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Cardiac Fibrosis: The Fibroblast Awakens

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Abstract

Myocardial fibrosis is a significant global health problem associated with nearly all forms of heart disease. Cardiac fibroblasts comprise an essential cell type in the heart that is responsible for the homeostasis of the extracellular matrix; however upon injury, these cells transform to a myofibroblast phenotype and contribute to cardiac fibrosis. This remodeling involves pathological changes that include chamber dilation, cardiomyocyte hypertrophy and apoptosis, and ultimately leads to the progression to heart failure. Despite the critical importance of fibrosis in cardiovascular disease, our limited understanding of this cell population impedes the development of potential therapies that effectively target this cell type and its pathological contribution to disease progression. This review summarizes current knowledge regarding the origins and roles of fibroblasts, mediators and signaling pathways known to influence fibroblast function after myocardial injury, as well as novel therapeutic strategies under investigation to attenuate cardiac fibrosis.

Keywords

Cardiac fibroblasts; cardiac fibrosis; heart failure; therapeutics

Introduction

Heart failure, the clinical manifestation of numerous forms of cardiovascular disease, is a devastating disorder characterized by interstitial fibrosis, chamber remodeling and reduced ventricular compliance. Heart disease remains the predominant cause of mortality in the United States, accounting for nearly 800,000 deaths per year.[1] Furthermore, it presents a considerable economic burden, with estimated direct and indirect costs in 2011 of about $320 billion and predictions suggesting that costs will rise to about $918 billion by 2030.[1] Despite substantial improvements in therapeutic strategies, cardiovascular disease remains the leading cause of death worldwide indicating an urgent need for innovative treatment strategies.

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None.

Nearly all etiologies of heart disease involve pathological myocardial remodeling characterized by excessive deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts (CFs), which reduces tissue compliance and accelerates the progression to heart failure. The CF is an essential cell type, predominantly of embryonic epicardial and endothelial origins,[2–4] that resides within the myocardial interstitium, epicardial and perivascular regions.[5, 6] Physiologically, CFs are responsible for homeostasis of the ECM, which provides a structural scaffold for cardiomyocytes (CMs), distributes mechanical forces through the cardiac tissue, and mediates electrical conduction.[6–8] Previously, identification of these cells was largely based on phenotypic observations; morphologically, fibroblasts are flat, spindle shaped cells with multiple processes when propagated on tissue culture plastic. The cardiac cellular milieu can vary greatly depending on the species being examined and between healthy and injured myocardium.[9] Although prior studies suggested that CFs accounted for the majority of cells within the adult rodent and human myocardium,[7, 9, 10] recent reports with more accurate delineation of the CF population have proposed that CFs may comprise less than 20% of the total cell population in the adult murine heart, substantially less than previously suggested.[2, 11] While it is now appreciated that a majority of resident fibroblasts originate from the embryonic epicardium,[12, 13] the contribution of various resident and infiltrating cells to the population of activated cardiac myofibroblasts (MFs) remains under active investigation, including further refinement of specific molecular markers for CFs.

Unlike other organs, the heart has very limited regenerative capacity following injury, and instead, repair processes involve the removal of necrotic CMs followed by fibrotic scar tissue replacement that acts to preserve myocardial structural and functional integrity. To perform these functions, CFs within the connective tissue convert to their activated form, often known as MFs, which secrete elevated levels of ECM proteins to promote a profibrotic environment. Cardiac fibrosis provokes pathological changes that culminate in chamber dilatation, CM hypertrophy and apoptosis, and ultimately lead to the development of congestive heart failure.[14–16] While the sources of these activated fibroblasts remain under intense investigation and debate, the refinement of molecular markers and the development of new techniques for lineage tracing are helping to enhance our understanding of their origins.[2, 4] Despite the pathophysiological importance of fibrosis in cardiovascular disease, the CF remains relatively mischaracterized and poorly understood; furthermore, there currently exist limited clinical interventions that effectively target this cell type and its pathological contributions to disease progression. While recent progress has improved our understanding of the CF, the absence of a universal marker or method for lineage mapping, combined with the heterogeneous nature of this cell population, have provided a formidable challenge in the interpretation of results.[17] This review summarizes current knowledge regarding the origin and role of the CF in development and disease, and presents recent advances toward novel therapeutic interventions targeting this critical cell population.

Role of CFs in Injury

The CF is now recognized for its fundamental contributions to the heart's response to various forms of injury (Figure 1). In general, the critical phases of this response consist of inflammation, proliferation of non-myocytes and scar maturation, with the CF intimately

involved in all of these processes. Following an acute myocardial injury, the expression of various pro-inflammatory cytokines and pro-fibrotic factors is upregulated in CFs, leading to increased proliferation of these cells and ultimately, the transition to the MF phenotype.[18] During this maturation phase, MFs begin to secrete elevated levels of collagens and other ECM proteins. The purpose of this adaptive fibrosis is to maintain the structural integrity and pressure generating capacity of the heart, as a loss of integrity in the mechanical strength of the ventricle may lead to myocardial dysfunction or rupture. In the advanced phases of fibrotic scar formation, the tensile strength of collagen increases within the site of injury.[19] In this setting, a subset of activated MFs acquire new phenotypic characteristics, including the expression of the contractile protein α smooth muscle actin (αSMA), and contribute to pathologic cardiac remodeling.[20, 21] While initially adaptive, these processes eventually lead to the development of adverse changes in ventricular structure and compliance, and a concurrent progression into overt heart failure.

Pathologic cardiac remodeling is characterized by fibroblast accumulation and excessive deposition of ECM proteins, which leads to distorted organ architecture and has significant consequences on cardiac function.[22] Fibrogenesis contributes to impaired cardiac function as fibrotic ECM increases ventricular stiffness and can lead to contractile dysfunction.[23, 24] In addition, excess ECM and fibroblasts impair mechano-electric coupling of CMs, thus reducing cardiac contraction and increasing the risk of arrhythmogenesis and mortality.[25, 26] This pathologic process can also include hypertrophy and dysfunction of CMs via paracrine mechanisms, which further contribute to impaired cardiac function.[27] Furthermore, inflammation and fibrosis within perivascular regions may decrease tissue availability to oxygen and nutrients and increase the pathological remodeling response.[28]

Following the initial wound healing process in many tissues, a majority of collagen-secreting fibroblasts undergo apoptosis and leave a mature scar composed of cross-linked collagen and other matrix components.[29] However, when this process persists in the heart, MFs within the cardiac scar tissue continue to release maladaptive proinflammatory and prohypertrophic signals, characterized by CM hypertrophy and necrosis followed by replacement fibrosis.[8, 30–32] Importantly, following an acute injury such as myocardial infarction (MI), activated fibroblasts not only increase the synthesis of ECM proteins at the site of injury, but also within the healthy tissue remote from the immediate infarct,[15, 33] commonly referred to as reactive fibrosis.[5, 34] This potentiates the pathophysiologic response after acute myocardial injury by reducing chamber compliance and increasing stiffness of the ventricles.[35] A portion of MFs remain embedded in mature cardiac scars long after the initial myocardial injury, likely due to a resistance to apoptosis, which perpetuates these pathologic processes.[34]

Cardiac fibrosis can be categorized into two forms, namely reactive interstitial fibrosis or replacement fibrosis, each which can be recapitulated by several models of heart failure.[36, 37] Transverse aortic constriction (TAC) is an animal model of left ventricular pressure overload (similar to aortic stenosis) characterized initially by reactive interstitial fibrosis, an adaptive response to preserve cardiac structure and function, and followed by replacement fibrosis in areas of CM necrosis.[38] In animal models of acute ischemic injury, such as MI or ischemia/reperfusion injuries, the initial injury is characterized by an

immediate, robust inflammatory response and extensive CM death; replacement fibrosis restores this region devoid of viable CMs to prevent cardiac rupture.[39] Cardiac fibrosis also occurs in the context of right ventricular (RV) volume overload that can result from congenital malformations, such as repaired tetralogy of Fallot or pulmonary hypertension. In a mouse model of RV volume overload, fibrosis is evident first in the subendocardial RV, but relatively little is known of the cellular origins or molecular mechanisms of fibrosis in this context.[40] These models represent critical yet differing recapitulations of human disease, and as such, show distinct functions of the fibroblast response to injury.[39]

Cardiac Fibroblast Markers and Limitations

One major area of study is an attempt to quantify relative contributions of the various cardiac cell lineages to the CF population following injury. Such studies rely upon molecular biomarkers, but experimental interpretation is difficult as this cell population can include fibroblasts, endothelial cells, pericytes and immune cells. Further complicating this effort, while lineage-specific markers have long been established for several cell types within this group, robust markers of both quiescent CFs and activated cardiac MFs remain elusive. With numerous molecular markers now being recognized for their ability to identify particular populations of CFs, the controversy surrounding this cell population remains unresolved. In the heart, as with other organs, there is increasing interest in the investigation of novel CF and MF markers, and transcriptomics is proving an effective method for the uncovering of new markers.[41] Aside from the immediate benefits for cell identification, biomarkers in use for other organs, for example the kidney, can be used as tools to diagnose the progression of disease and also as novel therapeutic targets. Here we present several commonly used molecular markers to identify quiescent and activated CFs, concerns regarding their use, and how the refinement of these markers may potentially provide for the identification of a purer fibroblast population (Table 1).

Among the first markers used to identify CFs was discoidin domain receptor 2 (DDR2), which acts as a receptor for several ECM proteins.[42] While expression is limited in other cell types, including endothelial cells, smooth muscle cells and myocytes, it does not appear that all CFs are positive for DDR2.[9] Fibroblast-specific protein 1 (FSP1, S100A4), one of the initial markers identified, was believed to be a reliable fibroblast indicator.[43] FSP1 was commonly used to designate quiescent and activated CFs in myocardial injury, including pressure overload and infarction-induced fibrosis models. However, recent in vivo work utilizing FSP1-GFP and FSP1 immunostaining has demonstrated that it is not fibroblast-specific, based on positive expression in several cell types, including a subset of immune and endothelial cells.[4, 44–46] Another marker used to identify CFs is Thymus cell antigen 1 (Thy1, CD90), a membrane glycoprotein expressed on the surface of CFs.[47] While this receptor does appear to be expressed on all CFs, other cell types are known to express Thy1, including immune cells, lymphatic endothelial cells and pericytes.[48–50] Vimentin is an intermediate filament protein that has been used extensively both in vitro and in vivo for the identification of fibroblasts, as it is expressed in the majority of fibroblasts both in the healthy and injured myocardium.[2] While CFs are positive for vimentin, concerns with its expression in other cell types, including the endothelium, restrict its efficiency and specificity.[51–53] In general, the major complications associated with many of the markers

currently in use involve either expression in other cell types or limited expression in all forms of CFs.[5, 54]

The transcription factor TCF21 has been used successfully to trace the development of resident CFs from their epicardial precursors.[47] In addition to its extensive expression during development, TCF21 is also broadly expressed in CFs both within interstitial and perivascular fibrotic regions in response to heart failure models of both pressure overload and ischemic etiologies.[55] Importantly, TCF21 is not expressed in infiltrating immune cells identified by positive CD45 expression.[55] Additionally, the TCF21 transcription factor appears to be required for CF cell fate determination in development.[47] The inducible TCF21^{mERCremER} transgenic mouse line represents one of the more powerful inducible lines for the study of CFs in development and disease.[56] In development, TCF21 is essential for the formation of CFs in utero,[47] and the TCF21 signal remains active in adult mice, which enables this line to provide significant insight into the contribution of resident fibroblasts to the development of fibrosis in the injured heart. Platelet derived growth factor receptor alpha (PDGFRα) has emerged as a reasonable marker of CFs both during development and in healthy and injured adult tissues.[47, 57] It is also expressed in multiple other organs including lung fibroblast lineages,[58] oligodendrocyte progenitors,[59] and bladder interstitial cells.[60] In the heart, PDGFRα is robustly expressed in fibroblasts, with relatively limited expression in other cell types, including smooth muscle cells[61] and possible expression in cardiac progenitor cells;[62] it has been utilized for the development of an inducible Cre system, the PDGFRα-GFPCreERT2 knock-in mouse line.[63] This will likely prove a useful tool in characterizing CFs and their role in fibrosis. PDGFRβ has also been used as a marker of fibroblasts. However, expression of PDGFRβ has been observed in numerous other cell types including smooth muscle cells, pericytes, neurons, kidney mesangium, myoblasts and muscle lineages, thus restricting its specificity and efficiency.[64]

As CFs are likely the principal cells responsible for the secretion of ECM proteins, a new line of thinking involves the identification of CFs by the expression of markers of ECM production. As the predominant protein of the myocardial ECM, Collagen Iα promoter sequences linked to Green Fluorescent Protein (GFP) has been used to create a reporter mouse for identification of CFs.[4, 47, 65] An inducible form of the Collagen1α2 Cre driver has also been developed, allowing for both genetic lineage tracing of Collagen1α2 Cre positive cells and conditional gene deletion for functional studies.[66] However, the expression of collagens is increasingly recognized as not being restricted solely to the fibroblast lineage. Positive Collagen 1a1-GFP expression has been reported within cells of both the epicardium and adventitia of large vessels,[4, 67] suggesting caution in the interpretation of fibroblast-centric conclusions from studies utilizing these lines.

In addition to the molecular markers discussed above, several are expressed following the transition to the activated MF. Identification of MFs in many tissues has almost universally relied on the expression of the contractile protein αSMA.[8, 46, 68] However, recent work using a pressure-overload model of heart disease showed that this marker is restricted to a subset of activated fibroblasts and is not expressed in all fibroblasts associated with fibrosis.[4, 46] Periostin (Postn) is an ECM protein expressed in development, which is also robustly upregulated by activated CFs following injury.[44, 69–72] A Postn-Cre transgenic

mouse line has been developed that possesses an EGFP/Cre fusion expression vector under the control of the endogenous periostin promoter that is expressed in resident CFs and epicardial cells in the developing heart,[17, 73–75] making it well suited to developmental investigations.[76] As Postn-Cre is robustly expressed within activated CFs/MFs and myocardial infarct sites following injury,[75–77] it presents one of the more promising tools for lineage tracing and genetically manipulating CFs and MFs.[75] The development of an inducible knock-in system in Postn expressing cells will likely be an enormously effective tool for the study of activated fibroblasts in disease. Of note, interstitial cells of the valves may also express some of the ECM proteins that mark fibroblasts, such as Col1α and periostin.[78] Overall, advancements in the identification and reliability of CF molecular biomarkers remain essential areas of research, and will eventually allow for a greater understanding of this critical cell type.

Sources of Cardiac Fibroblasts in Disease

The source(s) of activated CFs that accumulate in response to various pathological insults remains somewhat unclear. In other organs, including the lung, kidney and liver in particular, the cellular and molecular mechanisms of fibrosis, and the origins and characteristics of MFs have also been extensively studied, yet some ambiguity remains.[79–83] Lineage tracing has recently been utilized in cardiac fibrosis studies and numerous precursors to the fibroblast population in the injured heart have been proposed, including resident fibroblasts, cells of vascular origin, hematopoietic cells and pericytes (Figure 2).

Resident Fibroblasts

It is traditionally recognized that activated fibroblasts in the fibrotic heart derive from the proliferation and activation of resident fibroblasts, as these cells are remarkably sensitive to circulating pathologic stimuli.[2, 4, 84, 85] This theory coincides with numerous studies in the kidney, which propose that following renal injury a majority of MFs originate from nephrogenic progenitors that give rise to resident fibroblasts as well as pericytes, and also discount the contribution of other cell types.[79] Similarly, it is hypothesized that hepatic fibrosis is based on the activation of hepatic stellate cells and their transformation into MFs that express a broad spectrum of matrix components.[80] The majority of resident CFs appear to arise from the embryonic epicardium, and recent lineage tracing studies suggest that these resident epicardial-derived CFs account for the majority of fibroblasts responsible for fibrotic response.[2, 4] Studies have also revealed that cells of the endocardium, a specialized cardiac endothelial lining, undergo endothelial-mesenchymal transition (EndMT) during development to form the cardiac valves[86] and resident fibroblasts of the interventricular septum.[4] Proliferation of these endocardial-derived CFs has also been shown to contribute to the fibrotic response following pressure-overload.[2] These studies also discount the contribution of cells other than resident fibroblasts to the development of fibrosis in a pressure overload model of heart failure, as well as in a model of parabiosis.[2, 4] While these data contradict some prior studies, they indicate that MF accumulation following injury predominantly results from the proliferation of resident fibroblasts. In addition, resident fibroblasts have been suggested as a feasible source of matrix-producing cells during fibrosis, which could identify them as an important potential target for anti-fibrotic

therapies. Several studies have evaluated the contribution of alternative sources to the activated fibroblast population in pathologic cardiac remodeling as individually outlined below.

Vascular Endothelium

Endothelial cells of the coronary vasculature have been proposed as contributors to the MF population following injury through EndMT. Lineage tracing studies utilizing the constitutive endothelial/hematopoietic restricted Tie1Cre mouse line have suggested that FSP1 positive cells contribute substantially to the fibroblast population in a pressure overload model of heart failure.[46] These endothelial cells are believed to acquire a fibroblast-like phenotype and respond to pro-fibrotic stimuli in a manner similar to resident CFs.[87, 88] This phenotypic conversion involves the migration of these cells into the interstitium, where they exhibit typical MF markers and are believed to contribute to the fibrotic response.[46] However, recent studies have demonstrated that, in addition to fibroblasts, FSP1 also marks a substantial number of immune cells.[4, 43–45] Furthermore, Tie1Cre is not specific to the endothelium, with fibroblasts being labeled in addition to immune cells and other cell types.[89] In an attempt to resolve this controversy, several studies have since been performed using refined fibroblast markers, including tamoxifen-inducible Cre recombinase under the control of the vascular endothelial cadherin (VECad/Cdh5) promoter (VECad-Cre-ERT2), in concert with collagen-1α and PDGFRα as fibroblast markers.[4, 90] This was complemented by a study using the Tie2Cre mouse line to label and identify cells with endothelial origins.[2, 91] These studies concluded that, at least in the pressure overload model of heart failure, the endothelium does not significantly contribute to the MF population.

Epicardium

Resident fibroblasts of the cardiac interstitium are now generally recognized to derive predominantly from cells of the embryonic epicardium.[92] During development, these cells undergo epithelial-mesenchymal transition (EMT) under the influence of several growth factors;[12, 13, 93, 94] subsequently, a portion of these mesenchymal cells invade the myocardium to become the resident CFs.[12, 13] Signals regulating the transition of epicardial cells to fibroblasts are tightly regulated and factors known to influence this transition can include fibroblast growth factors (FGFs) and members of the transforming growth factor β (TGF-β) superfamily.[95] Once these cells have undergone EMT, platelet derived growth factor (PDGF) and TGF-β are thought to promote their transition into the CF phenotype.[57, 93] As seen during development, a subset of epicardial cells can undergo EMT to generate CFs following an acute cardiac injury.[96, 97] In pulmonary fibrosis, cells of the lung epithelium, particularly type II pneumocytes, play a key role in the initiation and propagation of fibrotic signaling in response to either endogenous or exogenous stress, leading to the activation of resident macrophages and fibroblasts.[98] In addition, several studies have suggested that a heterogeneous pool of MFs may originate from EMT of cholangiocytes in hepatic fibrosis, however new work has demonstrated that EMT may not play a significant role.[99, 100] Similarly, recent studies have indicated that cells of epicardial origin may contribute to MF accumulation following myocardial infarction to aid in the fibrotic response;[96, 97] however, EMT does not appear to be functionally significant in the

pressure-overload model of heart failure.[4] These cells may present an interesting source of activated CFs, but more work is needed to characterize the particular functional contribution(s) of this cell type in vivo.

Perivascular Cells

Pericytes, which lie in the perivascular space of cardiac vessels, can differentiate into collagen-producing cells in models of dermal scarring,[101] and may contribute to the fibroblast population following cardiac injury. This cell type has been defined in other tissues, including the retina and kidneys, where they have demonstrated phenotypical and functional overlap with fibroblasts,[102, 103] however they are more thoroughly characterized in the central nervous system.[104] Pericytes surround the neurovasculature and interact with other cell types including astrocytes and endothelial cells. Here they maintain the blood-brain barrier and regulate angiogenesis, immune responses and scar formation, which are affected by factors such as PDGF and TGF-β.[104] The integrity of retinal microvessels is particularly dependent on pericytes, as loss of retinal pericytes contributes to diabetic retinopathy through the formation of microaneurysms.[104] Recent work has revealed the presence of $Gli1^+$ mesenchymal stem cell (MSC) – like cells within cardiac perivascular regions. Lineage tracing studies demonstrated that the proliferation of these resident cells following kidney, lung, liver or cardiac injury generates MFs that contribute to organ fibrosis. Furthermore, ablation of these cells ameliorates cardiac fibrosis and preserves overall cardiac function in a pressure overload model of heart failure, and attenuates renal fibrosis following unilateral ureteral obstruction injury.[105] While these $Gli1^+$ cells may not represent a distinct mesenchymal cell population, as they are also positive for a number of other markers including PDGFRα, these studies do indicate that perivascular cells appear to be an important source of cardiac MFs and may represent a potential therapeutic target for ameliorating cardiac and general organ fibrosis.[105]

Hematopoietic Bone-Marrow Derived Progenitor Cells

Bone marrow-derived progenitor cells are considered a potential source of fibroblasts in the fibrotic heart. This assertion is based on studies of GFP-labeled bone marrow transplants, which identified GFP-expressing cells within fibrotic regions following both pressure overload and ischemic myocardial injuries.[46, 106, 107] It has been suggested that bone marrow-derived cells may account for up to 60% of all fibroblasts within the site of cardiac injury. However this cell population is significantly reduced after the initial reparatory process and therefore is unlikely to contribute to a persistent fibrotic response.[107] CD45-positive monocytes have been identified as a potential source of fibroblasts, as they appear to co-express MF markers[108] and inhibition of monocyte recruitment diminished the CF population and myocardial remodeling following MI.[109]

Several studies have proposed that infiltrating cells are instead likely to consist of a specific phenotype of inflammatory cells. Both a lineage tracing study using the hematopoietic specific Vav-Cre line and a study performing genetically labeled bone marrow transplants incorporating more specific markers of fibroblasts have refuted this idea, demonstrating that the contribution of circulating hematopoietic cells to the CF population is minimal.[2, 4]

Future studies will determine whether these data from a pressure-overload heart failure model will extend to models of ischemic heart failure etiology.

Fibrocytes

The contribution of cells of hematopoietic origin to the fibroblast population is also under intense investigation following the identification of fibrocytes in the circulation.[110] In fact, subsequent studies have reported the contribution of these fibrocytes to the CF population, as well as to the development of cardiac fibrosis in several injury models.[46, 107, 108, 111, 112] Their pathophysiologic relevance to cardiac fibrosis has been suggested in studies linking the inhibition of fibrocyte recruitment to reduced fibrosis and remodeling.[108] Fibrocytes represent a unique fibroblast progenitor population that co-express fibroblast markers, such as procollagen I and vimentin, along with typical hematopoietic markers.[113] These circulating fibrocytes appear to originate from hematopoietic stem cells in the bone marrow,[113] however their pathophysiologic relevance in cardiac disease and fibrosis has yet to be fully characterized.

Alternate Growth Substrates

Mechanical stretching of the CF, which potentially occurs secondary to cardiac dilatation or changes in the hemodynamic burden of the heart, may induce expression of pro-fibrotic cytokines and expression of ECM proteins and receptors on CFs.[114] Mechanical stress could play a contributory or preeminent role for the spontaneous activation observed when CFs are cultured on the stiff surface of standard tissue culture plastic. The elasticity (Young's Modulus) of most cell culture plastic is >1 GPa, and for glass, this measure exceeds 70 GPa. In contrast, the Young's Modulus of the normal heart is estimated to be less than 10 kPa. Thus, the natural growth environment for CFs is many orders of magnitude more elastic than standard cell culture conditions. Attempts to circumvent the issue of spontaneous activation in standard cell culture conditions have focused predominantly on the modulation of growth substrate tensile modulus. Polyethylene glycol-based hydrogels with a physiologically relevant elasticity (~7kPa) have shown promise in preserving the quiescent fibroblast phenotype of valvular interstitial cells when compared to standard tissue culture polystyrene. Cells grown on this substrate exhibited reduced levels of typical MF markers, including α-SMA and connective tissue growth factor (CTGF).[115, 116] The PI3K/AKT pathway is upregulated when these cells are mechanically activated by stiff substrates, and activation of this pathway was significantly attenuated in cells grown on the more compliant substrate.[116] Using tissue culture substrates with a physiologically relevant tensile modulus will aid in the characterization of fibroblast biology in vitro.

Therapeutic Targets in Cardiac Fibrosis

Numerous signaling pathways have been implicated in the early activation of CFs as well as in the pathologic remodeling that persists long after the initial injury. Modulation of these signals is of intense scientific interest as they represent potentially novel therapeutic targets or strategies. Here we explore mediators and signaling pathways known to influence fibroblast function after myocardial injury as well as developing therapeutic strategies to combat pathogenic cardiac fibrosis (Table 2; Figure 3).

Transforming Growth Factor β

The TGF-β family of growth factors is perhaps the most extensively studied mediator of fibroblast activation, of which TGF-β1 is likely to play the greatest role in pathological fibrosis.[117] TGF-β is known to play a major role in regulating fibrotic processes in many organs, and in fact, serum levels are utilized as diagnostic tools and also correlate with the severity of chronic liver and kidney diseases.[81] TGF-β1 is initially secreted in a complex with latent TGF-β binding proteins that restrict its activity; this complex is proteolytically cleaved and can be activated in an integrin-mediated process.[118, 119] The Type I TGF-β receptor, which is also known as activin receptor-like kinase (ALK) 5, represents the subtype thought to be predominantly responsible for the fibrotic activities of TGF-β1. The canonical pathway of TGF-β1 signaling involves the phosphorylation of Smad2/3, which subsequently bind Smad4 and translocate to the nucleus. The complex then acts as a transcription factor, inducing the activation of numerous pro-fibrotic genes.[120, 121] The ability of TGF-β to induce the production of ECM proteins in vitro is thought to depend upon Smad3,[120–122] and furthermore, fibroblasts isolated from Smad3-deficient mice appear resistant to TGF-β1-induced expression of ECM proteins.[123, 124] Perhaps equally detrimental to overall cardiac function as the initial infarct, the canonical TGF-β pathway is also activated in the infarct border and remote zones where it mediates the formation of pathological reactive fibrosis.[120–122] Finally, the persistence of activated fibroblasts is believed to be the result of perpetual TGF-β signaling, which may prevent CF apoptosis following the initial injury through Smad-mediated pathways.[125]

Inhibitors of the TGF-β receptor ALK5 are currently under investigation as potential antifibrotic mediators. ALK5 inhibitors can decrease TGF-β activity, rescuing cardiac dysfunction and ameliorating the remodeling that occurs post-MI.[126] These inhibitors can also attenuate the development of fibrosis and expression of collagen following pressure overload by transverse aortic constriction, and reduce the expression of collagen in response to TGF-β stimulation in vitro.[127] Several groups are also evaluating TGF-β neutralizing antibodies in experimental myocardial fibrosis models. While fibroblast activation and collagen transcript levels were reduced, there were no substantial improvements in overall cardiac function,[128] and there is evidence that this strategy may actually increase mortality.[129] While disparate, these results may have been the result of the disruption of the initial reparatory response leading to a reduction in cardiac function and increased mortality. Similar contradictory results have been observed in studies investigating TGF-β inhibition in animal models of chronic kidney disease (CKD). While there is a significant body of evidence in preclinical trials showing antifibrotic effects of TGF-β inhibition,[130] the clinical translation of these studies has been limited. Furthermore, recent data show that TGF-β plays a role in renal autophagy as a cytoprotective mechanism,[131] which suggests that therapies inhibiting TGF-β1 activity should be approached with caution. While inhibition of the canonical TGF-β signaling pathway remains appealing, this approach may require further investigation and refinement before it will have significant clinical impact.

In addition to the Smad-mediated pathways, TGF-β can also induce non-canonical signaling that involves several mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK) and p38.[132–134] This pathway involves the activation of TGF-β –

activated kinase (TAK) 1, which is believed to contribute to pathological cardiac remodeling, since cardiac overexpression of constitutively active TAK1 does induce cardiac hypertrophy and heart failure.[132–134] While the MF-activating properties of TGF-β have previously been attributed to the canonical signaling pathway, growing evidence suggests the non-canonical pathway may actually be the dominant driving force.[135, 136] Thus, attempts to prevent this non-canonical signaling may prove efficacious for the treatment of fibrosis and heart failure. The TGF-β non-canonical signaling pathway is believed to propagate primarily through the Type II TGF-β receptor (TGFβRII), given that CM-specific deletion of the TGFβRII has resulted in reduced fibrosis and remodeling in the TAC model of heart failure.[137] As the main effector of the TGF-β non-canonical pathway, TAK1 also represents a viable therapeutic target, as inhibition of TAK1 reduced TGF-β – induced fibroblast ECM protein production.[138] Further downstream, inhibition of p38 is being actively investigated for its antifibrotic potential. Inhibitors of p38 signaling can attenuate TGF-β-induced MF activation and reduce the expression of collagen, fibronectin and αSMA in mouse embryonic fibroblasts (MEFs); moreover, overexpression of p38 can induce the MF transition.[135] In vivo, inhibition of p38 reduces fibrosis and the expression of αSMA following MI.[139, 140] Several clinical trials evaluating the efficacy of p38 inhibition have begun in patients following acute MI. In the SOLSTICE Phase II trial, the p38 inhibitor losmapimod was well tolerated in non-ST segment elevation MI (NSTEMI) patients with no major liver toxicities observed. Although infarct size was non-significantly reduced, patients receiving losmapimod experienced fewer cardiac events.[141] These findings provide strong evidence for the therapeutic potential of targeting the TGF-β non-canonical signaling pathway, specifically through modulation of TAK1 and p38, in the treatment of cardiac fibrosis.

Renin Angiotensin System

The renin-angiotensin-aldosterone system (RAAS), of which Angiotensin II (AngII) appears to be the predominant effector, promotes many physiological and pathological functions, including the development of cardiac fibrosis.[142, 143] The AngII type 1 receptor (AT1R) mediates many of the effects of AngII in fibroblasts, including cell proliferation, migration and the induction of ECM protein synthesis.[144, 145] Cardiac AngII levels are quickly elevated following injury, and stimulation of CFs by AngII induces proliferation and the expression of collagen.[146, 147] Renal RAAS activity, which is reflected by urinary angiotensinogen levels, directly correlates with the extent of renal fibrosis and deterioration of renal function in patients with CKD.[148] AngII is also intimately involved with the inflammatory response, as it is expressed and activated by macrophages and MFs.[149] It is believed that AngII is also involved with TGF-β signaling, both in CMs and CFs. Specifically, AngII activation of ATR1 induces the expression TGF-β1, and it is thought that TGF-β is required for AngII to induce both cardiac hypertrophy and fibrosis.[150, 151] Furthermore, AngII-induced expression of collagen in CFs requires TGF-β/Smad and MAPK signaling.[146, 149]

Hemodynamic burden, chronic fibrotic remodeling and tissue stiffening following a myocardial injury can stimulate the release of endogenous AngII from CM stores, however it is now recognized that this mechanical stress can contribute directly to the activation of

the AT1R.[152] While HEK293 and COS7 cells do not natively respond to mechanical stretch, expression of the AT1R confers this ability, as seen by an increase in the activation of ERK, suggesting that the AT1R is itself a mechanical sensor. Mechanical stretching of CMs isolated from ATG null mice, which do not express endogenous AngII, also resulted in ERK activation. Additionally, cardiac hypertrophy in $ATG^{-/-}$ mice was induced by pressure overload, indicating that mechanical stress can induce hypertrophy even in the absence of AngII.[152] Of note, these mechanical sensing abilities appear to be specific to the AT1R, as other GPCRs do not respond to mechanical stretch.[152] A similar phenomenon occurs in CFs, as these cells are highly susceptible to variations in their mechanical microenvironment.[21] This stretch-induced activation of the AT1R can be inhibited by the AngII receptor blocker candesartan,[152] revealing a potential antihypertrophic and antifibrotic role for this class of drugs. As candesartan will reduce blood pressure and thus cardiac load, the mechanism by which it attenuates fibrosis remains unclear and may not necessarily be due to direct inhibition of pathologically activated cardiac fibroblasts.

While not approved for the treatment of cardiac fibrosis, inhibitors of angiotensin signaling, including angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), have already demonstrated significant efficacy in the treatment of heart failure, frequently reducing cardiac fibrosis both in humans and animal models.[153–155] One such angiotensin receptor inhibitor, losartan, is believed to reduce cardiac fibrosis through the inhibition of EndMT, as was demonstrated in mitral valve endothelial cells, by acting to block the AngII-elicited, TGF-β – induced phosphorylation of ERK1/2.[156] Losartan reduced collagen I synthesis and fibroblast activation in response to stimulation by AngII.[157] Stimulation of the AT1R promotes a pro-inflammatory environment, and losartan can decrease the expression of inflammatory markers, including TNF-α, IL-1β and IL-6.[158–160] Clinically, losartan is thought to attenuate the progression of myocardial hypertrophy and fibrosis in patients with hypertrophic cardiomyopathy.[161] These data present a compelling argument that AngII is a potent fibrotic mediator, and that the protective effects of AngII antagonists may partly be due to their ability to reduce cardiac fibrosis. Further studies will be needed to mechanistically establish the effects of AngII inhibition specifically in CFs, which may lead to refinement of ACE/AngII/receptor inhibitors and allow for more direct targeting of the pathway's pathologic activation of this cell population.

Endothelin

The endothelin family of peptides is typically recognized for its vasoconstrictive properties, and is now beginning to be appreciated for its potential role in promoting tissue fibrosis. While originally believed to be secreted predominantly by endothelial cells, Endothelin-1 (ET-1) is now understood as a significant pro-fibrotic peptide released by both inflammatory cells and fibroblasts in the lung.[162] The endothelin system also plays a major role in kidney disease, as ET-1 production is stimulated in disease downstream to both the sympathetic nervous system and RAAS.[163] There exist two known receptors for ET-1 in the heart, the Endothelin-A (ETA) and Endothelin-B (ETB) receptors, which have been shown to play differing and sometimes opposing roles. These receptors are primarily expressed by endothelial cells, however new data define expression on multiple cell types including CMs and CFs, along with some immune cells, such as macrophages.[164, 165] Importantly, ET-1

activation of the ETA receptor is known to increase collagen production in isolated human CFs.[166] Furthermore, MFs isolated from scar tissue following experimental MI possess elevated levels of ET-1, suggesting an important function for endothelin within these cells.[167] While known to have fibroblast-activating properties of its own, ET-1 also acts as a downstream mediator of both AngII and TGFβ to promote MF persistence in pulmonary fibrosis.[136, 162, 168] ET-1 signaling is thought to interact with AngII as well. For example, the development of cardiac fibrosis and hypertrophy in response to AngII stimulation is impaired in mice in which ET-1 is ablated in endothelial cells. It is believed these factors are mechanistically linked through the activation of ERK1/2 and the myocardin-related transcription factor (MRTF) A.[169–172]

Endothelin antagonists are currently approved for the treatment of pulmonary hypertension, and many believe they will be additionally efficacious in the treatment of pathologic fibrosis in the heart.[136] Endothelin receptor blockade has been incorporated extensively both experimentally and in the clinic for its salutary role in the treatment of hypertension. Bosentan, a nonselective endothelin receptor antagonist, while used clinically for the treatment of pulmonary hypertension, can improve cardiac function and reduce infarct size following myocardial ischemia/reperfusion injury in rats.[173] Preliminary trials in humans had demonstrated beneficial hemodynamic and cardiac effects in patients with end stage heart failure.[174] However subsequent clinical trials evaluating ET receptor antagonists on coronary artery disease and heart failure have not met primary endpoints, perhaps due to inefficient selection of potential responders prior to the initiation of major clinical trials.[175–178] While the reasons for these discrepancies remain unclear, manipulation of ET-1 signaling appears promising. Next generation ETR subtype-specific antagonists, such as ambrisentan and darustentan that are proving to be highly beneficial in the treatment of pulmonary hypertension, may prove more efficacious and help define receptor specificity that may be required for salutary effects. Further studies will be necessary to determine if ET-1 and its receptors will become clinically viable antifibrotic therapeutic targets.

RhoA-MRTF-SRF Signaling Pathway

Serum response factor (SRF), a member of the MADS box-containing family of transcription factors, is critically involved in the regulation of MF activation, given that numerous SRF binding sites are located within the promoter regions of genes responsible for fibroblast activation.[179, 180] Experiments in bleomycin-injured lung tissue have demonstrated upregulation of SRF gene expression in isolated MFs,[181] and overexpression of SRF can induce the transformation of both lung and cardiac fibroblasts.[135, 181] SRF interacts with cofactors such as members of the MRTF family of transcription factors, which translocate to the nucleus and bind SRF to regulate its transcriptional efficacy.[182] In CFs, MRTF-A overexpression alone can induce the MF transition and the expression of αSMA; conversely, MRTF-A ablation in vivo reduces the fibrotic response following MI.[182] SRF activation occurs downstream of stimulation by TGF-β, AngII and ET-1, and knockdown of SRF by siRNA can inhibit MF activation in response to these stimuli.[135] The Rho family of GTPases is believed to mediate the activation of the SRF-MRTF signaling pathway; specifically, RhoA signaling is activated downstream of the TGF-β receptor, several G-protein coupled receptors (GPCR) and mechanical tension.[183–185] Thus, TGF-β stimulation

in fibroblasts can simultaneously activate Rho-MRTF-SRF signaling along with its canonical and non-canonical signaling pathways. Perhaps the inhibition of RhoA, using compounds such as fasudil, presents a potential antifibrotic strategy that complements those described above.

Transient Receptor Potential Channels

Receptor-activated Ca^{2+} entry is induced following the activation of G protein-coupled receptors and receptor tyrosine kinases, including the AT1R or TGF-β receptor, leading to the activation of calcineurin and nuclear translocation of the nuclear factor of activated T-cells (NFAT) family of transcription factors.[186] The transient receptor potential (TRP) proteins are the channels responsible for this receptor-mediated Ca^{2+} entry; the canonical TRP (TRPC) channels specifically couple receptor-phospholipase C (PLC) signaling pathways to Ca^{2+} entry,[187] and are upregulated in mice subjected to myocardial pressure overload.[188] CM-specific overexpression of TRPC6 resulted in cardiomyopathy and promoted NFAT-dependent promoter activity. Furthermore, siRNA knockdown of TRPC6 reduced hypertrophic signaling in response to ET-1, suggesting a role of TRPC6 in the GPCR-dependent activation of the calcineurin-NFAT pathway.[188]

Recently, the TRPC6 channel was identified in an unbiased genome-wide screen as an important regulator of MF activation.[135] In primary fibroblasts, TRPC6 overexpression induced the expression of MF marker genes, and genetic ablation conferred resistance to TGF-β- and AngII-dependent MF transformation.[135] Furthermore, overexpression of a constitutively active calcineurin in CFs induced the MF transition, while inhibition of calcineurin blocked TRPC6-mediated transformation, indicating a functionally-relevant interaction of these proteins in CFs.[135] These findings have revealed TRPC6-mediated Ca^{2+} entry and activation of the calcineurin/NFAT signaling pathway as novel regulatory mediators in the activation of fibroblasts, and suggest that their inhibition may represent an important antifibrotic therapeutic strategy.

Connective Tissue Growth Factor

CTGF belongs to the CCN family (the acronym comes from the first three members of the family that were found: CYR61 (cysteine-rich angiogenic inducer 61 or CCN1), CTGF (connective tissue growth factor or CCN2), and NOV (nephroblastoma overexpressed or CCN3) of matricellular proteins, which are dynamically expressed non-structural proteins of the ECM.[189] This family of proteins is believed to play a significant regulatory role in the ECM to modulate cell surface receptors and their responses to cytokines, growth factors and proteins of the ECM.[189] CTGF is a well-characterized mediator of TGF-β activity during the fibrotic response, and its expression is induced in fibroblasts stimulated by TGF-β.[190, 191] Furthermore, CTGF is strongly upregulated both in human heart failure and animal models associated with myocardial fibrosis.[190, 192] Interestingly, induction of CTGF following injury has been observed prior to the upregulation of TGF-β or deposition of ECM proteins, suggesting an important role in these processes.[193] CTGF also appears to play a significant role directly in isolated CFs, as evidenced by a reduction of various chemokines, matrix metalloproteinases, ECM proteins and cell adhesion proteins in CFs in which CTGF is ablated using siRNA.[194] While studies targeting CTGF in animal models of heart failure are

relatively limited, preliminary studies in a pressure overload model have suggested efficacy of CTGF neutralizing antibodies in reducing cardiac remodeling and dysfunction, concomitant with attenuated collagen deposition.[195] These data indicate that the pursuit of therapies targeting CTGF may be of clinical interest for the treatment of cardiac fibrosis.

These data are in stark contrast to a recent study investigating the mechanism of CTGF and definitively characterizing its role in cardiac injury. Several transgenic mouse lines were developed that included heart-specific CTGF knockouts and overexpressors in combination with constitutively active TGF-β and fibroblast-specific CTGF knockout mice. Surprisingly, neither gain nor loss of CTGF affected overall cardiac function or the fibrotic response in multiple models of cardiac injury. However, modulation of CTGF did appear to slightly impact the response to TGF-β in a pressure overload model, suggesting a potential interaction between these factors.[196] As described above, CTGF is believed to play an important functional role with TGF-β to regulate the fibrotic response, but recent data suggest that CTGF is of limited importance as a TGF-β effector and thus may no longer represent an important therapeutic target for the treatment of cardiac fibrosis and heart failure.

Platelet Derived Growth Factor

The PDGF family of growth factors is a group of proteins originally recognized for their ability to regulate cell proliferation in smooth muscle cells. Expression of the PDGF family is significantly increased in endothelial cells, macrophages and MFs following MI.[197] Enhanced expression of both the ligands and receptors occur concomitantly with inflammatory and fibrogenic responses following MI, identifying a potential role in the regulation of cardiac repair.[197] Stimulation by PDGF is known to enhance CF proliferation, and also enhances TGF-β expression in vitro.[198, 199] Similarly, cardiac-specific overexpression of PDGF significantly elevates TGF-β1 transcription and promotes the development of cardiac fibrosis.[200]

The disruption of PDGF signaling is currently under active investigation for the treatment of myocardial fibrosis. The tyrosine kinase inhibitor Imatinib, typically recognized for its anti-tumorigenic properties, can also suppress PDGF signaling by acting as an antagonist against the PDGF receptor. In a MI model of heart failure, Imatinib reduced the expression of fibrogenic mediators, including TGF-β and collagen I, significantly reduced fibrotic scar formation and mildly rescued cardiac dysfunction.[201] A neutralizing PDGF receptor antibody has also been shown to attenuate atrial fibrosis in a pressure overload model of heart failure.[202] Collectively, these data implicate PDGF in the development of cardiac fibrosis, likely through the induction of TGF-β, and additionally present PDGF inhibition as a potential approach for antifibrotic therapy.

Integrins

Integrins are transmembrane proteins comprised of α and β subunits that mediate interactions between the extracellular environment and the actin cytoskeleton. CFs express a wide variety of integrin subtypes that mediate multiple cellular functions including proliferation, migration, adhesion, differentiation and apoptosis.[203–205] Furthermore, it has

been reported in cultured MFs that integrins are required for the activation of latent TGF-β.[119] The integrin-mediated transformation of MFs is believed to occur through activation of MAPK signaling cascades, including ERK1/2 and p38.[206] This regulation of TGF-β activation may be one potential mechanism causing the persistence of MFs within fibrotic scars, and may offer a target for ameliorating adverse myocardial remodeling following injury.[118, 125] Strategies aimed at blocking the function of integrins have shown preliminary success in limiting maladaptive remodeling. For example, small molecule targeting of the $\alpha_V\beta_1$ integrin, which is highly expressed in activated fibroblasts, attenuated pulmonary and liver fibrosis through a mechanism involving the inhibition of TGF-β activation.[207] It is possible that therapeutic approaches targeting various integrin subtypes could be therapeutically useful for the treatment of numerous diseases characterized by excessive tissue fibrosis.

Inflammation/Interferon Receptors

Inflammation is an essential mediator of the reparative response to an acute cardiac insult. Numerous inflammatory cells, including neutrophils and macrophages, infiltrate the site of injury where various pro-inflammatory cytokines are released, such as TNFα, Interleukin-1β (IL-1β) and IL-6, and play an important role in the initial induction of resident fibroblast proliferation and MF activation.[208] In addition to their direct release of ECM components, activated CFs also express numerous cytokines and growth factors that affect wound healing via autocrine and paracrine mechanisms.[209] Cytokine expression by CFs is markedly upregulated following acute myocardial injury, including the expression of the pro-inflammatory cytokines IL-1β and IL-6. IL-1β promotes fibroblast migration through increasing the expression of proteins involved in ECM turnover, including matrix metalloproteinases,[6, 210] and IL-6 is known to increase fibroblast proliferation and myocardial fibrosis.[211]

Interferons (IFNs) are a family of cytokines that cause a myriad of biological responses and, in addition to immune cells, they can be secreted by other cell types such as CFs.[212, 213] However, the role of IFNs in fibrosis, specifically IFN-γ, remains somewhat controversial.[214] In IFN-γ knockout mice challenged with AngII, there was a reduction in the MF marker α-SMA.[215] Similarly, mice null for the IFN-γ receptor (IFNGR) exhibit a decrease in cardiac hypertrophy and fibrosis, as well as a reduction in the infiltration of macrophages and T cells.[216] Since extensive data have demonstrated a critical role of inflammation in the fibrotic response, inhibiting these inflammatory mediators may prove efficacious in the treatment of cardiac fibrosis.

GPCR/Adrenergic Signaling

The adrenergic system plays a fundamental role in the physiologic regulation of the myocardium, but chronic overstimulation can induce both cardiac hypertrophy and fibrosis.[217] While several subtypes of the β-adrenergic receptor (β-AR) are expressed in the heart, the β2-AR appears to be the form that is predominantly expressed by CFs.[218] Chronic stimulation of this receptor can induce cell proliferation, collagen secretion, migration and transformation to the MF phenotype.[218]

It is well established that acute stimulation of the β2-AR increases the levels of cAMP, which can modulate proliferation[219] as well as inhibit the synthesis and secretion of various forms of collagen.[220, 221] In addition, elevated cAMP levels can inhibit the transformation of CFs to MFs induced by stimulation with TGF-β.[221, 222] Recent studies indicated that failing human CFs isolated from patients with heart failure had higher baseline collagen synthesis that was not inhibited by β-agonist stimulation. Furthermore, β-AR signaling was markedly uncoupled and associated with increased expression and activity of G protein-coupled receptor kinase 2 (GRK2).[223] Similarly, knockdown or inhibition of GRK2 restored β-agonist-stimulated inhibition of collagen synthesis and decreased collagen synthesis in response to TGF-β stimulation, indicating a significant role for GRK2 in the regulation of collagen synthesis and maladaptive ventricular remodeling.[223] GRK2 is also recognized for its role as a RhoA-activated scaffold protein for the ERK MAPK cascade, which may have implications in the development of hypertension,[224] and also suggests potential interactions with profibrotic mediators, including TGF-β, AngII and ET-1. GRK2 is typically recognized for its role in the downregulation of β-ARs, predominantly through the recruitment of β-arrestin, which is also significantly upregulated in adult human CFs isolated from failing left ventricles.[225] Enhanced β-arrestin signaling in CFs appears to be deleterious in that it promotes a pro-fibrotic phenotype via the uncoupling of β-ARs and potentiates ERK and Smad signaling downstream of TGF-β.[225] Targeting GRK2, RhoA and β-arrestin may represent plausible therapeutic strategies for the prevention of myocardial fibrosis.

Several approaches for specifically targeting GRK2 have been developed, all of which have shown some promise in reducing the fibrotic response concomitant with protection against overall cardiac dysfunction. The selective serotonin reuptake inhibitor paroxetine was recently identified as an inhibitor of GRK2.[226] Paroxetine is known to reverse left ventricular dysfunction following MI, and reduce both immediate infarct and remote region fibrosis, albeit at a dose substantially in excess of that used in humans.[227] These data suggest that targeting GRK2 signaling may be a potent anti-fibrotic approach. The idea of inhibiting the interaction between GRK2 and G-protein βγ subunits has been investigated in several models of heart failure, using either small molecules or a truncated form of GRK2 known as βARKct. The βARKct peptide has shown promise in preventing myocardial dysfunction in both small and large animal models of heart failure.[228, 229] The small molecule Gβγ-GRK2 inhibitor gallein has demonstrated an uncanny effect to improve cardiac function and reduce the fibrotic response in several murine models of heart failure.[230–233] Of further interest, the introduction of gallein attenuated pathologic renal abnormalities, including renal dysfunction, tissue damage and fibrosis, that occur secondary to myocardial pressure overload injury (in press). While the mechanism of GRK2 and Gβγ-GRK2 inhibition within CFs has yet to be fully delineated, GRK2 represents an exceptionally important target for therapeutic interventions directed against myocardial fibrosis.

Fibroblasts for CM Regeneration

With the advent of stem cell-based therapeutics for the treatment of cardiovascular disease, there is now a growing interest in the development of reprogramming techniques to induce the transformation of fibroblasts into functional CMs. While still in its infancy, this

technique relies on the creation of induced pluripotent stem (iPS) cells, which can be differentiated in vitro to potentially generate patient-specific CMs for subsequent introduction into the failing or infarcted heart.[234] Newer methods are beginning to allow for the direct reprogramming of fibroblasts into CMs in vivo through the administration of a specific combination of transcription factors.[234–237] Unfortunately, with these current approaches, the number of fibroblasts that successfully transition into healthy cardiac muscle cells in vivo still remains quite small, and some reports suggest this may not be a viable approach.[238] Additionally, there is arrhythmogenic potential resulting from newly generated CMs that do not correctly integrate with the surrounding cardiac muscle.[239] Perhaps with the development of more efficient delivery systems and identification of novel factors,[240] this technology may eventually allow for manipulation of chronic scar tissue as well as the development of new CMs and potentially attenuate or even reverse post infarct remodeling.[239]

Electrical Communication

A new area of study that has previously been thought to be of little physiological relevance is the electrical communication between CFs and CMs. The relatively large membrane resistance of interstitial CFs renders these cells suitable for the conduction of electrical signals. Seminal ex vivo studies have demonstrated a robust physical communication with adjoining CMs, although the physiological relevance of such interactions in vivo is still under investigation.[241, 242] It is now understood that CFs are capable of electrically coupling with CMs which influences their electrical properties,[243] and this likely occurs through the action of connexin 43 (Cx43), the subtype predominantly expressed by CMs.[244–246] The belief is this will allow CFs to form bridges linking regions of myocytes to aid in the synchronization of myocyte contractions.[247–251]

The development of fibrosis is known to disrupt the normal arrangement of myocytes and fibroblasts, and as such, has serious implications in the development of ventricular arrhythmias in patients following an acute myocardial injury. Modulation of the interactions between CFs and CMs may prove to be an interesting therapeutic approach.[243, 248, 252, 253] Of note, the risk of post-infarction ventricular tachycardia is significantly reduced following the engraftment of embryonic CMs engineered to express Cx43, likely due to the improved electrical coupling between the surrounding myocardium and the infarct region.[254] This therapeutic avenue will need to be approached cautiously though, as there are conflicting data regarding the effect of CF density on the impact of CF-CM coupling.[32] While the targeting of CF-CM electrical coupling holds potential for the effective treatment of cardiac arrhythmogenesis, the variable responses in different clinical settings and intricacies of this signaling will require full examination before these types of interventions can become clinically viable.

Conclusions

It is abundantly clear that a deeper understanding of CFs will be critical to achieving exciting advancements in the treatment of cardiac fibrosis and disease. A limited knowledge of the role of this essential and dynamic cell type continues to hinder progress in the design

and application of meaningful new therapies. The development of more specific in vivo approaches along with the identification of novel biomarkers will allow for greater investigation of this important cell population. Furthermore, unraveling the intricate mechanisms underlying this fibrotic signaling is of utmost importance for the development of effective therapies for the treatment of cardiac fibrosis. Therapeutic interventions targeting CFs and the pathologic fibrosis they promote will inevitably lead to significant advancements in the treatment of heart failure.

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Abbreviations

ALK	Activin receptor-like kinase
CF	Cardiac fibroblast
CKD	Chronic kidney disease
CM	Cardiomyocyte
CTGF/CCN2	Connective tissue growth factor
CYR61/CCN1	Cysteine-rich angiogenic inducer 61
DDR2	Discoidin domain receptor 2
EMT	Epithelial-mesenchymal transition
EndMT	Endothelial-mesenchymal transition
ET-1	Endothelin-1
ETA/ETB	Endothelin receptor A/B
FSP1	Fibroblast specific factor 1
IL-1β	Interleukin-1β
IFN	Interferon
iPS	Induced pluripotent stem cell
JNK	c-Jun N-terminal kinase
MF	Myofibroblast
MRTF	Myocardin-related transcription factor
MSC	Mesenchymal stem cell
NFAT	Nuclear factor of activated T-cells

NOV/CCN3	Nephroblastoma overexpressed
NSTEMI	Non-ST segment elevation MI
PLC	Phospholipase C
Postn	Periostin
αSMA	α-smooth muscle actin
SRF	Serum response factor
TAC	Transverse aortic constriction
TAK	TGF-β – activated kinase
Thy1/CD90	Thymus cell antigen 1
TRPC	Canonical transient receptor potential channel
VECad/Cdh5	Vascular endothelial cadherin

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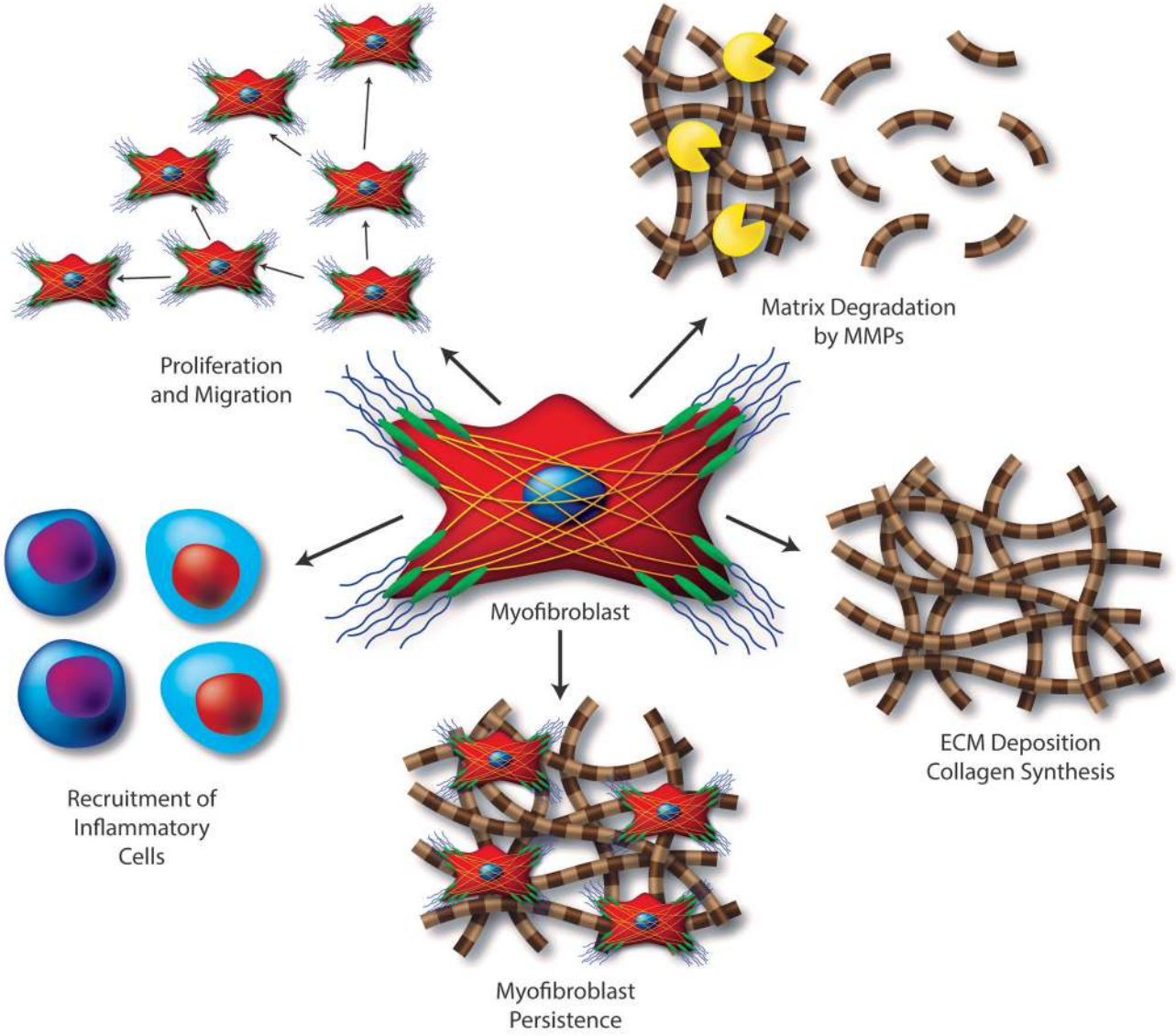

Figure 1. Characteristics and Functions of Activated Cardiac MFs

Cardiac fibroblasts respond to pathologic stress and environmental stimuli by transforming into MFs that (1) express elevated levels of various pro-inflammatory and pro-fibrotic factors that directly contribute to inflammatory cell infiltration and fibroblast proliferation, (2) secrete high levels of matrix metalloproteinases and other ECM degrading enzymes that facilitate fibroblast migration and (3) contribute to the deposition of collagen and other ECM proteins leading to scar formation. While this adaptive fibrotic scar tissue maintains the structural integrity and pressure generating capacity of the heart, MF persistence eventually leads to the development of adverse changes in ventricular structure and compliance, leading to the progression to heart failure.

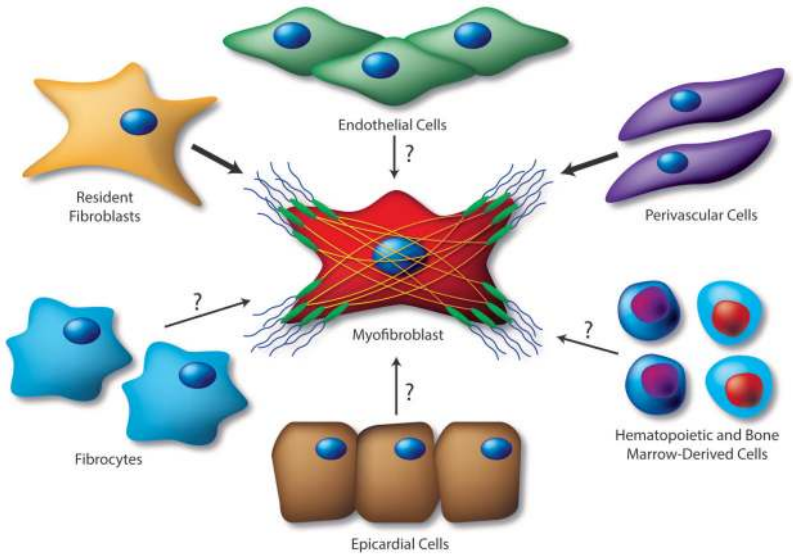

Figure 2. Proposed Sources of Cardiac MFs

The source(s) of activated CFs that accumulate in response to various pathological insults remains under active investigation. Mounting evidence suggests that activated MFs in the fibrotic heart derive from the proliferation and activation of resident fibroblasts, as these cells are remarkably sensitive to pathologic insult and represent a feasible source of matrix-producing cells during cardiac fibrosis. Numerous additional precursors to the fibroblast population in the injured heart have been proposed; these include endothelial and epicardial cells, through EMT and EndMT, respectively, hematopoietic bone marrow-derived cells, perivascular cells and fibrocytes. While supporting evidence exists for cellular sources of activated fibroblasts denoted by question marks, controversy remains regarding the functional contribution of these cell types to the cardiac MF population. Biomarkers with greater specificity will be required to fully characterize the pathophysiologic relevance of these cell types in the cardiac fibrotic response, which will enable potential targeting for anti-fibrotic therapies.

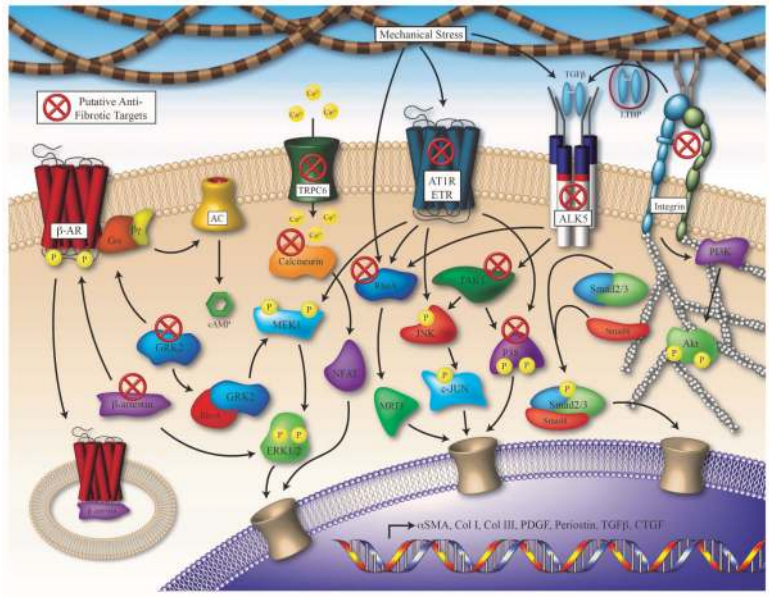

Figure 3. Selection of Signaling Pathways Regulating MF Activation and Potential Therapeutic Targets for Cardiac Fibrosis

Numerous signaling pathways have been implicated in the activation of cardiac fibroblasts and the induction of pathological remodeling; targeting these signals is of intense scientific interest toward development of novel therapeutic strategies. Cardiac injury promotes the activation of receptors such as the β-AR, ALK5, AT1R, ETR, TRPC6 and Integrins, which induces pathologic signaling through numerous mediators, leading to the transcription of factors that regulate MF activation and fibrotic remodeling. Proposed antifibrotic therapeutic targets are marked with a red X. (β-AR: β-adrenergic receptor, AC: adenylate cyclase, TRPC6: transient receptor potential channel C6, AT1R: type 1 angiotensin II receptor, ETR: endothelin receptor: TGF-β: transforming growth factor β, LTBP: latent TGF-β binding protein, ALK5: activin receptor-like kinase 5 / TGF-β receptor, GRK2: g protein-coupled receptor kinase 2, ERK1/2: extracellular regulated kinase 1/2, MEK1: mitogen activated protein kinase kinase 1, NFAT: nuclear factor of activated T-cells, MRTF: myocardin-related transcription factor, TAK1: TGF-β – activated kinase 1, JNK: c-JUN N-terminal kinase, PI3K: phosphoinositide 3-kinase).

Table 1
Fibroblast Markers and Mouse Models for Gene Targeting

This table lists a selection of molecular markers that have been utilized for the identification of quiescent cardiac fibroblasts and activated myofibroblasts; these markers have been translated into numerous mouse lines for lineage tracing and gene targeting studies. Limited expression in all cardiac fibroblasts or positive expression in alternate cardiac cells types are among the problems associated with the use of these markers. Multiple mouse lines exist for many of the markers listed with varying levels of specificity, and complications associated with some lines include Cre expression in the absence of induction, germ line expression, and low recombination efficiencies.

Marker / Mouse Line	Function	Expression in CFs	Expression in Other Cell Types Found in the Heart	References
α-Smooth Muscle Actin	Contractile intermediate filament-associated protein	Expression limited to subset of activated CFs in fibrotic regions	Epicardium, smooth muscle cells, pericytes and cardiac muscle cells	4, 8, 46, 68
Collagen 1a1	Extracellular matrix protein	Majority of CFs	Epicardium, adventitia of large vessels and valve interstitial cells	4, 47, 65–67
Discoidin Domain Receptor 2	Tyrosine kinase receptor for several ECM proteins	Limited expression in resident CFs	Limited expression in epicardium	9, 42
Fibroblast-Specific Protein 1	Calcium-binding protein Intermediate filament-associated protein	Activated fibroblasts in ischemic and non-ischemic heart failure models (typically perivascular)	Endothelial, smooth muscle and immune cells	4, 43–46
Periostin	Matricellular protein secreted by activated CFs	CFs in development Re-expressed in activated CFs following injury	Epicardium, vascular smooth muscle cells and valve interstitial cells	17, 44, 69–77, 255
Platelet-Derived Growth Factor Receptor-α	Mitogenic tyrosine kinase receptor	CFs during development and following injury	Epicardium	47, 57, 61, 63
The Transcription Factor 21	Regulates mesenchymal cell transitions	Resident CFs in development and following myocardial injury	Epicardium	47, 55, 56, 237
Thymus Cell Antigen 1	Membrane glycoprotein for cell adhesion	Limited expression in quiescent resident CFs	Endothelial cells, pericytes and immune cells	11, 47–50
Vimentin	Intermediate filament protein	CFs in healthy and injured heart	Endothelium	2, 51–53

Table 2

Therapeutic Targets and Approaches in Cardiac Fibrosis

Target	Approach	References
Transforming Growth Factor-β	TGF-β Receptor 1 (ALK5) Inhibition TGF-β Neutralizing Antibodies TGF-β-Activated Kinase (TAK) 1 Inhibition p38 Inhibition	126–129, 135, 138–140
Angiotensin System	Angiotensin II Receptor Blockers (ARBs) Angiotensin Converting Enzyme (ACE) Inhibition	152–161
Endothelin System	Endothelin Receptor Antagonists	136, 173–178
RhoA-SRF-MRTF Pathway	Serum Response Factor Inhibition RhoA Inhibition	135, 256
Transient Receptor Potential Channels	TRPC6 Inhibition Calcineurin Inhibition	135, 188
Connective Tissue Growth Factor	CTGF Neutralizing Antibodies	195
Platelet Derived Growth Factor	PDGF Receptor Antagonists PDGF Receptor Neutralizing Antibodies	201, 202
Integrins	$\alpha_V\beta_1$ Integrin Inhibition (Lung and Liver)	207
Inflammatory Signaling	Interferon-γ Inhibition	215, 216
Cardiomyocyte Regeneration	Induced Pluripotent Stem Cell Transplantation Fibroblast Reprogramming	234, 236, 237, 239
Electrical Communication	Cx43 – Expressing Cardiomyocyte Engraftment	254
Adrenergic System	Gβγ-GRK2 Inhibition (Paroxetine, βARKct and Gallein) RhoA Inhibition β-arrestin Inhibition	224–233