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Myofibroblasts: Trust your heart and let fate decide

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Abstract

Cardiac fibrosis is a substantial problem in managing multiple forms of heart disease. Fibrosis results from an unrestrained tissue repair process orchestrated predominantly by the myofibroblast. These are highly specialized cells characterized by their ability to secrete extracellular matrix (ECM) components and remodel tissue due to their contractile properties. This contractile activity of the myofibroblast is ascribed, in part, to the expression of smooth muscle α -actin (α SMA) and other tension-associated structural genes. Myofibroblasts are a newly generated cell type derived largely from residing mesenchymal cells in response to both mechanical and neurohumoral stimuli. Several cytokines, chemokines, and growth factors are induced in the injured heart, and in conjunction with elevated wall tension, specific signaling pathways and downstream effectors are mobilized to initiate myofibroblast differentiation. Here we will review the cell fates that contribute to the myofibroblast as well as nodal molecular signaling effectors that promote their differentiation and activity. We will discuss canonical versus non-canonical transforming growth factor- β (TGF β), angiotensin II (AngII), endothelin-1 (ET-1), serum response factor (SRF), transient receptor potential (TRP) channels, mitogen-activated protein kinases (MAPKs) and mechanical signaling pathways that are required for myofibroblast transformation and fibrotic disease.

Keywords

Fibrosis; Myofibroblasts; Smooth muscle alpha-actin; Extracellular matrix; TGF β ; Angiotensin II; Serum response factor; Rho signaling; TRP channel; Mechanical tension

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Disclosures

None declared.

1. Introduction

Indiscriminate fibrosis in the heart can occur with longstanding ischemic heart disease, inherited cardiomyopathy mutations, diabetes, and even aging. Excessive accumulation of extracellular matrix (ECM) that defines fibrotic heart disease profoundly impacts cardiac function due to the loss of myocardial compliance. In addition to these mechanical alterations, excessive scarring creates a perfect substrate for the initiation of ventricular arrhythmias leading to sudden cardiac death, as well as hastening heart failure progression [1–3]. Thus the clinical management of cardiac fibrosis presents a major challenge in positively influencing survival rates in heart failure patients. While the myocyte has been the overwhelming focus of most heart failure research to date, evolving evidence has increasingly implicated the cardiac fibroblast, or the myofibroblast, as a major pathological determinant in various forms of heart disease [4–7].

2. What is the Myofibroblast?

Fibroblasts are a highly prominent cell type within the myocardium that provides structural support [8]. Once activated these resident quiescent fibroblasts (*and other precursor cells*, Fig. 1) transdifferentiate into myofibroblasts, a fibroblast-smooth muscle cell hybrid that more effectively secretes and remodels the extracellular matrix (ECM) positioned between all myocytes. The healthy human heart is thought to be largely devoid of myofibroblasts but within days after an injury these cells appear in abundance [9, 10]. While the phenotypic features of the myofibroblast, which include their morphology, contractile properties, and ECM secreting ability, are well understood, there is limited understanding of the molecular markers for these cells. Morphologically, myofibroblasts are spindle shaped and can have protruding dendritic-like processes [5, 11, 12]. Transmission electron microscopy of cross sections from rat hearts that have undergone pressure overload demonstrate that cardiac myofibroblasts have elongated and serrated nuclei, extensive areas of rough endoplasmic reticulum and irregular non-sarcomeric myofibrillar structures [13]. Their defining protein markers include hyper-secretion of ECM proteins like periostin (Postn), collagen I (Col-I) and III (Col-III), fibronectin, and a more specialized ED-A isoform of fibronectin [14, 15]. In addition myofibroblasts begin to express smooth muscle genes including smooth muscle α -actin (α SMA), SM22 and caldesmon [5, 12, 15–20]. The type of myosin expressed in myofibroblasts is not entirely clear but both smooth muscle myosin [16, 18, 20, 21] and skeletal muscle myosins [22] have been detected *in vivo* in stromal myofibroblast and *in vitro* in transformed vascular interstitial cells respectively. Notably, in the injured myocardium most of the border zone myofibroblasts and about 50% of myofibroblasts within fibrotic lesions express embryonic smooth muscle myosin and α SMA in the absence of other smooth muscle markers, which differentiates them from smooth muscle cells [13, 23].

Myofibroblast transformation occurs in two stages [15]. In the first stage “proto-myofibroblasts” develop from fibroblasts (and possibly other precursor cells) that are characterized by the formation of cytoplasmic actin (*not* α SMA) stress fibers and small adhesion complexes that allows these cells to migrate into the wounded area [12, 15]. Proto-myofibroblasts begin secreting collagen and fibronectin rich matrix and then orientate

themselves along the primary stress axes within the injured tissue [15]. Around 20–30 hours after injury the second phase is initiated in accord with high levels of cytokines and mechanical stress that have accumulated within the wounded area, progressing these cells into mature myofibroblasts [15]. The defining marker of a fully differentiated myofibroblast is the *de novo* expression α SMA that forms a prominent stress fiber network underlying its new contractile function [12, 15, 17]. To aid in force transduction and enhance its tension generating capabilities, myofibroblasts develop mature super adhesion junctions made of cadherin-2 and -11 [24]. As the myofibroblast matures and more α SMA stress fibers form, cadherin-11 becomes the predominant cadherin isoform [24]. This newly formed contractile activity of a myofibroblast can be easily detected by measuring their ability to contract a collagen gel [22, 25]. In addition, myofibroblasts have an enhanced capability for migration that contributes to wound healing as well as tissue invasion [26–29].

While persistent cardiac fibrosis is clearly detrimental, myofibroblasts also play a protective role in the heart by producing a necessary acute wound healing response. For instance, after a myocardial infarction myofibroblasts facilitate the generation and remodeling of a fibrotic scar that prevents wall rupture as the ischemia-affected myocytes die over several days [9, 10, 25, 30–32]. In most physiologic wound healing situations myofibroblasts eventually disappear, but this process is less understood particularly in the heart after acute injury.

A critical therapeutic consideration is the degree to which cardiac fibrosis is reversible and if myofibroblasts can revert to a quiescent state or dedifferentiate back to their original cell type. There is evidence that as the injury event stabilizes and a permanent scar forms, some of the myofibroblasts undergo senescence [33] and/or apoptosis [34–36]. TUNEL staining and electron microscopy have shown that 2 weeks after myocardial infarction about a third of cardiac myofibroblasts are indeed undergoing apoptosis [36]. While the molecular mechanisms that govern this process are poorly understood, the inflammatory cytokine TGF β acts as both a primary initiator of myofibroblast transformation (*see discussion in subsequent sections*) but also as a prosurvival ligand. Fibroblasts treated with TGF β are resistant to apoptotic cell death initiated by Fas ligand (TNF α pathway) [37, 38], interleukin-1 (IL-1) [39], and serum starvation [40, 41]. TGF β treatment of human lung fibroblasts and mesenchymal cells caused an induction of focal adhesion kinase (FAK) and the prosurvival phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)/AKT pathway through canonical and non-canonical TGF β signaling respectively [40, 42]. As only a third of the myofibroblast population regresses by apoptosis the fate of the remaining cells is still in question. One possibility is that myofibroblasts could dedifferentiate back to their original cell type. To date this question remains untested in cardiac fibroblasts but *in vitro* valvular interstitial cells (VICs) toggle between the activated myofibroblast and quiescent state in response to altered matrix rigidity [43]. In a more definitive *in vivo* study genetic mapping was used to show that approximately 50% of liver myofibroblasts avoid cell death and return back to a hepatic stellate cell (HSC), which is the primary source for myofibroblasts in the liver [44].

Finally, myofibroblasts can also persist in the heart long after an acute injury has resolved due to ongoing neuroendocrine dysregulation or possibly aberrantly high wall stress that creates an environment leading to chronic cardiac fibrosis and/or hypertrophic scarring [5,

10, 12, 45–49]. For instance, with intermittent bouts of ischemia the heart chronically experiences a wound healing milieu that likely contributes to the permanent activation state of the myofibroblast [32]. The current interventions for fibrosis are directed at inhibiting inflammatory pathways or angiotensin signaling, which can diminish myocardial fibrosis but probably not due to the most direct molecular effectors [50]. Thus, better understanding the pathobiology of cardiac fibrosis and the direct underlying effectors of this process will be critical towards developing more potent therapeutic approaches. Given the prominent role myofibroblasts play in the fibrotic response this review will focus on emerging signaling pathways that appear to directly facilitate the differentiation and persistence of these cells.

3. Multiple Cell Types Transdifferentiate into Myofibroblasts

During embryonic development fibroblasts can be derived from local mesenchymal tissue by a process of endothelial or epithelial mesenchymal transition (EMT), or by transdifferentiation of circulating bone marrow derived stem cells (fibrocytes) [51–55] (Fig. 1). However, with respect to myofibroblast generation in the adult heart, or even other tissues, it is difficult to assemble a cohesive listing of bona fide lineage sources and percentages from each. This lack of cohesiveness in the published literature likely reflects heterogeneity in the genetic signature of the fibroblast itself that may indeed vary depending on its developmental lineage source or based on its differentiation status along a continuum. Not only do fibroblasts differ between tissues in their genetic signatures, but they likely vary based on their topographical location within any given tissue [56–58]. Even dermal fibroblasts have unique genetic signatures based on their anterior-posterior positioning throughout the skin of an organism [56].

During embryonic development, cardiac fibroblasts are primarily derived from the proepicardium that surrounds the heart. The proepicardium yields cells that help generate the cardiac cushions and valves, the vessels, and the fibrotic skeleton of the heart [59]. Recently, a lineage tracing study using a tamoxifen inducible Cre knocked into the *Tcf21* (encodes POD1 / epicardin) locus demonstrated that the majority of resident mesenchymal cardiac fibroblasts are derived from TCF21 positive epicardial cells, which commit to being a fibroblast before EMT occurs [60]. This result is supported by previous studies in chick embryos that also identified epicardial cells as the source for cardiac fibroblasts in the mature heart [61–63]. Notably, *Tcf21* null mice do not develop cardiac fibroblasts providing strong evidence that at least in the healthy adult heart the vast majority of fibroblasts come from this source [60].

During injury the longstanding paradigm has been that the altered cytokine, neurohumoral, and mechanical environment of a damaged tissue initiates locally residing fibroblasts to transform into myofibroblasts [7, 58]; however, myofibroblasts have also been shown to originate from alternative cellular sources including EMT, vascular pericytes or smooth muscle cells, a myeloid lineage, and fibrocytes (Fig. 1) [9, 58, 64, 65]. One hypothesis is that resident fibroblasts are designed to maintain tissue structure under normal homeostatic states, while during acute injury or in response to altered neuroendocrine signaling fibroblasts from these other sources are recruited and are responsible for pathophysiologic fibrosis [58, 64, 66]. It is also conceivable that the resident TCF21-positive mesenchymal

tissue fibroblast gives rise to myofibroblasts in the heart. To date the precise contribution of each cellular source to the myofibroblast population is still widely unknown as rigorous fate mapping studies in the heart have yet to be published. Also adding to this challenge is that the manifestation of different forms of fibrosis (chronic versus acute) may be due to the transformation of differential cellular sources into myofibroblasts or that the myofibroblast itself is substantially more heterogeneous than at first appreciated. Indeed, myofibroblasts likely display a continuum of phenotypic conversion based on cumulative signals, position in the heart, and the amount time they have been exposed to various signals.

4. Combinatorial Signaling Pathways that Regulate Myofibroblast Transformation

Myofibroblast transformation is initiated by the integration of neurohumoral, cytokine, growth factors and mechanical signals from the extracellular environment. Indeed, the injured myocardium is known to provide an environment rich in both biochemical and mechanical signals that can induce myofibroblast formation [67]. The subsections below will review several of the more well-described molecular regulators of this transformation process.

4.1 Canonical and Non-canonical TGF β Signaling

TGF β has been identified as a primary and potent mediator of myofibroblast transformation [4, 5, 12, 68] and fibrotic remodeling in the heart [69]. TGF β induction of myofibroblast transformation appears to be a universal paradigm that extends to fibroblasts isolated from most, if not all tissues [25, 68, 70–72]. During injury TGF β gene expression is upregulated and the protein is subsequently secreted into the ECM by surrounding mesenchymal cells, tissue macrophages and monocytes, and resident fibroblasts. TGF β protein is additionally regulated through either proteolytic cleavage or tension-mediated release from the ECM [12, 73]. TGF β 1 binds to its cell surface receptor, a heterodimeric complex consisting of TGF β receptor type I (TGF β RI) and II (TGF β RII) [74] (Fig. 2). In canonical TGF β signaling the transcription factors SMAD2/3 become phosphorylated by TGF β RI, which permits SMAD4 interaction and translocation into the nucleus where they are thought to participate in the transcriptional induction of myofibroblast genes (Fig. 2). ECM genes like *Colla*, *Col6a* and inhibitors of matrix metalloproteases (MMPs) are strongly upregulated by canonical TGF β -SMAD2/3 signaling in dermal fibroblasts [75]. This upregulation can be blocked by both a dominant negative SMAD3 and also by overexpression of an inhibitory SMAD (SMAD7) factor [75]. In support of this mechanism, isolated *Smad3* null cardiac fibroblasts showed impaired α SMA expression and stress fiber formation [76], and SMAD3 can directly bind sites in the α SMA promoter by electrophoretic mobility shift assay (EMSA) [77]. *Smad3* null mice have reduced collagen deposition and fibrotic remodeling following bleomycin-induced lung injury [78] and myocardial infarction injury [76]. Surprisingly, *Smad3* null mice had increased numbers of α SMA positive myofibroblasts in the infarcted heart relative to wildtype controls despite attenuated collagen production. These data might suggest that SMAD3 is only required for enhanced ECM secretion, but not transdifferentiation of the myofibroblast. We also observed that adenoviral-mediated overexpression of canonical inhibitory SMADs (SMAD6/7) were unable to block α SMA stress fiber formation [25]

suggesting that SMAD2/3 may only underlie select aspects of the myofibroblast functional phenotype.

TGF β can also initiate non-canonical signaling through TGF β RII, in part, by recruitment of TGF β activated kinase (TAK1) and TAK1 binding protein (TAB) that then activate the mitogen-activated protein kinase (MAPK) signaling branches such as c-Jun N-terminal kinase (JNK) and p38 kinase (Fig. 2). While much of the myofibroblast literature focuses on the canonical TGF β signaling pathway, there is mounting evidence suggesting that non-canonical TGF β pathway signaling through p38 may play a more central role [4, 25, 67]. For example, inhibition of the non-canonical TGF β signaling pathway using *Tgfb2* genetically targeted mice preferentially showed reduced fibrosis and remodeling in response to pressure overload [79]. While the loss of *Tgfb2* was specifically in myocytes, these data demonstrate the importance of non-canonical TGF β signaling in the cardiac injury response. Inhibitors of p38 signaling can also block TGF β -induced myofibroblast markers like Col-Ia, fibronectin, α SMA positive stress fiber formation, and collagen gel contraction in mouse embryonic fibroblasts (MEFs), cardiac, and human ocular fibroblasts [25, 80]. Genetic deletion of the downstream p38 target gene MK2 (MAPK-activated protein kinase 2) in MEFs also blocked TGF β induction of α SMA stress fiber formation [81]. Gain-of-function experiments for p38 signaling either in glomerular mesangial cells [82] or cardiac fibroblasts [25] showed phenotypic markers of myofibroblast transformation, as well as induction of the α SMA promoter [83]. In support of these observations, pharmacologic inhibition of p38 can reduce fibrosis and α SMA expression in rodent models of lung, kidney and cardiac injury [84–88]. Collectively, these data provide strong evidence that non-canonical TGF β -p38 signaling potentially regulates myofibroblast transformation and fibrosis.

4.2 Angiotensin II Signaling

Angiotensin II (AngII) is an additional neuroendocrine factor that is induced with disease where it appears to dominantly affect the cardiac fibrotic response and myofibroblast formation/activity [89]. In patients with end stage renal disease or heart failure pharmacologic inhibitors of AngII signaling, such as losartan, reduce or at least delay fibrotic remodeling of the heart [50, 90]. AngII is thought to affect the fibrotic response, in part, by serving as an upstream inducer of TGF β signaling in the cardiovascular system [4]. For example, studies on cardiac fibroblasts and valvular interstitial cells (VICs) have demonstrated that AngII treatment induces TGF β expression and initiates the expression of downstream fibrotic genes like *Colla*, which is blocked by losartan treatment [91–93]. AngII treatment of cardiac fibroblasts and kidney epithelial cells also activates SMAD2/3 and MAPK activity [94–96]. However, epithelial cells lacking TGF β were refractory to AngII-dependent increases in collagen gene expression [96]. A similar result was observed in cardiac fibroblasts [94]. In vascular smooth muscle cells AngII activates canonical SMAD transcription factors independent of TGF β [95] through a direct and sustained activation of p38. Both SMAD2 activity and fibrotic gene transcription (fibronectin and Col-I) increased as a result of the sustained p38 activation [95].

Interestingly, there are very few reports that directly test the role myofibroblasts play in AngII mediated fibrosis. While it is clear that AngII induces the expression of TGF β and

ECM genes like, collagen and fibronectin, these studies did not address a role for AngII in myofibroblast transformation or function. CD34/CD35 positive fibrocytes that also express Col-I have been identified in cardiac tissue after chronic AngII infusion, and these fibrocytes appear to be partially responsible for the AngII dependent upregulation of TGF β 1 and collagen type I and III [89]. However, AngII treated cardiac fibroblasts develop α SMA stress fiber and contractile function with or without TGF β R1 (ALK5) suggesting that AngII can stimulate myofibroblast transformation independent of TGF β [25]. In fact, pharmacologic inhibition of p38 blocked AngII-mediated myofibroblast transformation in cardiac fibroblasts showing the centrality of this “non-canonical” signaling branch [25].

In aortic smooth muscle cells AngII has been shown to activate α SMA transcriptional activity by enhancing the DNA binding strength of the transcription factor SRF [97]. In corroboration, shRNA knockdown of SRF in cardiac fibroblasts inhibits AngII-mediated myofibroblast transformation [25]. Collectively these results suggest that AngII signaling can initiate the myofibroblast differentiation program by two routes: 1) enhancement of TGF β 1 expression and activation of canonical and non-canonical signaling effectors, or 2) directly activating MAPK-SRF signaling (Fig. 2). Thus, AngII is clearly an important fibrotic mediator and effector of the myofibroblast program through more than one effector pathway.

4.3 Endothelin 1 Signaling

Endothelin 1 (ET-1) is a potent bioactive peptide that is produced during various forms of cardiac injury [4] where it affects the wound healing and fibrotic response, as also observed in other tissues [98–100]. For instance, injury to the liver causes endothelin converting enzyme transcript to stabilize, which in turn stimulates production of ET-1 [101]. ET-1 has been described as an essential mediator of fibroblast contraction, wound closure, and scar formation in the skin and lung [102–105]. Direct *in vivo* assessment of ET-1 mediated myofibroblast activation has not been well studied in the heart, although it can cause myofibroblast formation in primary cardiac fibroblasts in culture [106]. An emerging hypothesis in the field is that ET-1 acts synergistically with other profibrotic agonists to constitutively promote myofibroblast transformation in an injured tissue [4, 67]. AngII for example has been shown to increase ET-1 expression in cardiac fibroblasts, a function that can be blocked with losartan treatment [107], and in lung fibroblasts ET-1 expression is induced through non-canonical TGF β /TAK1/JNK signaling [108]. Pretreatment of lung fibroblasts with an endothelin receptor antagonist, bosentan, partially inhibited TGF β -mediated myofibroblast gene expression and the ability to contract a collagen gel matrix suggesting that ET-1 can act in tandem with TGF β to induce myofibroblast transformation [109]. Taken together, ET-1 appears to function synergistically and downstream of both TGF β and AngII to promote and maintain the myofibroblast phenotype [4, 67].

4.4 SRF-MRTF-RhoA-GTPase Pathway

Serum response factor (SRF) is a member of the MADS (stands for, MCM1, Agamous, Deficiens, and SRF) box-containing family of transcription factors that binds DNA in A/T rich regions flanked by double C/G regions called CArG boxes (CC(AT₆)GG) [110]. To enhance SRF's transcriptional efficacy, it can interact with cofactors like myocardin or

others members from the myocardin-related transcription factor family (MRTF) [111]. The promoter regions of genes underlying myofibroblast or smooth muscle cell differentiation and functionality appear to be highly enriched for SRF binding sites [110, 112, 113]. For example, the promoter of the α SMA gene contains multiple conserved CArG binding elements [110]. Also by inference, many genes primarily associated with smooth muscle differentiation contain SRF binding sites within their regulatory regions and some of these are induced in the myofibroblast to underlie its contractile phenotype [114–118]. The necessity for SRF-induced expression of these contractile genes *in vivo* is supported by the observation that mice with smooth muscle-restricted *Srf* deletion (using an SM-22 Cre transgene) failed to develop smooth muscle cells and died before birth due to cardiac insufficiency [119]. While *Srf* has yet to be deleted from primary fibroblasts *in vivo*, SRF gene expression becomes upregulated in myofibroblasts from bleomycin injured lung tissue [120]. In a rat ulcer model delivery of antisense SRF nucleotides blocked myofibroblast formation while overexpression of SRF promoted myofibroblast formation [121]. These results has been corroborated *in vitro*, as overexpression of SRF alone converts cardiac, esophageal, and pulmonary fibroblasts into myofibroblasts [25, 120, 121]. In loss-of-function studies, adenoviral overexpression of a short hairpin RNA (shRNA) directed at SRF fully inhibited both TGF β and AngII mediated myofibroblast transformation (Fig. 2 [25]). Thus, SRF appears to function as a central mediator of the myofibroblast gene program.

SRF transcriptional activity is dominantly regulated by the MRTF family. MRTFs are ubiquitously expressed and composed of two members MRTF-A (MAL, MKL1, or BSAC) and MRTF-B (MKL2 or MAL16). The MRTFs are regulated by G-actin monomers, which retain MRTF molecules within the cytosol [122, 123]. Once actin polymerizes, G-actin levels drop and MRTFs are released and translocated to the nucleus where they bind with SRF to induce transcription of target genes (Fig. 2). In pulmonary fibroblasts the mere inhibition of actin polymerization with lantrunculin B prevents TGF β from fully activating SRF gene transcription [124]. By contrast, stabilizing filamentous actin (less free G-actin) induces α SMA expression in the absence of a myofibroblast activating ligand, collectively demonstrating the importance of cytoskeletal feedback to the expression of myofibroblast genes, likely through a MRTF-SRF mechanism [124]. Indeed, adenoviral-mediated overexpression of MRTF-A transforms cardiac fibroblasts into myofibroblasts as evident by a significant increase in SM22 gene expression and α SMA positive stress fibers [111]. *In vivo*, *Mrtfa* null mice undergoing an AngII challenge showed negligible amounts of cardiac fibrosis [111]. Thus, the MRTF-SRF signaling module appears to provide critical feedback between the structural aspects of the fibroblast cytoskeleton, which may play a role in tension sensing and the need to initiate and maintain the myofibroblast program.

The Rho family of GTPases may provide the final linkage in explaining how cytoskeletal structural alterations, such as stretch and strain, affect actin polymerization and ensuing MRTF nuclear occupancy. RhoA signaling can be activated by many extracellular ligands including TGF β , signaling through G-protein coupled receptors, and mechanical tension (Fig. 2) [125]. Once activated, RhoA can initiate RhoA associated kinase (ROCK) or mDia to directly affect actin dynamics and cytoskeletal reorganization that then initiates cellular

migration, adhesion, membrane outgrowths and contraction [122, 123]. In fibroblasts derived from heart, lung, or skin pharmacologic inhibition of ROCK was able to block TGF β -dependent ECM gene transcription and α SMA expression [111, 124, 126]. TGF β stimulation of fibroblasts simultaneously activates Rho signaling and multiple other pathways including canonical and non-canonical signaling as described above [126].

In TGF β -mediated fibroblast to myofibroblast transformation a multi-phase/multi-pathway activation of canonical (SMAD) and non-canonical (MAPK-p38) TGF β signaling occurs early to initiate the transcription of ECM and smooth muscle contractile genes, but the Rho-MRTF-SRF pathway is part of a later phase helping to cement the contractile and migratory phenotype of the myofibroblast [124]. This later phase activation of Rho-MRTF-SRF signaling could explain, at least in part why *Mrtfa* null mice survive MI without rupture, suggesting that they have aspects of the fibrotic response still largely in tact [127]. It is also noteworthy to mention that TGF β -SMAD signaling transcriptionally upregulates many of the genes involved in Rho-MRTF-SRF signaling including SRF itself [19, 128, 129] suggesting it is later in the transformation process. Collectively, these data depict a model of TGF β driving myofibroblast differentiation and maintenance by switching on mutually reinforcing and complimentary pathways that all appear to converge on SRF-mediated transcription of myofibroblast genes (Fig. 2).

4.5 Transient Receptor Potential Channels Signaling

Recently, transient receptor potential (TRP) channels have been identified as another important activator of myofibroblast differentiation. TRP channels comprise a superfamily of cation channels (TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPP (polycystin), TRPA (ankyrin), and TRPML (mucolipin)). Most TRP channels are permissive to Ca²⁺ and Na⁺ entry and are activated by a variety of ligands as well as mechanical stretch. Both TRPM7 and TRPV4 have been shown to be involved in myofibroblast activation [130, 131], although the functional effects of TRPM7 seems to be specific to atrial fibroblasts [130]. In an unbiased genome-wide screen for novel activators of myofibroblast transformation the plasmid for TRPC6 was identified [25]. Adenoviral overexpression of TRPC6 in a variety of fibroblasts induced myofibroblast transformation as defined by the enhanced expression of several myofibroblast target genes (α SMA, Fn-ED-A and Col-I) and the functional ability to contract a collagen matrix [25]. Genetic ablation of *Trpc6* in fibroblasts made them refractory to both TGF β - and AngII-dependent myofibroblast transformation, and *Trpc6* null mice were defective in skin wound healing with reduced myofibroblast numbers, as well as greater death rates after myocardial infarction injury due to defective scar formation with wall rupture [25]. Further examination of where TRPC6 might function within known pathways for myofibroblast formation revealed a linkage with non-canonical TGF β -p38-SRF signaling, as SRF directly binds the TRPC6 promoter to induce its expression with TGF β stimulation.

The immediate effect associated with TRPC6 induction was the appearance of enhanced Ca²⁺ entry, which is also known to occur in fibroblasts with agonist stimulation, suggesting that Ca²⁺ is likely yet another nodal regulatory effector of myofibroblast transformation and function [25]. TRPC channels regulate many of their biological effects through Ca²⁺

dependent signaling pathways like calcineurin / nuclear factor of activated T-cells (NFAT) (Fig. 2)[132]. The activation of liver myofibroblasts is highly correlated with increased Ca^{2+} influx [133], and a significant population of cardiac fibroblasts converted to myofibroblasts just by the overexpression of a constitutively activated form of calcineurin (CnA) [25]. Inhibition of calcineurin signaling by genetic deletion of the gene or the overexpression of inhibitory molecules like Cain or VIVIT blocked TRPC6 induction of myofibroblast transformation suggesting that calcineurin signaling is activated by TRPC6 in this process. *In vivo* topical application of adenovirus expressing CnA restored dermal wound healing kinetics in *Trpc6* null mice. In addition CnA and TRPC6 can both override the loss of SRF and induce myofibroblast transformation [25] remarkably suggesting that NFAT is alone sufficient. Lending support to this hypothesis are data showing that the contractile phenotype of pulmonary myofibroblasts has also been linked to calcineurin-NFAT and MEF2 transcriptional activity [22]. Furthermore, $\text{TGF}\beta$ induces NFAT nuclear translocation and activity in both fibroblasts and mesangial cells [25, 134–136], while calcineurin inhibitors cyclosporine A (CsA) and FK506 block both ECM production and α SMA transcriptional activity [135–137]. NFAT and SRF have been shown to cooperatively regulate α SMA expression through a conserved intronic CARG element in smooth muscle cells [137]. Finally, mechanical stretch can initiate the expression of myofibroblast genes, collagen III and MRTF, through calcineurin-NFAT [136]. Together these data have added an entirely new signaling module to our understanding of the global network that underlies myofibroblast differentiation and function through TRPC- Ca^{2+} -calcineurin-NFAT (Fig. 2).

4.6 Mechanical-Transcriptional Coupling

A predominant aspect of chronic fibrotic remodeling is an increase in tissue stiffness that can have a negative influence on the functional properties of the heart [73]. This very event of altered tissue compliance may exacerbate or cause a feed-forward phenomenon that further enhances the fibrotic response and activity of myofibroblasts. For example, the composition of the ECM that develops in granulation tissue post injury contributes to the number of fibrotic precursor cells that differentiate into myofibroblasts [12, 73], and α SMA expression and myofibroblast formation increases when wounds receive additional tension [70]. Both fibroblasts and myofibroblast are highly sensitive to their mechanical microenvironment, and in general a greater induction of myofibroblast transformation is achieved with increased matrix tension [12, 73, 138]. For instance, fibroblasts or VICs differentiate into myofibroblasts when they are cultured in substrates with high tensile strengths that approximate the rigidity of the scarred tissue from which the fibroblasts were derived. By contrast, maintaining fibroblasts on highly elastic or soft matrices retains them in the quiescent state [139–145]. In some instances reculturing fibroblasts on a soft matrix can permit regression of the myofibroblast phenotype [43]. It has even been suggested that significant tensile forces are required for actin stress fibers to form [140, 146]. Notably, tension-based induction of myofibroblast differentiation appears to be a global mechanism, as precursors cells from multiple tissues including heart, valves, lung, liver, and skin will all differentiate when cultured on stiff substrates [139, 140, 142–145, 147].

The process of how mechanical signals are transduced into a molecular signal is still under investigation, although as described earlier, RhoA is one such possible effector [148]. Other

possible effectors include the activation of stretch-sensitive ion channels like the TRPs (also described earlier [149–151]) and the release or activation of stored growth factors like TGF β from the ECM by force-transducing matrix adhesion receptors or cellular contractility [152]. Of these mechanisms the activation of stretch-sensitive ion channels has the least amount of supporting evidence, although Ko *et al.* [153] demonstrated that stretching fibroblasts causes Ca²⁺ influx and actin assembly, which could be blocked by adding a stretch channel inhibitor like gadolinium or Ca²⁺ chelators. In this study cadherins were implicated as the stretch sensitive channel, but with the techniques employed alternative Ca²⁺ permeable ion channels could have also contributed. The TRP channels also represent an interesting candidate because their mechanical sensitivity is linked to G-protein-coupled receptors like the angiotensin receptor 1 [151].

Much of the mechanical transduction literature has focused on the ECM itself as the basis for sensing and then releasing soluble fibrosis-inducing ligands. TGF β 1 is synthesized and secreted by both fibroblasts and myofibroblasts. When secreted TGF β 1 resides in the ECM in a latent form complexed with a latency associated peptide (LAP), one of four latent TGF β binding proteins (LTBP 1–4) [154], and fibrillin. Active TGF β is liberated by either proteolytic cleavage or tension induced release of TGF β from the integrins [73, 154]. Both the enhanced rigidity of the ECM and α SMA-dependent contraction provide enough tension to mechanically activate latent TGF β providing a positive feedback signal for the continued induction of myofibroblast transformation and maintenance [12, 73]. That latency is involved in TGF β mediated fibrotic remodeling and pathologic processes in the heart was recently demonstrated by the identification of a genetic linkage with *Ltbp4* [155]. Mutations in LTBP4 also showed enhanced disease severity in Duchenne muscular dystrophy patients [156]. This holds equally true in disease models of Marfan syndrome in which latent TGF β activation is disrupted due to mutations in the gene encoding fibrillin, which is also likely part of a stretch-dependent effector pathway [154, 157, 158].

While the contribution of tension-mediated activation of TGF β is clearly important, there is evidence that α SMA stress fibers can form in the absence of TGF β release from the ECM [141]. The current paradigm to explain TGF β -independent mechanoactivation of the myofibroblast phenotype revolves around the actin cytoskeleton physically linking to focal adhesions that then sense mechanical tension to activate RhoA-MRTF-SRF [141, 159–161]. Culturing healthy pulmonary fibroblasts in stiffer matrices promoted actin fiber formation and MRTF nuclear translocation, which enhanced α SMA expression [141, 159], but fibroblasts devoid of MRTF or expression of a dominant negative form of MRTF showed loss of tension-induced α SMA gene expression [141, 159]. Similarly, matrix rigidity induced RhoA activity and α SMA expression, but pharmacologically blocking a primary aspect of RhoA signaling with a ROCK inhibitor prevented tension-induced myofibroblast transformation [141, 159, 162]. Alternatively, force-dependent activation of p38 MAPK in fibroblasts was also causally associated with α SMA expression [163]. Another aspect of these studies was the remarkable observation that α SMA itself was likely part of the transduction signal. For example, eliminating α SMA with antisense approaches blocked force-mediated p38 induction suggesting that the cytoskeleton and its contraction play a critical role in regulating the myofibroblast gene program [12, 161, 163].

5. Conclusion

The growing concern for remediating fibrotic disease has brought myofibroblast biology to the forefront of many diverse areas of biomedical research. Myofibroblasts clearly have an important physiologic role in wound healing (and prevention of rupture after myocardial infarction) and homeostatic tissue remodeling and maintenance. However, their continued presence through the self-reinforcing molecular signaling pathways described here can result in tissue pathology. Once the vicious cycle of myofibroblast activation is set in motion through on going bouts of matrix stiffening and enhanced neuroendocrine signaling, it may become increasingly difficult to dedifferentiate these cells back into quiescent fibroblasts. Thus, there is an impetus for understanding the molecular effector pathways that underlie not only the initial formation of myofibroblasts, with the hopes of identifying drugs that might stop their formation in chronic disease states, but also finding therapeutic strategies that could spare the protective and adaptive functions of these cell types such as during wound healing. We might also attempt to identify molecular targets that short-circuit the feed-forward signaling of the differentiated fibroblast in the hopes of better preserving tissue integrity long-term with chronic disease. Clearly the myofibroblast has emerged as a substantially more complex cell type than was originally appreciated. Based on accounts in the literature, albeit from different groups, these cells could arise from as many as 7 different lineages, which could easily underlie its heterogeneity. Even within one potential lineage myofibroblasts show substantial phenotypic differences based on tissue of residence, position in that tissue, age of the cell, and a complex integration of neurohumoral cues that collectively impart a graded phenotypic output and associated “marker” gene expression profile (i.e. ECM and contractile genes). In the end, the search for better pharmacologic agents to inhibit or at least modulate the myofibroblast phenotype may be more challenging than initially envisioned, though still certainly highly desirable.

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Highlights

- Cardiac fibrosis contributes to heart failure and the demise of this organ
- The myofibroblast is a specialized celltype that mediates chronic cardiac fibrosis
- We will review the molecular pathways that mediate myofibroblast transformation
- We will review the molecular pathways that control myofibroblast function
- Defining myofibroblast formation and function will suggest treatments strategies

Fig. 1. Myofibroblast precursor cell fates

Myofibroblasts can originate from several cellular sources during tissue injury. Cells resident to the tissue of interest including fibroblasts, smooth muscle cells (SMCs) and vascular pericytes can all differentiate into a myofibroblast, which can then facilitate acute injury repair processes and tissue remodeling. The hypothesized cellular sources for myofibroblasts during chronic injury include endothelial and epithelial cells that can undergo mesenchymal transition (EMT) and circulating bone marrow derived cells including fibrocytes and myeloid cells, although resident fibroblast sources that generate myofibroblasts could also participate in long-term chronic disease states.

Myofibroblast Target Genes: α SMA, TRPC6, Col-I, Col-III, Postn, Fn-ED-A, Cadherin-11

Fig. 2. Integrated signaling pathways in myofibroblast transformation

Canonical (purples) and non-canonical (blues) TGF β signaling are illustrated as converging on SMAD2/3 or SRF mediated transcription of myofibroblast genes, respectively. Neurohumoral signaling via AngII or ET-1 binding to its membrane localized G-protein-coupled receptor (GPCR) is depicted initiating myofibroblast gene transcription through p38/SRF (dark line) but also possibly leading to RhoA signaling (dashed line), similar to a presumed linkage between TGF β RI/II and RhoA signaling. The RhoA-MRTF-SRF signaling axis (red-shaded) is depicted as regulating smooth muscle actin (α SMA) dynamics and MRTF translocation to the nucleus to serve as a synergistic cofactor for SRF transcriptional activity of myofibroblast genes. Non-canonical TGF β -p38-SRF activates TRPC6-Ca²⁺-calcineurin-NFAT signaling is also mediating myofibroblast gene transcription. Mechanical tension activates myofibroblast differentiation through stretch-sensitive ion channels, activation of latent TGF β , RhoA-MRTF and p38 through signals from the cytoskeleton. Together these signaling events amplify and reinforce the transcriptional pathways responsible for myofibroblast differentiation.