclic mechanical stretch (10%, 1 Hz, 72 h) reduced the effectiveness of TGF-β1 in promoting cardiac myofibroblast differentiation, as indicated by the decreased expression of α-SMA and collagen, by attenuating the phosphorylation of smad2. Notably, their finding was associated with a novel observation that mechanical stretch can increase BNP and natriuretic peptide receptor A (NPRA) expression in cardiac fibroblasts. BNP acted via NPRA and further reduced the potency of TGF-β1 in inducing myofibroblast differentiation on mechanically stretched cardiac fibroblasts (Fig. 2*D*). Besides that, Watson and his group (99) have also studied the impact of various matrix substrates on cardiac fibroblast responses (including myofibroblast differentiation) to TGF-β1 and mechanical stretch. They found that differential responses of cardiac fibroblasts in terms of α-SMA and collagen expression to mechanical stretch (10%, 1 Hz, 72 h) were observed depending on the type of matrix substrates to which the cells adhered. For instance, cardiac fibroblasts grown on collagen type I and laminin were more sensitive toward myofibroblast differentiation induced by TGF-β1. Furthermore, mechanical stretch inhibited myofibroblast differentiation on cardiac fibroblasts seeded on collagen type I but promoted myofibroblast differentiation on those seeded on collagen IV and V and laminin. Overall, these findings may give insight into the impact of selective pathological deposition of ECM proteins on myofibroblast differentiation within different heart disease states.

Recently, pellino-1 (a protein called E_3 ubiquitin ligase) was found triggered in cardiac fibroblasts subjected to cyclic stretch (15%, 1 Hz, 24 h), stimulating proliferation, cardiac myofibroblast differentiation, and collagen synthesis. Transfection of pellino-1 silencer, adenovirus-mediated delivery of sh-pellino-1, into the stretched cardiac fibroblasts has negated these effects. Furthermore, silencing the activity of pellino-1 reduced the expression of TGF-β1 and binding activity of nuclear factor-κB and activator protein 1 to the promoter region of TGF-β1, which in turn activates TGF-β1, suggesting the role of pellino-1 in mediating cardiac myofibroblast differentiation though TGF-β1 signaling pathway in response to mechanical strain (85).

Instead of using the Flexcell tension system, a cardiac bioreactor system has been recently developed to apply strain on cardiac fibroblasts to investigate myofibroblast differentiation (52). The PDMS substrate and top and bottom glass slides of the bioreactor chamber are all transparent, thus allowing

optical instruments to be coupled to the system for the ease of optical inspection and real-time analysis. Furthermore, microfluidic devices can be integrated into this system to replicate the human body's natural hydrodynamic environment, i.e., flow velocity and shear stress (23), as interstitial fluid flow has been implicated in myofibroblast differentiation (25). This kind of integration may greatly enhance the versatility of the bioreactor system. Overall, various magnitudes of strain should be applied on cardiac fibroblasts to study myofibroblast differentiation, by considering the ECM to which the cells attached and the physiological niches in which the cells resided. The assessment of cardiac fibroblast responses under such forces are critical in furthering our understanding of both the regulation of normal cellular functions and pathological responses associated with heart diseases involving mechanical alteration.

3-D In Vitro Mechanoregulation of Cardiac Myofibroblast Differentiation

Studies on cardiac myofibroblast differentiation have been vigorously conducted in the 2-D mechanical microenvironment, but relatively few existing studies concerning the 3-D mechanical microenvironment. Although 2-D studies have provided valuable insights into cytoskeletal mechanics and the mechanisms by which cells interact with their physical surroundings, however, they do not represent a good approximation of the in vivo, 3-D cell microenvironment with its complex cell-cell and cell-ECM interactions (68). For example, cells cultured in a 2-D microenvironment usually appear to be flattened with most of the cell surfaces exposed to fluid and the culture substrate, thus limiting cell-cell contact. This reduces the communication and signaling among neighboring cells, which might affect their biological response and function (66). It has been observed that fibroblasts grown on 2-D culture plate spread with prominent cellular extensions, whereas those embedded within 3-D collagen matrixes favored spindle or stellate shape (68). This altered phenotype has been shown to affect their biological response such as proliferation and biosynthesis (74). Therefore, it is clear that 3-D studies mimicking native microenvironment are needed to get insight into mechanical cues in regulating cardiac myofibroblast differentiation in vivo (53). However, the findings of 2-D studies may provide a good platform for guiding 3-D studies to elucidate mechanotransduction pathways involved in cardiac myofibroblast differentiation.

Effect of ECM stiffness on cardiac myofibroblast differentiation in 3-D culture. METHODS OF ENGINEERING 3-D HYDROGELS WITH TUNABLE STIFFNESS. Most of the 3-D culture platforms include hydrogels as ECM mimics for cell encapsulation (25, 38). The mechanical environment (e.g., ECM stiffness) of the encapsulated cells can be controlled by several methods. For instance, by changing the polymer concentration, hydrogels with stiffness (ranged from ~Pa to ~MPa) can be fabricated, especially for synthetic hydrogels such as PEG diacrylate (84). The stiffness of MeHA was tuned (1–10 kPa) using this method to study myofibroblast differentiation in aortic valve interstitial cells (19). Another method to modulate hydrogel stiffness is to change the cross-linking density via modulating the concentration of hydrogen peroxide or sequential UV polymerization, without adjusting polymer concentration (56, 97, 98). For example, aortic valvular interstitial cells encapsulated in soft PEG-based hydrogels (0.24 kPa) were stiffened in situ via a second photopolymerization to generate stiff hydrogels (13 kPa) for evaluating the effect of matrix stiffness on myofibroblast differentiation in 3-D culture (56). Apart from the chemical adjustment method, the stiffness of the gels can be mechanically altered by adjusting the gel boundary condition (rigidly attached to the boundary or freely floating in media). Gels rigidly attached to the boundary (e.g., anchored to the sides and bottom of culture dish) exhibit higher stiffness compared with gels freely floating in media (2, 43).

EFFECT OF ECM STIFFNESS ON CARDIAC MYOFIBROBLAST DIFFERENTIATION. The effect of matrix stiffness on cardiac myofibroblast differentiation was evaluated by encapsulating cardiac fibroblasts in physically constrained (anchored to the sides and bottom of culture dish) collagen gels and in free-floating collagen gels in media (27). The floating gels offered little resistance to deformation as they were physically separated from the culture dish, whereas constrained gels offered increased resistance to deformation (2). Cardiac fibroblasts showed increased α-SMA expression in 3-D constrained collagen gels, suggesting that increased matrix stiffness promoted cardiac myofibroblast differentiation (27) (Fig. 3*A*) through the same mechanism as mentioned earlier. It has been suggested that transcription of α-SMA in fibroblasts is regulated by β_1 integrin, in where collagens bind and activate myofibroblast phenotype (10).

Effect of mechanical strain on cardiac myofibroblast differentiation in 3-D culture. METHODS OF APPLYING MECHANICAL STRAIN ON CARDIAC FIBROBLASTS CULTURED IN 3-D. The initial study on the effect of mechanical strain toward cardiac fibroblast responses, including myofibroblast differentiation in 3-D culture, was performed by applying static stretch on cardiac myofibroblasts encapsulated in a 3-D scaffold (e.g., 3DTC) using magnets (69). Thereafter, cardiac fibroblasts encapsulated in collagen gels were subjected to cyclic mechanical stretch and interstitial fluid flow using a self-designed bioreactor (25, 26). Furthermore, engineered heart tissues (EHTs) were produced consisting of cardiac fibroblasts and cardiomyocytes and which have been subjected to a cyclic stretching device developed by Zimmermann et al. (114) to optimize the culture conditions to generate functional EHTs for cardiac regeneration (62). Thus this platform has the potential to be used to study the cellular response of cardiac fibroblasts (including myofibroblast differentiation) in the presence of cardiomyocytes and mechanical strain, as it mimics the natural microenvironment of heart. However, this platform has not been used for studying cardiac myofibroblast differentiation to date.

EFFECT OF MECHANICAL STRAIN ON CARDIAC MYOFIBROBLAST DIFFERENTIATION. As a result, static stretch (30%, 1 wk) induces phenotypic conversion from myofibroblasts to cardiac fibroblasts with a concomitant reduction in collagen secretion by decreasing the expression levels of α-SMA (69). On the other hand, cardiac fibroblasts encapsulated in collagen gels showed different responses in myofibroblast differentiation when exposed to cyclic mechanical stretch and interstitial fluid flow, respectively (25, 26) (Fig. 3*B*). Cyclic mechanical stretch (5%, 1 Hz, 48 h) was found to attenuate the phosphorylation of smad2 (downstream effector of TGF-β1 signaling), thus reducing myofibroblast differentiation as indicated by decreased expression of collagen and α-SMA. Meanwhile, interstitial

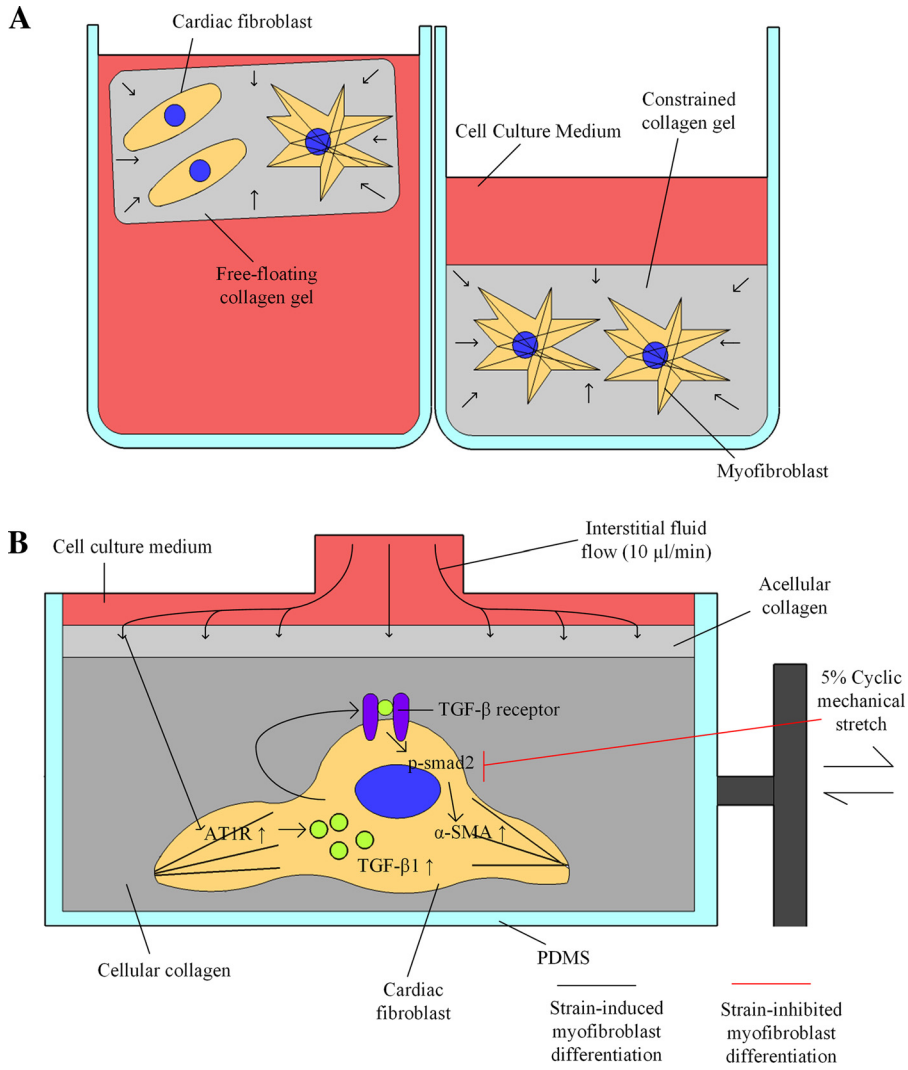

Fig. 3. Methods for engineering 3-dimensional cell mechanical microenvironment and the mechanisms proposed for cardiac myofibroblast differentiation. *A*: evaluation of matrix stiffness effect on myofibroblast differentiation in cardiac fibroblasts encapsulated in free-floating and physically constrained collagen gel. Arrow indicates cell traction force. *B*: bioreactor was designed to apply mechanical stretch and interstitial fluid flow on cardiac fibroblasts encapsulated in 3-dimensional collagen gels to elucidate the mechanisms for mechanical strain in regulating cardiac myofibroblast differentiation. AT_1R, angiotensin II type 1 receptor; PDMS, polydimethylsiloxane.

fluid flow (10 μl/min) increased the expression of collagen and α-SMA, thus promoting myofibroblast differentiation. Blocking of ANG II type 1 receptor (AT_1R) using losartan or short hairpin RNA negated this effect, indicating that fluid-induced shear stress stimulates the production of TGF-β1 though activation of AT_1R, which in turn promoted myofibroblast phenotype (25). In short, these results showed that mechanical stretch and interstitial fluid flow have distinct effects on cardiac fibroblast phenotype. Overall, the development of 3-D engineered microtissues (3-D EMTs) (21), EHTs (62, 79, 114), and 3-D microscale culture systems (e.g., hanging drop plates, microwell plates, and magnetic cell levitation) (59) shows the possibility of deeper insight into the cellular responses in vivo, which would help in furthering our understanding of mechanically regulated cardiac myofibroblast differentiation. This will encourage more 3-D studies on mechanoregulation of cardiac myofibroblast differentiation, which are presently sparse.

In Vivo Mechanoregulation of Cardiac Myofibroblast Differentiation

Mechanical stress-induced cardiac myofibroblast differentiation also has been studied using various in vivo heart diseased models, including animal-based models of myocardial infarction, diabetic cardiomyopathy, or pressure-overloaded heart. In normal hearts, cardiac fibroblasts are generally protected from mechanical stimuli by a stable cross-linked ECM network. In diseased hearts, the structural integrity of the ECM is disrupted because of prolonged cardiac remodeling, and this disorganized matrix causes exposure of cardiac fibroblasts to increased mechanical stress, thus contributing to myofibroblast differentiation (17). With the use of in vivo heart disease models, many inducers, integrin receptors, and signaling pathways involved in cardiac myofibroblast differentiation have been explored. For instance, concurrent elevations of collagen VI and myofibroblast content were observed in the infarcted rat myocardium 20-wk post-myocardial infarction, indicating the potential of collagen VI to induce cardiac myofibroblast differentiation (63). Furthermore, collagen-VI disruption has been shown to improve cardiac function and attenuate cardiac fibrosis in in vivo models of myocardial infarction (6, 54). Collagen IV is seen to interact with α_3-integrin possessed by cardiac fibroblasts to mediate myofibroblast differentiation (6, 81). On the other hand, expression of α-SMA and α11-integrin were found to be upregulated in rat models of diabetic cardiomyopathy. Disorganized glycated collagens formed in the diabetic hearts disrupt the structural integrity of ECM, increase mechanical

stress in the hearts, and interact with α11-integrin to induce cardiac myofibroblast differentiation (87).

In the pressure-overloaded heart, cardiac fibroblasts experience increased mechanical stress and strain, which activate them to differentiate into myofibroblasts, and produce excessive amounts of ECM, thus leading to cardiac fibrosis and failure (57). Mechanosensor proteins (e.g., syndecan-4) or pellino-1 has been found activated in the cardiac cells of in vivo models of pressure-overloaded heart. Syndecan-4 mediates cardiac myofibroblast differentiation through calcineurin/nuclear factor of activated T-cell (NFAT) signaling pathway, which is involved in the development of cardiac hypertrophy. Furthermore, α-SMA expression was found markedly reduced in the models of pressure-overloaded heart that lack of syndecan-4, further implicating the potential of syndecan-4 to induce myofibroblast differentiation (33). On the other hand, pellino-1 enhances the binding activity of nuclear factor-κB and activator protein 1 to the promoter region of TGF-β1, which activates the release of active TGF-β1 to promote cardiac myofibroblast differentiation. Silencing the activity of pellino-1 by infecting the pressure-overloaded rat hearts with adenovirus-mediated delivery of sh-pellino-1 (pellino-1 silencer) has notably reduced the expression of α-SMA and TGF-β1 (85). Although studies involving animal-based models may provide deep insights into the cellular responses in vivo, because of limited experimental parameters to be assessed, these studies were often undertaken following the in vitro studies for further investigations. Therefore, in vitro models, particularly 3-D tissue models that better mimic the in vivo microenvironment, are considered advantageous. When compared with animal-based models, in vitro models are more conducive to systematic and repetitive investigation of cell or tissue physiology, less expensive, and less time consuming. Furthermore, the use of in vitro models allows high-throughput testing and avoids the ethical issue of pain or discomfort caused to animals (20).

Conclusion and Future Perspectives

Cardiac fibrosis is a substantial problem in managing multiple forms of heart diseases. At the moment, cardiac fibrosis cannot be reversed or even stopped by surgeries and currently available drug therapies (e.g., antifibrotic agents) once it has begun. To treat fibrotic disease, fibroblast and myofibroblast survival becomes the main target. Further research on controlling the activities (e.g., myofibroblast differentiation) and survival of these cells should eventually lead to new, effective treatments. Myofibroblast differentiation is a complex and highly regulated process. The understanding of the regulation mechanism of this process offers several possible targets for intervention in cardiac fibrosis. For instance, TGF-β and its signaling pathway (AT_1R and NPRA), syndecan-4 and its signaling pathway (NFAT), or integrin receptors (e.g., α_3 and α_{11}), which interact with mechanical cues, could be interesting targets in the search of novel treatment agents against cardiac fibrosis. To date, mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) hold great potential in cardiac fibrosis treatment because of their paracrine effects (e.g., antifibrotic and cardiac regeneration) and cardiomyogenic differentiation ability, respectively. However, MSC and CSC therapeutic effects were greatly reduced under abnormal mechanical conditions in cardiac fibrosis. Therefore, integrating the mechanical factors into MSC and CSC therapies will help to improve the therapeutic efficacy and delivery mode of MSCs and CSCs for cardiac fibrosis treatment in future (72, 106). Furthermore, understanding of mechanobiology in cardiac myofibroblast differentiation may lead to many potential mechanotherapies (therapeutic interventions that recover damaged tissues by mechanical means at the molecular, cellular, or tissue level). Mechanobiology-based mechanotherapy is a promising future medical therapy, but its development is hindered by many current challenges, e.g., specificity (precise amplitude, duration, and frequency of mechanical stimuli specifically to certain cells), selectivity (therapeutic intervention on selective target without causing adverse effects on nontarget), and timeliness (40). To address these challenges, the mechanisms for mechanically regulated cellular processes (including cardiac myofibroblast differentiation) should be comprehensively and clearly defined.

Despite remarkable advances, the comprehensive mechanisms for mechanoregulation of cardiac myofibroblast differentiation remain elusive. Most of the related in vitro research were undertaken in 2-D cell culture systems, which have provided valuable insights into how cytoskeletal mechanics and cells interact with their physical surroundings. Yet, they do not represent a good approximation of the in vivo, 3-D cell microenvironments with complex cell-cell and cell-ECM interactions. Certain challenges in engineering 3-D mechanical microenvironments still remain. For instance, the properties of hydrogels (e.g., porosity, ligand density, stiffness, and strain) for engineering 3-D cell mechanical microenvironments are usually coupled to each other, making it difficult to distinguish independent effects of these factors on cardiac myofibroblast differentiation. New hydrogel fabrication and microengineering methods are needed to decouple these factors while maintaining 3-D cell encapsulation. In addition, the effects of mechanical cues on cardiac myofibroblast differentiation are also affected by the presence of other factors such as myocytes, growth factors, and oxygen tension. Therefore, it is necessary to include these factors into hydrogels to mimic natural microenvironment of cardiac fibroblast when studying the mechanisms of mechanically regulated cardiac myofibroblast differentiation in vivo. Furthermore, mechanical loading applied to or provided by hydrogels does not necessary represent the actual strain received by cells in hydrogels. The development of in situ high-resolution cellular and mechanical imaging techniques may help to quantify the mechanical dose transferred to cells. Finally, traditional models based on large-scale (several millimeters to centimeter) tissue constructs still have limitations in terms of mass delivery and distribution control of mechanical stimulations. The development of 3-D EMTs, EHTs, and 3-D microscale culture systems could encourage research into mechanoregulation of cardiac myofibroblast differentiation in a more accurate and controlled manner by overcoming the limitations of the existing methods.

GRANTS

This work was financially supported by National Natural Science Foundation of China Grant 11372243; International Science & Technology Cooperation Program of China Grant 2013DFG02930; Key Program for International S&T Cooperation Projects of Shaanxi Grant 2013KW33-01; High Impact Research Grant UM.C/HIR/MOHE/ENG/44 from the Ministry of Education Malaysia; China Young 1000-Talent Program and Program for New Century Excellent Talents in University Grant NCET-12-0437.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.W.Y. prepared figures; K.W.Y. drafted manuscript; K.W.Y., Y.H.L., G.Y.H., T.J.L., W.K.Z.W.S., B.P.-M., and F.X. edited and revised manuscript; K.W.Y., Y.H.L., G.Y.H., T.J.L., W.K.Z.W.S., B.P.-M., and F.X. approved final version of manuscript.

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