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Molecular Networks Underlying Myofibroblast Fate and Fibrosis

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Abstract

Fibrotic remodeling is a hallmark of most forms of cardiovascular disease and a strong prognostic indicator of the advancement towards heart failure. Myofibroblasts, which are a heterogeneous cell-type specialized for extracellular matrix (ECM) secretion and tissue contraction, are the primary effectors of the heart's fibrotic response. This review is focused on defining myofibroblast physiology, its progenitor cell populations, and the core signaling network that orchestrates myofibroblast differentiation as a way of understanding the basic determinants of fibrotic disease in the heart and other tissues.

Keywords

Myofibroblast; Fibrosis; Differentiation; Actin Cytoskeleton; TGF β ; Mitogen Activated Protein Kinases; TRP Channels; RNA Binding Proteins

1. Introduction

Fibrosis is a hallmark of heart disease independent of its etiology. Managing cardiac fibrosis is a major clinical concern given its correlation to a heightened risk of ventricular arrhythmias, sudden cardiac death, and rapid progression towards failure [1-4]. After an ischemic insult or myocardial infarction fibrotic remodeling is a critical phase of physiologic wound healing because the enhanced secretion of ECM structurally supports the injured myocardium. However, permanent fibrotic scarring ensues due to the heart's limited regenerative capacity. Similarly, with chronic injury that is associated with hypertension, aging, diabetes, or primary genetic disease, fibrotic matrix accumulates in perivascular and interstitial spaces without myocyte necrosis and further impairs myocardial function.

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Disclosures
None.

Decades of work have identified the myofibroblast as the major cellular effector of the fibrotic response [5-8]. To date research in the fibrosis field is dominated by studies from *in vitro* experimentation. However, the development of multi-dimensional biomaterials for cell culture studies that better mimic a tissue's microenvironment makes it clear that physical surroundings markedly influence the molecular determinants of a myofibroblast precursor cell's fate [9-13]. Moreover, new fate mapping strategies have evolved that allow a deeper understanding of the complexities of identifying myofibroblast phenotypes and the regulatory networks governing this process. Together these factors likely contribute to the plethora of confounding data regarding key molecular pathways and cell populations responsible for fibrosis. Hence, this review places special emphasis on curating these data into a refined molecular signaling network based on *in vivo* validation as well as emphasized genetic fate mapping efforts as a means of understanding how cellular heterogeneity impacts myofibroblast function and hence fibrotic outcomes in response to various cardiac injuries.

2. The Myofibroblast Identity

2.1 Characteristics of the Myofibroblast

Myofibroblasts are typically found in the interstitium of the injured heart with spindle shaped appearance, dendritic processes, and expanded golgi and endoplasmic reticulum organelles. Contractile bodies in myofibroblasts are electron dense and contain embryonic smooth muscle myosin and α -smooth muscle actin (α SMA) [14]. Occasionally alternative skeletal myosin isoforms have also been detected in myofibroblasts [15]. The emergence of α SMA stress fibers is the primary marker for a fully matured myofibroblast, and α SMA underlies the ability of the myofibroblast to contract, migrate, and impart traction forces onto the ECM. Along with acquiring contractile function, myofibroblasts can secrete large amounts of matrix specialized for reinforcing the structural integrity of the heart including variants of Collagen 1 (Col1a1), Collagen 3, Collagen 4, Periostin (Postn), and Fibronectin (**Figure 1**). During the activation process TGF β initiates the incorporation of the ED-A splice variant into the matrix, which in is required for both latent TGF β activation and the incorporation of α SMA into stress fibers that together creates a feed-forward loop for reinforcing the myofibroblast phenotype [16-18]. Indeed, mice lacking the ED-A variant have reduced numbers of α SMA+ myofibroblasts and fibrosis after myocardial infarction [19].

Myofibroblasts physically connect to the ECM through integrins that are linked to highly developed focal adhesions. These focal adhesions are 4-5 times longer than those observed in quiescent fibroblasts [20]. Increased expression of factors like α v β 3 or α v β 5 integrin, cadherin 11, vinculin, tensin, paxillin, and activated focal adhesion kinase (FAK) can also be used to discriminate between myofibroblast and other cell types [10, 20, 21]. Myofibroblast maturation is marked by the appearance of "supermature" focal adhesions in tandem with α SMA incorporation into stress fibers [10, 20, 21]. The dual expression of these two structures in a myofibroblasts sustains contractile tension, which potentiates wound closure [20]. These unique functional properties can be quantified with various mechanical assays or by assessing wound closure rates *in vivo* [18, 20, 22].

Myofibroblast differentiation goes through multiple stages in which intermediate phenotypes or proto-myofibroblasts form. At this stage stress fibers containing β -cytoplasmic actin, rather than α SMA, appear with underdeveloped focal adhesions [10]. Proto-myofibroblasts, while structurally immature, synthesize new matrix components like fibronectin ED-A, which are necessary for the final transition into the fully differentiated state [6, 7]. To date signals that mature proto-myofibroblasts and the relative contribution of mature versus immature myofibroblast to fibrotic remodeling has not been defined. Moreover, the challenge of defining the cellular underpinnings of fibrosis is surely impacted by the coexistence of both immature and mature myofibroblasts.

2.2 Sources of the Myofibroblast

Healthy tissue is normally devoid of myofibroblasts, but injury or stress induces their appearance. Diverse sources of cardiac myofibroblasts have been proposed including: locally residing fibroblasts [23-27], circulating fibrocytes of a hematopoietic lineages [5, 28-32], and tissue resident cells undergoing endothelial (EndoMT) or epithelial (EMT) to mesenchymal transition (**Figure 1**) [33-35]. As we discuss the data for these sources below, it is important to note several limitations to fate mapping studies of fibroblasts. First, all of these cells are derived from multiple developmental sources, and thus they carry many markers that might be passed down to myofibroblast such as: TCF21 (POD1 or Epicardin), platelet-derived growth factor α receptor (PDGFR α), thymus antigen 1 (THY1, CD-90), Wilm's tumor gene 1 (WT1). However, many of these factors fail to extensively mark or solely identify the heart's fibroblast population [23-27]. For instance, after aortic banding only a small fraction of WT1+ fibroblasts co-label with Colla and even more rarely with α SMA [26, 36]. Similarly, THY1+ fibroblasts can co-express Colla, but again only a fraction become α SMA positive in response to hypertensive injury suggesting that these cells rarely differentiate into myofibroblasts at least in response to pressure overload (**Figure 1**) [24, 26]. There is still another fibroblast population labeled by Colla, PDGFR α , in addition to TCF21, which is a transcription factor required for ventricular fibroblast development that robustly marks a majority of ventricular fibroblasts in the adult heart [23].

Given this genetic diversity, uniformly labeling mature myofibroblasts is extremely challenging. Colla has been used extensively to mark both quiescent and activated (myofibroblast) cardiac fibroblast populations [23-25, 37]. However, with pressure overload only 15% of Colla+ fibroblasts fully matured into α SMA+ myofibroblasts indicating that a hypertrophic stimulus only mildly induces myofibroblast differentiation or another myofibroblast precursor may exist that is not labeled by Colla [24]. To overcome this limitation, several labs use the matricellular protein periostin (Postn) (**Figure 1**) [38]. Postn is vital for the development and organization of the ECM and is a hallmark of myofibroblast differentiation [38, 39]. During embryonic development Postn is strongly expressed in cardiac fibroblasts from the TCF21 lineage as well as in some of the valves [38]. With the transition to adulthood Postn is expressed in the periosteum, periodontal ligament, metastatic cancer cells, cells undergoing mesenchymal transition, injured valves, and ~10% of quiescent cardiac fibroblasts [25, 38, 40]. In areas of injury, Postn is robustly expressed in cardiac fibroblasts [25, 41, 42] and corresponds with conversion to a myofibroblast phenotype [22, 41, 43]. Postn-Cre transgenic mice have been extensively used in several

organs including the heart to either label or manipulate myofibroblast genes [25, 38, 40, 43-45].

Despite the strong indication that resident fibroblasts are the primary source for myofibroblasts [23-27], there is evidence that other cells contribute to the myofibroblast pool. Endothelial to mesenchymal transition was implicated in contributing to both development and injury repair in the heart [34], although recent studies by independent labs indicate that <5% of myofibroblasts come from this mechanism during pressure overload (**Figure 1**) [23-26]. Bone-marrow derived circulating fibrocytes can also adopt characteristics of the myofibroblast in response to ischemia-reperfusion injury [31, 46]. These cells retain the hematopoietic markers CD45, CD34 and CD14 while taking on myofibroblast characteristics. Lineage-traced bone marrow transplant studies confirmed bone marrow as a source for myofibroblast-like cells in ischemic injury and a neurohumoral overload models of fibrosis [29]. By contrast transplantation-parabiosis studies indicated hematopoietic fibrocytes do not contribute to the myofibroblast population following pressure overload [26].

As perivascular fibrosis is commonly observed with many cardiac diseases including hypertension, myocarditis and ischemia, many groups have sought out a source for myofibroblasts in the perivascular niche. Pericytes are located near the endothelial basement membrane and express chondroitin sulfate proteoglycan 4 (NG2) and PDGFR α . These cells act as a support structure for the endothelium in the vasculature. Pericytes have been associated with fibrosis in the kidney, lungs, and liver (stellate cells) [47, 48]. In the heart, direct expansion of pericytes into myofibroblasts was shown in a mouse model of muscular dystrophy [47]. Recently glioma associated oncogene-1 (Gli1) was found as a marker of resident perivascular stem cells in kidney, lung, liver, and heart (**Figure 1**) [48]. After pressure overload injury Gli1+ cells differentiated into α SMA+ myofibroblasts that were causal for fibrosis. In this same model fibrogenesis was blocked by ablating Gli1+ cells; providing convincing evidence that this population contributes fibrotic remodeling in hypertensive injury.

As is clear from the data described above the mixed developmental origins of fibroblast presents a huge hurdle for singularly targeting cardiac fibroblasts *in vivo*. This is reminiscent of the heterogeneity described in fibroblasts populations of other organs like the skin where fibroblasts isolated from various anatomical locations have distinct genetic profiles [49]. Furthermore, we have yet to understand how the genetic profile of myofibroblast progenitors change during differentiation, how genetic heterogeneity affects myofibroblast function, and whether specific types of injury recruit particular populations.

3. The Myofibroblast Differentiation Regulatory Network

The injured myocardium is extremely dynamic as the cellular, chemical, and mechanical milieu fluctuates with the stages of wound healing. These environmental changes activate the programed differentiation of resident fibroblasts into myofibroblasts (**Figure 2**). Their appearance is triggered by several cytokines, chemokines, neurohumoral factors, and vasoactive peptides circulating throughout the injured environment. These chemical signals

include profibrotic (TGF β) and inflammatory cytokines (TNF α , IL-1 and IL-6), as well as the heart failure hormones angiotensin II (AngII) and endothelin-1 (ET-1). The changing mechanical environment also induces fibroblasts to differentiate. To date researchers are still trying to discover how these environmental signals are integrated within the fibroblast and transduced into the differentiation and persistence of myofibroblasts in the heart. Our current knowledge these mechanisms fall under 4 fundamental signaling branches of a regulatory network that are reviewed below and illustrated in **Figure 2**.

3.1 Canonical and Non-canonical TGF β Signaling

TGF β and its downstream effectors encompass one of the most potent regulatory cascades of myofibroblast differentiation and the fibrotic response. Several cell populations including fibroblasts, inflammatory cells, and resident mesenchymal cells, contribute to the enhanced levels of extracellular TGF β after an injury. Typically, cells secrete TGF β in its latent form where it pools in the matrix and becomes activated by enzymatic cleavage events or through mechanical liberation [50]. With injury the heart attains a level of stiffness necessary for activating TGF β prior to incorporating α SMA into the actin cytoskeleton of differentiating myofibroblasts [20]. This mechanism creates a positive feedback loop that maintains TGF β in its active state thereby reinforcing the differentiation signal. There are 3 TGF β ligands; however, TGF β 1 is the most highly studied in the context of fibrosis. Indeed silencing of TGF β receptor type 1 (TGF β R1) blocks myofibroblast differentiation *in vitro* [22]. TGF β 1 binds to its heteromeric receptor made up of TGF β receptor type 1 (TGF β R1) and 2 (TGF β R2), which activate canonical SMAD or non-canonical signaling cascades [51]. In the canonical pathway SMAD2/3 is phosphorylated by TGF β R1, which permits complex formation with SMAD4 and the transcription of myofibroblast target genes. There's growing evidence in the literature to suggest that SMAD2 is not a mediator of cardiac fibrosis [52, 53]; whereas, SMAD3 regulates the secretory phenotype of myofibroblasts including the following ECM genes: Col1a1, Col1a2, Col3a1, Col5a2, Col6a1, Col6a3, Fibronectin and TIMP-1 [54]. Furthermore, adenoviral overexpression of inhibitory SMADS 6 and 7, which are specific to canonical SMAD 2/3 signaling, fails to block TGF β -dependent α SMA stress fiber formation in isolated cardiac fibroblasts [22]. These data are consistent with the attenuated fibrotic scarring observed in heart in SMAD3 knockout mice after myocardial infarction without a decrease in α SMA+ myofibroblast numbers [54]. However, the α SMA promoter contains SMAD3 binding sites and TGF β response elements suggesting that SMAD signaling regulates the transition to a contractile α SMA+ myofibroblast. *In vitro* SMAD3 deficient fibroblasts fail to form α SMA-stress fibers, contract, or migrate in response to TGF β [54], and recent *in vivo* evidence has demonstrated that fibroblast-specific loss of glycogen synthase kinase 3 β (GSK3 β) hyper-activates SMAD3 signaling causing cardiac fibroblasts to become α SMA+ and promote fibrotic scar expansion [55]. Moreover, this phenotype could be blocked by treating these mice with small molecule inhibitors of SMAD3 [55] supporting the notion that SMAD3 is involved either directly or indirectly with regulating several features of the myofibroblast phenotype.

Non-canonical TGF β signaling, which works through activated TGF β R2 (*rather than 1* [56-59]) has also been strongly implicated as a regulator of myofibroblast differentiation [60]. TGF β R2 recruits intermediate factors that turn on several downstream signaling

cascades including mitogen-activated protein kinases (MAPKs), Rho-GTPase-Actin, phosphoinositide 3 kinase (PI3K) / protein kinase B (PKB or AKT), and TNF Receptor-Associated Factor (TRAF) 4 and 6 [60]. The best characterized non-canonical pathways for myofibroblast differentiation are activated downstream by TGF β receptor kinase 1 (TAK1), which phosphorylates several intermediate MAPKs and then activates p38 or c-Jun N-terminal kinase (JNK) signaling [22, 61-68]. Using alternative signaling intermediates, TGF β R2 can also induce classical MAPK-extracellular related kinase (ERK) or Rho-GTPase-actin cytoskeletal signaling. While not tested directly in the cardiac fibroblast, activating TAK1 in the heart exacerbates cardiac hypertrophy and induces fulminant interstitial fibrosis [69, 70]; whereas, genetic ablation of TGF β R2 does the opposite in a pressure overload model underscoring the importance of non-canonical TGF β signaling to cardiac remodeling [56]. Dual specificity MAPK Kinases 3 and 6 (MKK3 and MKK6) transduce the signal from TAK1 to p38, and in kidney fibroblasts TAK1 overexpression activated MKK3 and p38 resulting in enhanced ECM gene expression [71, 72]. By contrast the expression of a dominant negative TAK1 mutant or genetic ablation of MKK3 reduced p38 activity causing a down-regulation of collagen genes [71]. While markers for the fully matured myofibroblast were not examined in these studies, expressing constitutively active variants of MKK6 or p38 in rodent fibroblasts induces α SMA transcriptional activity and α SMA⁺ stress fibers [22, 68]. Notably, MKK6-mediated myofibroblast differentiation occurs independent of TGF β R1 activity demonstrating that the MKK6-p38 signals reside downstream of the TGF β receptor [22]. More proximal targeting of the p38 pathway has been performed using specific pharmacologic inhibitors of p38 itself. This strategy dramatically blocked the fibrotic response in injured hearts, lung and kidneys coinciding with a reduction in both collagen and α SMA immunoreactivity [65, 73, 74]. Moreover, p38 inhibition in cardiac fibroblasts or genetic ablation of MAPK-activated protein kinase 2 (MK2), which is a downstream target of p38 blocked α SMA stress fiber formation, suggesting that p38-mediated myofibroblast transformation is causal for the fibrotic response [22]. However, bleomycin-induced lung injury in MK2 knockout mice causes a fulminant fibrotic response in the absence of α SMA⁺ myofibroblasts. In this case MK2 regulated contractility and motility through α SMA⁺ stress fiber formation [75, 76]. These data indicate that various p38 targets modulate select phenotypic characteristics and underscore that the components of these molecular networks likely control independent myofibroblast functions. To date we do not fully understand how myofibroblast contractility and ECM secretion independently alter wound healing and fibrosis, or the ramifications of singularly blocking one function or the other. Because TGF β signaling is not confined to SMAD-dependent or MAPK signaling, many other pathways and their interactions have yet to be explored with respect to myofibroblast differentiation and the heart's fibrotic response. The dynamic interplay between these pathways and their temporal activation will ultimately determine the fibroblast's differentiated state.

3.2 Ca²⁺-dependent Signaling

There is a growing list of transient receptor potential (TRP) cation channels associated with cellular differentiation including the conversion of fibroblasts into myofibroblasts. These channels are part of a family composed of several subtypes that include the following classifications: TRPC (canonical), TRPM (melastatin) TRPV (vanilloid), TRPP (polycystin),

TRPA (Ankyrin), and TRPML (mucolipin). Most of the TRP channels are activated by a variety of ligands, the mechanical environment, and/or changes in internal Ca^{2+} stores. Three different laboratories have demonstrated that cardiac fibroblasts utilize a Ca^{2+} signal from these channels to transform quiescent fibroblasts into myofibroblasts. Of those channels TRPM7, TRPV4, and TRPC6 have been linked to myofibroblast differentiation. TRPM7 appears to be highly specific to human atrial fibroblasts where it represents the dominant source of Ca^{2+} initiated during TGF β -dependent myofibroblast differentiation [77]. TRPV4 appears to act as a mechanosensor in ventricular fibroblasts as loss of TRPV4-mediated Ca^{2+} entry blocked TGF β and tension-dependent myofibroblast transformation [78]. These particular TRP channel mechanisms have not been tested in the intact heart, but TRPV4 deficient lungs fail to fibrose in response to toxic stimuli like bleomycin [79]. Moreover, loss of TRPV4 in this lung injury model impaired actin cytoskeletal reorganization suggesting that fibroblasts failed to activate and transform.

TRPC6 was identified in a genome-wide screen for regulators of fibroblast to myofibroblast differentiation [22]. This result was validated in cardiac fibroblasts by overexpressing TRPC6 and several other TRPC channels [22]. Interestingly, TRPC6 alone was the only TRPC channel that robustly induced both myofibroblast target gene expression (α SMA, fibronectin ED-A, periostin, and Col1a) and contractile activity [22]. These phenotypic changes were absent or extremely weak with the overexpression of other TRPC family members suggesting that TRPC6 has unique properties beyond Ca^{2+} entry that may facilitate myofibroblast differentiation. Moreover, fibroblasts derived from TRPC6 null mice were unable to differentiate when stimulated with various profibrotic agonists including TGF β and AngII demonstrating a requirement for TRPC6 in this process [22]. These agonists through a p38 mechanism cause a coordinated increase in TRPC6 gene expression and store-operated Ca^{2+} entry, which is absent in TRPC6 null fibroblasts explaining their impaired ability to differentiate [22]. Accordingly, TRPC6 null mice have poor dermal wound healing and high mortality rates post myocardial infarction due to reduced matrix deposition and a lack of differentiated myofibroblasts [22].

TRP channels create spatially discrete microenvironments with distinct Ca^{2+} -dependent signaling [80]. Several potent signaling factors have been associated with TRP channels including Rho-GTPase, protein kinase C, receptor for activated C kinase-1, calmodulin, and calcineurin. Calcineurin signaling via nuclear factor of activated T-cells (NFAT) is the predominant pathway regulating fibroblast differentiation by TRPC6. This was proven independently by genetic ablation of calcineurin activity, overexpressing genetic calcineurin-NFAT inhibitors, and treatments with pharmacologic inhibitors of calcineurin activity like cyclosporin-A (CSA) and FK506, which all impaired matrix formation, α SMA transcription, and antagonized TRPC6-dependent myofibroblast transformation. In the converse experiment expressing constitutively active calcineurin variants in fibroblasts converted them into myofibroblasts independent of a Ca^{2+} signal. NFAT's role in myofibroblast differentiation is further supported by data demonstrating that TGF β induces its nuclear translocation in fibroblasts from several sources and its requirement for pulmonary myofibroblast contraction [15, 22]. These data suggest that NFAT alone is capable of initiating the transcriptional cascade causal for the myofibroblast phenotype at least *in vitro*. More striking evidence of the impact of the TRPC6-Calcineurin-NFAT signaling axis was

observed *in vivo* as the topical delivery of adenovirus expressing constitutively active calcineurin rescued the poor dermal wound healing of TRPC6 null animals [22]. Together these data demonstrate that TRPC6-Calcineurin-NFAT signaling is a critical branch of the molecular network governing myofibroblast differentiation. Ca^{2+} -sensitive signaling mechanisms are just now being rigorously explored with respect to the fibrotic response *in vivo* and elucidating Ca^{2+} -transcriptional coupling in myofibroblast progenitors could provide exciting new pathways for modulating fibrosis.

3.3 Actin Cytoskeleton & Mechanotransduction Mechanisms

Fibroblasts are highly sensitive to the physical stimuli present in their microenvironment. Changes in tissue rigidity, shear forces, and strains are mechanical cues sensed by fibroblasts and transduced into new phenotypes. For instance, fibroblasts cultured on silicone membranes and subjected to repeated bouts of cyclical strains convert into myofibroblasts in the absence of any secondary soluble growth factors, demonstrating the fibroblast's sensitivity to mechanical cues [81, 82]. In fact the rigidity of standard tissue culture plates can induce a fraction of otherwise quiescent fibroblasts to activate. Hence, multi-dimensional bioactive matrices (e.g. synthetic hydrogels or silicone substrates) and micro/nano-patterned surfaces provide improved *in vitro* methods for recapitulating a tissue's physical environment. For instance, culturing fibroblasts, hepatic stellate cells, or mesenchymal cells in matrices of different elastic moduli demonstrate a positive correlation between tissue rigidity and myofibroblast differentiation. These *in vitro* data correspond to the finding that an injured tissue stiffens prior to signs of histopathologic fibrosis or matrix deposition, and likely precedes the emergence of fully differentiated myofibroblasts [83]. While a certain level of tissue stiffness must be achieved before α SMA incorporates into filamentous actin bundles, other quiescent fibroblast markers, like vimentin, are unresponsive to changes in the mechanical environment [10, 12, 84]. In comparison, culturing differentiated myofibroblast in compliant matrices that mimic healthy tissue causes partial reversion to a quiescent mesenchymal state [12, 13]. Fate-mapping studies in two different hepatic injury models revealed that myofibroblasts revert back to their original cell fate, but with a modified genetic signature permissive of accelerated conversion to a fibrotic myofibroblast upon additional injury [37]. These data suggest that myofibroblasts dedifferentiate to their original progenitor phenotype in response to trauma rather than regress by apoptotic mechanisms as previously proposed, suggesting they have genetic memory of their previous mechanical environment [37, 85].

Beyond direct manipulations of tissue mechanics, fibroblasts also respond to changes in matrix architecture, which has only recently been uncovered through nano-patterned scaffolds. Here matrices fabricated as an array of ridges that vary in depth, spacing, and directionality mimic the environmental topography created by the ECM. Scaffolds with densely packed grooves directed along a single axis, which is analogous to injury-induced ECM, increased focal adhesion maturity, cytoskeletal organization and migration speeds in fibroblasts [86-88]. Notably the ECM after infarct varies in terms of maturity but also isotropic organization [89], and these data suggest that the varied topography of the ECM impacts the maturation and density of differentiated myofibroblasts found in the injured myocardium.

Clearly, fibroblasts sense and interpret their mechanical surroundings. Uncovering how these signals integrate and transduce into their biologic outcome is an area of active research. The fibroblast's physical interaction with the extracellular environment occurs via a contiguous structural complex comprised of the ECM, integrin-associated focal adhesions, and the actin cytoskeleton. These interactions convey multi-dimensional geometric information. Relative to chemical signaling, mechanical inputs can last for longer durations and extend over longer distances, often overriding chemical inputs that are also present [90]. In the context of myofibroblast differentiation, the actin cytoskeleton is a hub for integrating mechanical inputs into transcriptional events. Rho GTPases like Rho, Rac, and CDC42 reorganize the actin cytoskeleton into stress fibers that, in a myofibroblast, contain smooth muscle isoforms. They are activated by a number of profibrotic ligand-receptor complexes including TGF β R2, G-protein coupled receptors (GPCRs), Integrins, PDGFR α , and possibly TRP channels [91]. Rho factors, particularly RhoA, appear to govern α SMA stress fiber formation in fibroblasts and other mesenchymal cells, although targeted genetic approaches directed at RhoA, B, and C have not yet been used to support this hypothesis. A single study in human atrial fibroblasts found that the SMAD3-dependent myofibroblast transformation works through RhoB leaving undetermined which Rho-GTPase actually promotes a myofibroblast phenotype [92]. Downstream of Rho are 2 regulatory branches mediated by the Rho associated kinases (ROCK1 & 2) and the diaphanous family of formins (mDia), which are both required for polymerization and bundling stress fibers. RNAi-mediated knockdown of mDia in fibroblasts blocks tension-dependent α SMA transcription and myofibroblast differentiation [93]. Similarly, pharmacologic inhibition of ROCK blocks cytoskeletal remodeling and matrix gene expression in TGF β stimulated fibroblasts [94-96]. Moreover, ischemic hearts from ROCK1 knockout mice fail to form myofibroblasts and have less fibrosis [97].

The Rho-GTPases and actin itself are directly linked to myofibroblast gene expression through SRF. SRF's primary DNA target sequences are CArG boxes (CC(AT)₆GG), which are found in many myofibroblast gene promoters including α SMA and Col1a [96, 98]. SRF-mediated myofibroblast gene transcription is sensitive to actin dynamics. Lantrunculin-B inhibits actin filament formation in response to profibrotic stimulants due to a loss of SRF transcriptional activity [95]. Both myocardial infarction and bleomycin lung injury in mice increase SRF expression. Accordingly, when mimicking this result *in vitro*, SRF overexpression has been shown to promote the differentiation of cardiac, lung, and esophageal fibroblasts into α SMA⁺ myofibroblasts [22, 43, 99, 100]. Conversely, SRF deletion from cardiac fibroblasts and MEFs blocks AngII and TGF β -mediated myofibroblast conversion [43]. Beyond its actin-sensing mechanism SRF is a nodal convergence point for many of the signaling networks described above. In fact, the induction of Ca²⁺-Calcineurin-NFAT signaling by TGF β requires SRF-mediated transcription of TRPC6 [22], yet surprisingly the ectopic expression of TRPC6 or a constitutively activate form of calcineurin in fibroblasts lacking SRF can still induce myofibroblast differentiation despite the loss of such a powerful transcription factor [22]. Notably, α SMA expression in smooth muscle cells requires a cooperative interaction between NFAT and SRF, which could be applicable to myofibroblast development. Recently, SRF was excised specifically in TCF21⁺ cardiac fibroblasts *in vivo*. At baseline the loss of SRF was innocuous [43]; however, myocardial

infarction caused cardiac rupture in a substantial fraction of these mice, while those receiving a milder ischemic injury survived with nearly a 30% reduction in total myocardial fibrosis [43]. In a separate model, SRF was deleted in Postn+ dermal fibroblasts, which significantly delayed wound closure kinetics and elicited dermal rupture in some mice creating a larger wound [43].

SRF's transcriptional activity is potently enhanced by the myocardin related transcription factor family (MRTF). MRTF-A (MAL or MKL1) and MRTF-B (MAL16 or MKL2) are widely expressed and directly regulated by actin cytoskeletal dynamics. MRTFs bind to G-actin monomers, which sequester them in the cytosol, but as F-actin bundles into stress fibers, the G-actin monomer pool depletes, thereby freeing MRTFs to translocate to the nucleus. Once in the nucleus MRTF-A and B bind SRF, which potentiates SRF target gene transcription. Overexpression of MRTF-A converts cardiac fibroblasts into α SMA+ myofibroblasts [96]. Similarly mechanical induction of myofibroblast transformation correlates with the nuclear accumulation of MRTF-A [101]. Conversely, myofibroblast differentiation is significantly blocked by knocking down MRTF-A or pharmacologically inhibiting actin regulators like the Rho-GTPases [96, 102]. In line with these data the global loss of MRTF-A in mice antagonizes fibrotic scarring after myocardial infarction and chronic neurohumoral stimulation [96]. Tissue collected from the border zone of infarcted MRTF-A null mice have reduced ECM (Col1a, Col3a, and Elastin) and contractile gene expression (α SMA and SM-22) [96]. The sensitivity of this pathway to actin dynamics suggests that Rho/MRTF-SRF signaling predominate during late myofibroblast differentiation, perhaps to reach full maturation when compared to TGF β signaling which appears more critical to induction. This hypothesis could explain why MRTF-A null mice are not susceptible to cardiac rupture after myocardial infarction MI [96, 103]. Because TGF β and Rho signaling rely on SRF mediated myofibroblast gene transcription, these pathways likely run in parallel to reinforce each others signals.

Additional signaling pathways that transduce mechanical stimuli into myofibroblast differentiation are continuing to emerge. For instance, the transcriptional coactivators of the HIPPO pathway yes-associated protein (YAP) and transcriptional coactivator with a PDZ domain (TAZ) translocate to the nucleus in response to mechanical stimuli [85]. In lung fibroblasts from patients with idiopathic pulmonary fibrosis there is enhanced nuclear localization of both factors [104]. Conversely, knockdown of YAP or TAZ in skin, liver, and kidney fibroblasts decreases TGF β and α SMA expression [105-107]. Similarly, TAZ deficient mice have a blunted fibrotic response to bleomycin-induced lung injury [108], while pharmacologic inhibition of YAP impairs liver stromal cells from activating and becoming fibrotic in response to toxic stimuli [106]. The role of YAP and TAZ has not been examined specifically in cardiac fibroblasts, but loss of YAP/TAZ function in myocytes promotes fibrosis [109, 110].

Other mechanically sensitive factors include several MAPK family members like ERK, JNK, and p38 that were reviewed earlier. Passively stretching rodent cardiac fibroblasts activates ERK and JNK; whereas, p38 is activated by perpendicular traction forces that are transmitted through the integrin-actin cytoskeletal complex [67]. These forces send p38 to the nucleus where it synergizes with transcription factors to promote actin expression.

Conversely, p38 inhibitors block the tension-dependent actin remodeling that is characteristic of myofibroblast transformation. In non-fibroblast cell types, p38 regulates actin polymerization and stress fiber formation, suggesting a global role for p38 in transducing mechanical signals into cytoskeletal changes [111-114]. Taken together, p38 and SRF appear to be the primary integrators of signals from several growth factors and mechanically-activated pathways that drive myofibroblast differentiation (**Figure 2**). The PI3K/AKT pathway is also mechanically responsive in VICs, which like fibroblasts, convert into myofibroblasts when cultured in stiff matrices [13]. This response is antagonized by preventing PI3K/AKT signaling. In lung tissue, focal-adhesion mediated myofibroblast differentiation also depends on AKT signaling, supporting the role of PI3K/AKT in fibroblast mechanotransduction [115].

3.4 Transcriptome Regulation by RNA Binding Proteins

RNA binding proteins (RBPs) have emerged as potent downstream components of signaling networks that regulate disease and differentiation. RBPs regulate all facets of RNA maturation including alternative splicing, stability, polyadenylation, and transcript localization [116-118]. This function coupled with having pleiotropic RNA targets make RBPs powerful differentiation factors as they can completely alter a cell's phenotype just through modulating their activity or expression. Until recently mechanisms involving RNA maturation have not been associated with fibrosis or the myofibroblast. However, genome wide screening for regulators of myofibroblast differentiation uncovered the RBP, muscleblind-like 1 (MBNL1)[43]. MBNL1 is part of a conserved family of proteins encoded by 3 different genes that use zinc finger domains to interact with CUG repeat regions of RNAs [119, 120]. When MBNL1 proteins are functionally sequestered from their normal RNA targets inappropriate maturation of gene products ensue resulting in myotonic dystrophy [121, 122], blocking cardiac myocyte maturation [123], or inhibition of embryonic stem cell differentiation [124].

In the cardiac fibroblast MBNL1 is normally expressed at extremely low levels, but with myocardial infarction or profibrotic agonists MBNL1 expression markedly increases [43]. Overexpression of MBNL1 in a variety of fibroblasts promotes myofibroblast differentiation as defined by α SMA⁺ stress fibers, contractility, and ECM gene transcription; whereas, MBNL1 null fibroblasts fail to differentiate in response to TGF β treatment [43]. Moreover, MBNL1 knockout mice have impaired dermal wound healing and poor survivability in response to myocardial infarction [43]. Post-mortem examination revealed that MBNL1 null mice had a markedly diminished fibrotic response and significantly fewer myofibroblasts when compared to their injured wild-type littermates. To directly examine whether MBNL1 underlies myofibroblast differentiation *in vivo*, an MBNL1 transgene was conditionally overexpressed in TCF21⁺ cardiac fibroblasts, which caused mild fibrosis to develop in otherwise healthy hearts [43]. With chronic AngII infusion the fibrotic area nearly doubled when compared to non-transgenic controls demonstrating that MBNL1 is a *bona fide* promoter of myofibroblast differentiation. Notably, TCF21⁺ interstitial cells are also in the kidney and lung, and like the heart these organs turned fibrotic with MBNL1 overexpression. MBNL1-regulated transcripts were identified by RNA-immunoprecipitation (RIP) and RNAseq. Here MBNL1 interacted with ~2500 transcripts that clustered into functional

pathways associated with chemokine signaling, TGF β signaling, MAPK signaling, actin cytoskeletons, focal adhesion, adheren junctions and cell cycle regulation [43]. Molecular constituents of several of these pathways were already known as fundamental regulators of myofibroblast differentiation [39]. MBNL1 promoted myofibroblast differentiation in part by stabilizing several of those known transcripts including TGF β R2, SRF, and calcineurin. Interestingly, MBNL1 stabilized all of the spliced variants of calcineurin, one of which is constitutively active. As discussed above activated calcineurin is sufficient for converting fibroblasts into myofibroblasts, which was reconfirmed in this study [43]. Given the important role SRF plays in myofibroblast differentiation the regulation of SRF mRNA by MBNL1 was also examined in greater depth. TGF β and MBNL1-mediated myofibroblast differentiation was occurring through a distal MBNL1 binding site in SRF's 3' UTR that stabilizes the SRF transcript [43]. These results defined an entirely new regulatory mechanism involving the maturation of select transcripts that serve as integral effectors within the key signaling networks orchestrating myofibroblast differentiation. MBNL1's regulation of the fibroblast transcriptome represents a rapid mechanism for simultaneously activating several nodal signaling axes that transition fibroblasts into the myofibroblast cell fate.

4. Conclusions and Future Directions

Fibrosis is the pathologic consequence of a chronically active wound healing response that is a universal signature of disease in most organs. Despite this tissue diversity, myofibroblast function is a unified determinant of fibrosis regardless of the inciting stimulus for disease. Our understanding of the cellular and molecular underpinnings of cardiac fibrosis is still in its infancy with many important questions regarding the basic tenets of fibroblast and myofibroblast biology left unknown. While it's now clear that the genetic signature or lineage of a fibroblast varies within tissues including the heart, how this heterogeneity effects their function and potential to differentiate into myofibroblasts has yet to be studied leaving open the question of whether all fibroblast populations contribute to the fibrotic response. Myofibroblast differentiation also invokes a powerful feed forward loop that reinforces the fibrotic response through recurring bouts of tissue stiffening and matrix deposition. Whether this recurrent stiffening of the heart causes fibroblasts to terminally differentiate particularly in an environment like the myocardium where the functional tissue fails to renew is still unknown, but underscores the importance of temporally defining *in vivo* the stages of myofibroblast differentiation in response to different cardiac pathologies. Along this same line the heart's fibrotic response varies by injury in which pathologies like hypertension, diabetes, and aging cause reactive interstitial fibrosis versus ischemia and myocardial infarction, which cause replacement fibrosis and scarring. The basis for this differential fibrotic response has not been rigorously studied, but is important to understand for translating basic research into clinically viable therapies for fibrosis. As for now manipulating the existence of myofibroblasts through promoting or eliminating regulators of its cell fate *in vivo* demonstrates that targeting this cell effectively modulates wound closure kinetics and the fibrotic response providing strong rational for studying myofibroblast biology and identifying therapies directed at halting myofibroblast formation or regressing myofibroblasts back into its precursor once the initial injury response has subsided. Here we

detailed 4 key branches of the myofibroblast differentiation network, and at any of these points pharmacologic inhibition would be expected to entirely short circuit external cues to differentiate and secrete fibrotic matrix. Several of the molecular regulators discussed here are already pharmacologically tractable and include p38, MRTF, ROCK1/2, Calcineurin, and TRPC6. Despite these prospective targets cellular specificity as well as myocyte renewal remain confounding variables when considering targeting these pathways for clinically managing fibrosis in patients with cardiovascular disease or heart failure.

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Highlights

- Myofibroblasts are primarily derived from resident fibroblasts.
- MAPK signaling regulates fibroblast to myofibroblast differentiation.
- Ca^{2+} signals from the TRP channel family initiates myofibroblast differentiation.
- The cytoskeleton senses the environment and imparts signals to differentiate.
- RNA binding proteins mature the transcriptome to promote differentiation.

Figure 1.

Cellular progenitors of cardiac myofibroblasts. Fate mapping approaches have identified that locally residing TCF21 positive epicardially derived fibroblasts are responsible for the majority of myofibroblasts that secrete collagen (Col1a) and periostin (Postn) and express αSMA. A majority of TCF21 fibroblasts are also positive for PDGFRα and can also express THY1 and/or WT1. Circulating fibrocytes that are hematopoietic (CD45+, CD34+, CD14+) and GLI1+ pericytes can also give rise to myofibroblasts in the heart, but their contribution to the myofibroblast population seems to be primarily associated with ischemic injury. Endothelial and epithelial to mesenchymal transition has also been proposed as a mechanism for myofibroblast formation although recent studies suggest that less than 5% of Col1a+ myofibroblasts come from this population in response to aortic banding.

Figure 2.

The signaling network orchestrating fibroblast to myofibroblast differentiation. Injury-induced chemical (TGFβ, AngII, ET-1 and others) and mechanical stimuli (tissue rigidity, shear and tensile forces, and matrix isotropy) activate 4 potent signaling axes: canonical TGFβ (red), non-canonical TGFβ-MAPK (orange), actin cytoskeletal (green), and Ca²⁺-pathways (purple) to induce myofibroblast gene transcription. MBNL1 selectively matures transcripts of the myofibroblast differentiation transcriptome including most components of all 4 axes listed above. Several signaling arms converge on p38 and the transcription factor SRF, suggesting their nodal role in this differentiation process. The combinatorial activation of these regulators potentiate and reinforce the transition of a fibroblast into a myofibroblast. *Factors in gray have links to these pathways but have not been directly proven as regulators myofibroblast conversion.