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Entanglement of GSK-3 β , β -catenin and TGF- β 1 signaling network to regulate myocardial fibrosis

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Abstract

Nearly every form of the heart disease is associated with myocardial fibrosis, which is characterized by the accumulation of activated cardiac fibroblasts (CFs) and excess deposition of extracellular matrix (ECM). Although, CFs are the primary mediators of myocardial fibrosis in a diseased heart, in the traditional view, activated CFs (myofibroblasts) and resulting fibrosis were simply considered the secondary consequence of the disease, not the cause. Recent studies from our lab and others have challenged this concept by demonstrating that fibroblast activation and fibrosis are not simply the secondary consequence of a diseased heart, but are crucial for mediating various myocardial disease processes. In regards to the mechanism, the vast majority of literature is focused on the direct role of canonical SMAD-2/3-mediated TGF- β signaling to govern the fibrogenic process. Herein, we will discuss the emerging role of the GSK-3 β , β -catenin and TGF- β 1-SMAD-3 signaling network as a critical regulator of myocardial fibrosis in the diseased heart. The underlying molecular interactions and cross-talk among signaling pathways will be discussed. We will primarily focus on recent *in vivo* reports demonstrating that CF-specific genetic manipulation can lead to aberrant myocardial fibrosis and sturdy cardiac phenotype. This will allow for a better understanding of the driving role of CFs in the myocardial disease process. We will also review the specificity and limitations of the currently available genetic tools used to study myocardial fibrosis and its associated mechanisms. A better understanding of the GSK-3 β , β -catenin and SMAD-3 signaling network may provide a novel therapeutic target for the management of myocardial fibrosis in the diseased heart.

Keywords

cardiac fibroblast; fibrosis; GSK-3 β ; SMAD-3; β -catenin; TGF- β 1

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1. Introduction

About 5.7 million adults in the United States have heart failure (HF) and half of the people who develop HF die within 5 years of diagnosis [1]. HF costs the nation an estimated \$30.7 billion each year [2]. Clinical studies have identified a strong association of ongoing myocardial fibrosis and development of HF [3–5]. Almost every form of heart disease is associated with expansion and activation of the cardiac fibroblast (CF) compartment, suggesting fibrotic remodeling as a major cause of HF [6]. Circulating levels of the collagen I and III N-terminus propeptides in the blood plasma are considered strong predictive markers for HF [3–5]. Furthermore, approaches to prevent the excessive ECM turnover leads to a survival benefit in human HF patients. However, all these clinical studies are associative in nature and fail to answer the critical question of cause versus consequence. Thus, until very recently, there was a lack of direct evidence for the role of activated CFs and fibrosis in driving myocardial pathology. In the absence of direct evidence, CFs and fibrosis were often considered secondary effects of the disease process. The primary reason behind the lack of definitive evidence was the unavailability of well-characterized animal models to genetically manipulate the target gene specifically in the CFs. Thus, most of the existing knowledge about cardiac fibroblast biology was generated either from *in vitro* culture models or mouse models in which genetic manipulation was only made in cardiomyocytes (CM).

Recent studies from our lab and others have challenged this concept by demonstrating that fibroblast activation and fibrosis are not only the secondary consequences of a diseased heart but plays an essential role in mediating various myocardial disease processes [7–15]. Takeda et al [8] were the first to demonstrate the driving role of CFs in the myocardial disease process. In this pioneering work, Takeda et al [8], for the first time, used mouse lines in which Cre recombinase was driven by the periostin (*Postn*) promoter (CF-specific gene targeting) to demonstrate that CFs are essential for the adaptive response of the heart to pressure overload. Haploinsufficiency of the Krüppel-like factor 5 (*Klf5*) suppressed TAC (transaortic constriction)-induced cardiac fibrosis and hypertrophy, whereas CM-specific *Klf5* deletion did not lead to any cardiac phenotype. Furthermore, CF-specific *Klf5* knockout mice developed severe heart failure when subjected to high-intensity pressure overload, suggesting a cardioprotective role of CFs. Thus, this report was the first to provide compelling evidence that CFs play a pivotal role in the myocardial pathophysiology. At this end, we employed periostin promoter driven cre model to demonstrate that CF-specific GSK-3 β -knockout (KO) leads to hyperactivation of canonical TGF- β 1-SMAD-3 signaling, resulting in adverse fibrotic remodeling and cardiac dysfunction in the ischemic heart. Acharya et al [16] have developed a mouse model expressing Cre recombinase protein fused to two mutant estrogen receptor ligand-binding domains (MerCreMer) under the control of the endogenous *Tcf21* (transcription factor 21) gene locus. This mouse model has been successfully utilized for the fibroblast-specific gene manipulation and lineage tracing in multiple studies [12, 15, 17]. Studies with CF-specific genetically manipulated mouse models are listed in Table 1. Importantly, in several of these studies, the cardiac phenotype was only observed in CF-specific genetic manipulation and CM-specific genetic manipulation of the same gene did not lead to any cardiac phenotype, suggesting the driving role of CFs in mediating various myocardial disease processes [8, 14]. Taken together, these

studies have demonstrated successful utilization of CF-specific mouse models and also recognized the concept that CFs and fibrosis plays a driving role in the myocardial disease process. The emergence of these newly optimized CF-specific mouse models has led to an explosion of interest in fibroblast biology and fibrosis research in the past few years (Fig. 1).

In terms of a molecular mechanism, most studies have implicated the direct role of canonical SMAD-2/3-mediated TGF- β signaling as the primary mediator of fibrogenesis process. However, recent studies with newly optimized CF-specific mouse models have implicated the significant role of other signaling cascades in myocardial fibrosis or fibrosis in general [7, 10, 13, 15, 17]. As the new studies with CF-specific mouse models are emerging, it is becoming clear that the profibrotic pathways operate in a network, instead of being isolated entities. Recent studies have uncovered the multiple molecular interactions of the GSK-3 β , β -catenin pathway with the canonical TGF- β 1-SMAD-2/3 cascade to regulate the strength and duration of the profibrotic signal [7, 10, 18, 19]. At the molecular level, several mechanisms have been proposed to define the biological effect of this signaling network, which includes: **i)** GSK-3 β mediated regulation of SMAD-3 phosphorylation, **ii)** β -catenin dependent regulation of SMAD-3 activity and **iii)** SIRT3 mediated regulation of GSK-3 β acetylation and TGF- β 1 signaling. Herein, we will discuss the emerging role of the GSK-3 β , β -catenin and TGF- β 1, signaling network as a critical regulator of myocardial fibrosis in the diseased heart. The underlying molecular interactions among signaling pathways will also be discussed.

2. Current status of fibroblast-specific genetic animal models

Even though the critical role of CFs in myocardial pathophysiology is now generally accepted, direct *in vivo* studies to understand the role of CFs in myocardial pathophysiology are just starting to emerge, largely due to the recent availability of CF-specific genetic mouse models. Table 2 lists published markers of fibroblasts with their specific advantages and limitations. These markers have been targeted to create numerous mouse lines for fibroblast-specific gene deletion and lineage tracing. Several studies have employed fibroblast-specific protein 1 (FSP1) as a fibroblast-specific marker [20, 21]. Transgenic mice with green fluorescent protein (GFP) expression driven by the FSP1 (FSP1.GFP mice) have been used to map cell fate of fibroblast population and FSP1-Cre animals have been used as a tool to dissect the effect of fibroblast-specific gene targeting in the fibrogenesis process [20, 21]. However, in recent years, a growing body of evidence has challenged the specificity of FSP1 as a fibroblast marker [22, 23]. Recently, Kong et al [22] reported that FSP1 is not a specific marker for CFs in the myocardium. In fact, the majority of FSP1⁺ cells in the infarcted myocardium were identified as hematopoietic or endothelial cells, not myofibroblasts. They used both pressure overload and myocardial infarction models to demonstrate that FSP1 was primarily expressed by hematopoietic and vascular cells, and is not specific to the myofibroblast population. Thus, these reports suggest that use of FSP1 as a fibroblast marker or FSP1-Cre driven gene deletion is of limited value to dissect the role of CFs in the pathophysiology of cardiac diseases [22].

Mice with an inducible Cre under the control of a regulatory sequence from the pro α 2(1)collagen gene (Col1a2-cre) have been extensively used for lineage tracing and

fibroblast-specific gene targeting [32, 54, 55]. Duan et al [10] were the first to utilize this model to explore the role of cardiac fibroblast β -catenin in the myocardial ischemia-reperfusion (I/R) injury model. To generate CF-specific β -catenin KO mice, Col1a2Cre^{ER} mice were crossed with β -catenin^{flox/flox} mice and thereafter treated with tamoxifen. Deletion of β -catenin signaling in CFs led to defective wound healing and accelerated cardiac dysfunction, post-I/R injury [10]. Recently, the same group further employed Col1a2Cre^{ER} for CF-specific targeting of p53 to demonstrate that endothelial-mesenchymal transition (EMT) contributes to cardiac neovascularization [11]. Specific deletion of p53 in CFs resulted in decreased formation of fibroblast-derived endothelial cells, reduced post-infarct vascular density, and worsened cardiac function [11]. We employed Col1a2Cre^{ER} to determine the role of CFs-GSK-3 β in the ischemic heart. Loss of GSK-3 β in CFs leads to excessive fibrosis and adverse ventricular remodeling, post-MI [7]. Recently, Woodall et al [13] employed Col1a2Cre^{ER} to target fibroblasts G protein-coupled receptor kinase 2 (GRK2) in the setting of I/R injury. CF-GRK2 knockout mice displayed decreased infarct size and preserved cardiac function, post I/R injury. These functional benefits were associated with decreased fibrosis and decreased neutrophils infiltration. Although Col1a2Cre^{ER} allows deleting the target gene as required (pre- or post-injury), as opposed to the periostin-Cre model (please see below), one of the major limitations of Col1a2-cre is that it targets fibroblasts globally and thus not specific to CFs. Furthermore, several recent studies have raised a concern regarding the specificity of Col1a2-cre to fibroblasts, since colla1, 1a2 and 3a1 are also expressed by many other parenchymal cell types, epicardial cells, and valvular interstitial cells [12, 16, 24]. Moreover, cardiomyocytes also express colla1 in an injury-inducible manner [12]. Taken together, the above studies raise a significant concern about the specificity of Col1a2 as a fibroblast marker or Col1a2-cre for CF-specific gene targeting. Due to these limitations, Col1a2 should not be the primary model of choice to study cardiac fibroblast biology and fibrosis.

Numerous reports have shown that periostin expression is exclusive to the fibroblasts [39, 45, 56]. In the healthy adult heart, periostin expression is negligible; however, fibrotic remodeling is consistently associated with induction of periostin expression specifically in the fibroblast population [45, 56]. Takeda et al [8] employed periostin-Cre mediated gene deletion model to demonstrate that cardiac fibroblasts are critical for the adaptive response to pressure overload. Kong et al [39] compared FSP1 and periostin-Cre, head-to-head for fibroblast specificity and further confirmed that periostin expression in the heart is restricted to the cardiac fibroblast population and not expressed in cardiomyocytes, endothelial cells or vascular smooth muscle cells in the normal or injured myocardium (infarcted or pressure-overloaded). Recently, we employed periostin-Cre for CF-specific deletion of GSK-3 β in a myocardial infarction model [7]. CF-specific deletion of GSK-3 β led to excessive fibrosis and cardiac dysfunction in the ischemic heart. Mohamed et al [14] utilized periostin-Cre for CF-specific deletion of plasma membrane calcium ATPase 4 (PMCA4) and demonstrated that calcium signaling in CFs is important in the regulation of cardiac hypertrophy. These effects are specific as CM-specific deletion of PMCA4 does not lead to any cardiac phenotype [14]. Recently, the Molkentin group generated a knock-in mouse line containing tamoxifen-inducible Cre recombinase (MerCreMer) expression under the control of the *Postn* genetic locus (*Postn*^{MRM}) for tamoxifen-regulated Cre activity [12]. In this elegant

study, Kanisicak et al [12] demonstrated that essentially all the activated fibroblasts (myofibroblasts) in the heart, regardless of their prior lineage, expressed periostin and could be directly traced using the *Postn*^{MCM} allele. Furthermore, RNAseq analysis demonstrated that periostin-traced activated fibroblasts have a characteristic gene expression profile consistent with activated cardiac fibroblasts. Kaur et al [9] generated transgenic line containing periostin promoter elements (periostin-Cre^{ERT2}). This transgenic line was used to trace the activated fibroblasts and the effect of their ablation in the healthy and injured heart. In the healthy heart, periostin expression was limited to valvular fibroblasts only, however, robust expression was observed in ventricular fibroblasts only after injury. Analysis of CF characteristic marker genes at the single cell level identified *Postn* as the marker with the best correlation to an activated CF phenotype [9]. As discussed above, multiple models of periostin-Cre have been reported, these include constitutive [7, 8] and two different inducible lines [9, 12]. The inducible lines are; i) transgenic Periostin-Cre^{ER2} [9], and targeted knock-in of MerCreMer (MCM) at the periostin genetic locus (periostin^{MCM}) [12]. Since these lines have never been compared head to head, one would speculate that most of the advantages and limitations of these genetic tools will be shared with the fundamental characteristic of the applied genetic strategy itself. For example, constitutive periostin Cre can avoid the complexity of acute tamoxifen toxicity; however, as the periostin expression is not exclusive to fibroblast during the development, this line may lead to deletion in other cells/organs. With that said, by employing this line, we have not observed noticeable non-specific deletion in cardiomyocytes at baseline and post-MI [7]. Importantly, non-specific Cre activity in other organs was not examined. Among the inducible lines, targeted knock-in (periostin^{MCM}) will have an advantage over the transgenic approach (Periostin-Cre^{ER2}). The crucial difference between a transgenic Cre driven by a fragment of the periostin promoter versus a Cre cassette knocked into the periostin locus is the expression of the Cre in the knock-in model represents true endogenous regulation of periostin gene expression; whereas, the periostin-promoter driven Cre transgenic may not have a complete set of regulatory elements for specific locus and thus doesn't fully represent the periostin lineage of cells. Once again, the above statement regarding inducible periostin-Cre is based on the fundamental characteristics of employed genetic strategy itself. Nevertheless, the periostin-cre mouse is a very useful tool for lineage tracing and CF-specific gene targeting in the activated fibroblast. However, because periostin expression is very low to nonexistent in quiescent fibroblasts, these animals are of limited value for CF-specific gene targeting in the healthy heart [7, 12, 30, 40].

Transcription factor 21 (TCF21) (also known as pod-1, capsulin, or epicardin), is a member of the bHLH (basic helix-loop-helix) family of transcription factors and critical for the development of several cell types during embryogenesis of the heart, spleen, kidney and lung [16, 57, 58]. Recent studies have suggested TCF21 as a powerful tool for tracing resident CFs and CF-specific gene targeting [6, 12, 16, 59]. TCF21 is essential for the development of cardiac fibroblasts as TCF21 null hearts fail to form cardiac fibroblasts and are unable to undergo EMT[16]. TCF21 expression is activated during fibrogenesis following cardiac injury [59]. Importantly, TCF21 activation in the injured heart is specific to fibroblasts and is not expressed in infiltrating inflammatory cells [59]. In the healthy heart (quiescent fibroblasts), lineage-tracing strategy labeled large numbers of resident fibroblasts

as TCF21⁺ throughout the heart [12]. These resident fibroblasts were positive for fibroblast markers vimentin and PDGFR α , as well as negative for the myofibroblast marker α SMA (alpha-smooth muscle actin), endothelial cell marker CD31, and proposed non-specific fibroblast marker FSP1 (please see above) [12]. These observations were further confirmed by quantitative FACS analysis. In the injured heart, TCF21 lineage-traced (eGFP⁺) fibroblasts displayed a characteristic profile of periostin-labelled myofibroblasts, suggesting that TCF21⁺ cells are the primary source of myofibroblasts in the injured heart. Taken together, these recent reports propose TCF21 as a powerful genetic tool for reliable fibroblast-specific lineage-tracing or gene targeting [6, 12, 30, 40].

Collectively, there are limited resources and approaches available for fibroblast-specific gene targeting or lineage-tracing. We believe that one can reach a reasonable conclusion by employing a combination of the inducible periostin and TCF21 models. These complimentary models have key advantages: periostin-Cre specifically targets activated fibroblasts in injured hearts, allowing deletion of the target gene specifically in activated fibroblasts, whereas tamoxifen-inducible TCF21-Cre allows to delete the target gene at desired time points (pre or post injury). Thus, a combination of these two distinct and complementary models could be used to delineate the specific functions of the target gene in CFs.

3. Myofibroblasts in fibrotic remodeling of the diseased heart

Myofibroblasts are activated fibroblasts with a hybrid characteristic of fibroblasts and smooth muscle cells [60, 61]. These cells have a unique ability to produce ECM, characteristic of a typical fibroblast and are able to contract like a typical smooth muscle cell. A cardiac injury (eg, MI) leads to a significant loss of cardiomyocytes, resulting in a crisis to maintain the myocardium structural integrity. In this special adversity, fibroblasts quickly re-acquire an activated synthetic phenotype (activated fibroblast or myofibroblasts) to initiate a reparative fibrosis in order to preserve the structural integrity of the myocardium by forming a scar tissue [60, 61]. This scar formation is essential to prevent the expansion of the infarct area and maintain the cardiac structural integrity. While protective at the early onset of injury, persistent activation of myofibroblasts leads to excessive deposition of ECM, resulting in cardiac stiffness, adverse myocardial remodeling and heart failure [60, 61]. Indeed, numerous studies have implicated the myofibroblast mediated mechanisms in the pathological remodeling of the injured heart [9, 12, 17, 60–62]. The activated myofibroblasts are absent in the healthy heart [7].

The origin and source of the myofibroblasts in the injured heart are highly debated [12, 20, 63]. Myofibroblasts are believed to originate from different precursor cells, the major contributor being the pre-existing residential fibroblasts. Additionally, epithelial and endothelial cells can acquire a myofibroblast phenotype through the processes of epithelial–mesenchymal transition (EMT) [20, 64–66]. Indeed, EMT at the epicardium is the largest initial source of resident fibroblasts and it is suggested that this population may transdifferentiate in response to cardiac injury [65]. Many other cell types have been suggested as a major source of myofibroblast in the injured heart, these include pericytes, fibrocytes, bone marrow-derived myeloid cells, and other infiltrating immune cells [17, 60,

61]. Kanisicak et al [12] created multiple tamoxifen-inducible CF-specific mouse models to define the myofibroblast origin and function in the injured heart. The lineage tracing studies demonstrated that resident fibroblasts are the primary source of the myofibroblast in the diseased heart [12]. Furthermore, lineage tracing studies also excluded any significant contribution of endothelial, immune/myeloid or smooth muscle cells as the significant source of myofibroblast in the injured heart. Myofibroblasts were essential for the adaptive healing and fibrosis in the ischemic heart as its deletion leads to diminished collagen production and compromised scar formation [12]. Myofibroblasts have been shown to persist for a long time, months and even years [67, 68]. To examine the ability of differentiated myofibroblast to revert back into a quiescent fibroblast-like phenotype, cardiac fibrosis was introduced with fibrotic agonist (Ang II/PE) through minipumps, thereafter, animals were allowed to recover in the absence of the fibrotic agonist [12]. A combination of multiple analyses suggested that myofibroblasts have the ability to revert back to a less-activated state upon injury resolution [12]. Kaur et al [9] employed transgenic mice containing periostin promoter elements (periostin-Cre^{ERT2}) to trace the myofibroblasts (periostin expressing cells) and the effect of their ablation in the healthy and injured heart. In a healthy heart, ablation of the Postn expressing population does not affect the cardiac morphology and function. Importantly, ablation of this population in the injured heart prevented excess myocardial fibrosis and preserved ejection fraction without reducing the scar stability [12]. The source of origin, heterogeneity, and the role of myofibroblasts in cardiac pathophysiology are currently under rigorous investigation, however, based on the available evidence, it is reasonable to conclude that myofibroblasts primarily originate from the residential cardiac fibroblasts and play a critical role in the fibrotic remodeling of injured myocardium. Taken together, specific inhibition of this population in the setting of chronic fibrotic remodeling may represent a promising target for future therapies.

4. Circulating fibrocytes and myocardial fibrotic remodeling

Accumulating evidence suggests the involvement of circulating fibrocytes in the regulation of myocardial fibrogenesis [69–73]. This unique cell population is of hematopoietic origin, which circulates in the peripheral blood and expresses the markers of both hematopoietic and mesenchymal lineages. The numbers of circulating fibrocytes were found to be significantly increased in multiple myocardial disease conditions [69–73]. Furthermore, circulating fibrocytes have been implicated in several models of myocardial fibrosis including myocardial infarction, aging human hearts, hypertension, heart failure and atrial fibrillation [69–73]. However, the molecular mechanism of circulating fibrocytes mediated regulation of cardiac fibrosis is largely unknown and therefore, further mechanistic studies are warranted to expand our understanding of fibrocytes mediated regulation of cardiac fibrosis. Herein, we will focus on the profibrotic signaling pathways primarily characterized in cardiac resident fibroblasts.

5. The core components and basic regulation of the TGF- β 1 signaling pathway

Transforming growth factor- β 1 (TGF- β 1) is the most potent pro-fibrotic cytokine identified to date and a key mediator of fibroblast activation and fibrosis in the diseased heart. The TGF- β superfamily consists of three subfamilies, **1)** TGF- β pathway, **2)** activins/inhibins/Nodal pathway and **3)** bone morphogenetic proteins (BMPs) pathway [74–77]. The details of the components and mechanism of TGF- β superfamily signaling has been recently reviewed elsewhere (Table 3) [74, 76, 78]. For the purpose of this review, we will only introduce the TGF- β pathway. The TGF- β pathway is known to regulate many cellular processes including proliferation, growth inhibition, cell migration and invasion, myofibroblast transformation, and ECM remodeling [79, 80]. There are three highly homologous isoforms of the TGF- β ligand in humans: TGF- β 1, TGF- β 2, and TGF- β 3 [74–76]. While all three ligands share the same receptor complex and signaling pattern, their distinct biological function has been suggested by knockout mouse studies [75, 81–84]. The TGF- β s signal via Type I and Type II serine/threonine kinase receptors [77]. A TGF- β ligand initiates signaling by binding to the Type II receptor and facilitating the formation of a heteromeric complex of Type I and Type II receptor kinases. This facilitates receptor II to trans-phosphorylate the receptor I kinase domain, which then spreads the signal through phosphorylation of the SMAD proteins (canonical TGF signaling) or through other signaling mediators (non-canonical or SMAD-independent pathways) [74, 75, 77]. There are eight distinct SMAD proteins, constituting three functional classes: **1)** receptor-regulated SMAD (R-SMAD), **2)** Co-mediator SMAD (Co-SMAD), and **3)** inhibitory SMAD (I-SMAD). In the canonical TGF- β signaling (SMAD-dependent), activation of TGF- β receptor leads to phosphorylation of SMAD-2/3 (R-SMAD for TGF- β subfamily) [74–77]. Activated R-SMADs then form heteromeric complexes with Co-SMAD (SMAD-4) and translocate into the nucleus. Once in the nucleus, the R-SMAD-Co-SMAD Complex binds to SMAD-binding element (SBE) to regulate the expression of target genes [74, 75, 77]. In the non-canonical pathway, the activated TGF- β receptor complex transmits a signal through other signaling mediators (other than SMADs), such as TGF- β -activated kinase 1 (TAK1), tumor necrosis factor (TNF), MAP kinases (ERK, p38 and JNK), RHO, phosphoinositide 3-kinase (PI3K), AKT and NF- κ B. The I-SMADs (SMAD-6 and SMAD-7) negatively regulate TGF- β signaling by competing with R-SMADs for the receptor or Co-SMAD interaction and also by targeting the receptors for degradation [74–77].

6. GSK-3 β and TGF- β 1-SMAD-3 crosstalk to regulate myocardial fibrogenesis in the diseased heart

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed and highly conserved serine/threonine kinase. GSK-3 was first identified in 1980 for its role in regulating glycogen synthase, the rate-limiting enzyme in glycogen synthesis [85]. The GSK-3 family consists of two isoforms, α and β . These isoforms have very high homology in their kinase domains (98% identical) but differ substantially in their N- and C-terminal sequences [86, 87]. Unlike most protein kinases, GSK-3 is typically active in unstimulated cells and is inhibited in response to a variety of inputs [86]. Since GSK-3-mediated phosphorylation of

substrates usually leads to inhibition of those substrates, the end-result of stimulus-induced inhibition of GSK-3 is typically activation of its downstream targets. GSK-3 is involved in a number of central intracellular signaling pathways, including glucose metabolism, cellular proliferation, migration, inflammation and immune responses, and apoptosis. Furthermore, GSK-3 has been implicated in a number of diseases, including bipolar disorder, diabetes, Alzheimer's disease, inflammation, and cancer. However, in the present review, we will focus on its role in fibroblast biology and fibrosis with a specific emphasis on myocardial fibrosis.

Emerging evidence suggests that GSK-3 β exerts its anti-fibrotic effect by directly interacting with SMAD-3 and negatively regulating its protein stability and enzymatic activity. Guo et al [88] were the first to demonstrate that GSK-3 β controls SMAD-3 protein stability to modulate TGF- β 1 signaling. In this elegant study, Guo et al [88] demonstrated that reduction in the expression or activity of GSK-3 β leads to increased SMAD-3 protein stability and transcriptional activity. These effects were specific to SMAD-3, as expression and activity of TGF- β receptors and SMAD-2 was unchanged. These findings were further confirmed with the gain-of-function approach demonstrating that overexpression of GSK-3 β promotes SMAD-3 basal degradation and desensitizes cells to TGF- β signaling [88]. These effects were also specific to GSK-3 β isoform, as specific deletion of GSK-3 α did not alter the SMAD-3 protein stability and activity. Furthermore, exogenous expression of wild type GSK-3 β (WT-GSK-3 β) in GSK-3 β KO mouse embryonic fibroblasts (MEFs) leads to decreased SMAD-3 levels. Conversely, shRNA-mediated GSK-3 β knockdown reduced SMAD-3 ubiquitination and blocked its basal turnover. Pharmacological inhibition of GSK-3 by the selective GSK-3 β inhibitor, SB-216763, prevented SMAD-3 degradation. Consistently, expression of a constitutively active form of GSK-3 β (S9A) leads to enhanced SMAD-3 ubiquitination. Thus, GSK-3 β regulates the constant turnover of SMAD-3 protein. GSK-3 β specifically regulates the stability of SMAD-3 but not SMAD-2, as TGF- β 1 treatment of GSK-3 β knockout (KO) cells leads to increased phosphorylation of SMAD-3 at the C-terminal but not of SMAD-2. Furthermore, TGF- β -induced, SMAD binding element luciferase (SBE-Luc) activity was increased in GSK-3 β KO cells, while the SMAD-2-dependent activin response element luciferase (ARE-Luc) activity was comparable to WT cells. The combination of several approaches identified that GSK-3 β phosphorylates SMAD-3 at Thr66, which is sufficient to trigger SMAD-3 ubiquitination and degradation. Importantly, the phosphorylation status of Thr66 directly correlates with SMAD-3 stability, suggesting that its phosphorylation by GSK-3 β controls SMAD-3 protein stability and predetermines SMAD-3 activity.

However, all of these studies were carried out either in immortalized cell lines or isolated primary cells. Thus, until very recently, the *in vivo* role and physiological significance of this signaling crosstalk was unknown. In reference to myocardial biology, the role of GSK-3 family of kinases in cardiomyocyte biology is well established [89–95]. However, *in vivo* studies supporting the role of GSK-3 family of kinases in myocardial fibrosis are just starting to emerge [7, 89]. Several recent studies have implicated this pathway in regulating the transition of fibroblasts into myofibroblasts and in fibrotic signaling [7, 96, 97]. Recently, we employed fibroblast-specific GSK-3 β KO mouse models and identified a novel cross-talk of GSK-3 β and TGF- β 1-SMAD-3 signaling in myocardial fibrotic remodeling

post-MI [7]. We reported [7] that GSK-3 β is phosphorylated (inhibited) in fibrotic tissues from ischemic human and mouse hearts. We employed both periostin-Cre and Col1a2-Cre for CF-specific gene targeting to demonstrate that CF-specific deletion of GSK-3 β leads to excessive fibrogenesis, left ventricular dysfunction, and profound scarring in the post-ischemic heart. Furthermore, expansion of scar tissue and border zone fibrosis was also profoundly increased in the CF-GSK-3 β deficient hearts. At the cellular level, fibroblast-specific loss of GSK-3 β induces a pro-fibrotic, myofibroblast phenotype in isolated CFs, in MEFs and in post-MI hearts. Taken together, GSK-3 β actively protects from fibrosis in a healthy heart, and injury leads to its inhibition, resulting in activation of a pro-fibrotic gene program.

We employed both a pharmacological inhibitor and GSK-3 β KO fibroblasts to determine the molecular mechanism of GSK-3 β mediated regulation of pro-fibrotic signaling [7]. Loss of GSK-3 β activity leads to increased TGF- β 1-induced C-terminal phosphorylation of SMAD-3 (Ser423/25), indicating that GSK-3 β negatively regulates the profibrotic SMAD-3 pathway. Most of the literature on TGF- β signaling pathways has predominantly focused on the TGF- β receptor-mediated phosphorylation of SMAD at the C-terminal domain [74, 76]. However, recently there is an increasing interest in the alternate serine and threonine phosphorylation sites within the linker region of SMADs, which control a number of cellular responses including SMAD-3 transcriptional activity and EMT [98–100]. The linker region is defined as the domain which lies between the MH1 and MH2 domains of a SMAD protein [101]. In contrast to C-terminal domain phosphorylation (which leads to activation), linker region phosphorylation leads to inhibition of SMAD transcriptional activity [98, 102]. GSK-3 β mediated phosphorylation of the linker region at Ser²⁰⁴ was proposed as an alternative mechanism of GSK-3 β mediated SMAD-3 inhibition [102, 103]. We employed both GSK-3 β deficient MEFs and a specific GSK-3 pharmacological inhibitor to determine the role of GSK-3 β in the regulation of SMAD-3 linker region phosphorylation. TGF- β 1 induced phosphorylation of SMAD-3^{Ser204} was significantly decreased in GSK-3 β deficient cells. Consistently, pharmacological inhibition of GSK-3 β resulted in a significantly reduced response of TGF- β 1 on SMAD-3^{Ser204} phosphorylation [7]. Reporter assay analysis reveals that TGF- β 1-induced transcriptional activity of SMAD-3 was significantly increased in the GSK-3 β KO MEFs and GSK-3 β deleted adult cardiac fibroblasts [7]. Thus, GSK-3 β exerts dual control on TGF- β 1-SMAD-3 signaling, specifically by regulating the post-translational modification of both the C-terminal domain and the linker region.

Finally, to confirm that hyper-activation of SMAD-3 is really responsible for excessive fibrotic response observed in CF-GSK-3 β KO mice, we performed a rescue experiment with a small molecule pharmacological inhibitor of SMAD-3 (SIS3). Treatment with SIS3 efficiently rescued the detrimental phenotype of CF-GSK-3 β at multiple levels. At the functional level, pharmacological inhibition of SMAD-3 nearly abolished the detrimental phenotype of cardiac fibroblast-specific GSK-3 β deletion, as evidenced by restored ventricular function and chamber dimensions. Thus, the rescue experiment provided strong evidence that hyper-activation of SMAD-3 is the primary mechanism underlying the observed phenotype in CF-GSK-3 β KO mice. At the molecular level, we demonstrated that GSK-3 β directly interacts with SMAD-3 in the mouse cardiac fibroblasts and in human

hearts. Collectively, when GSK-3 β is deleted in cardiac fibroblasts, SMAD-3 is hyper-activated, resulting in excessive fibrosis that leads to adverse ventricular remodeling.

The cooperation of GSK-3 β and β -catenin signaling was also demonstrated in the cutaneous fibrosis model. Bergmann et al [96] employed small molecule pharmacological agents to elucidate the role of GSK-3 β and β -catenin signaling in dermal fibrosis. Incubation of cultured dermal fibroblasts with specific GSK-3 inhibitor SB216763 leads to an increased expression and release of collagen. In an *in vivo* setting, administration of SB216763 alone was sufficient to induce progressive dermal fibrosis. siRNA-mediated knockdown of β -catenin abolished the stimulatory effects of GSK-3 inhibition on ECM production, suggesting that inhibition of GSK-3 induces collagen synthesis by activating β -catenin signaling [96]. However, this study was heavily limited by exclusively relying on non-isoform specific GSK-3 inhibitor and its systemic administration. Nonetheless, direct crosstalk of the GSK-3 β / β -catenin and the SMAD-3 signaling pathways is critical to regulate fibrotic remodeling in the heart and various other fibrotic diseases.

7. β -catenin and TGF- β 1-SMAD-3 crosstalk to regulate myocardial fibrosis

It is well recognized that GSK-3 β is an essential component of the Wnt/frizzled signal transduction pathways that regulate the balance between cytoplasmic accumulation and nuclear translocation of β -catenin [104–106]. The components and molecular mechanism of Wnt signaling have been recently reviewed [107]. In brief, at the quiescent stage, β -catenin levels are maintained low by a phosphorylation event that is mediated by GSK-3 β , which targets β -catenin for ubiquitination and proteasomal degradation. Since GSK-3 β is an essential component of this degradation complex, its deletion leads to β -catenin accumulation and nuclear translocation, resulting in activation of the β -catenin dependent gene program. There is growing evidence which suggests that activation of the β -catenin dependent gene program leads to fibroblast activation and fibrogenesis in multiple organ systems [108–110]. Recently Mizutani et al [111] employed neonatal mouse heart cryoinjury model to demonstrate that epicardial cells and myocardium are Wnt-responsive in the neonatal uninjured heart. Furthermore, in response to injury, these epicardial cells give rise to cardiac fibroblasts and myofibroblasts and contribute to scar formation. Importantly, Wnt signaling was specifically activated in the cardiac fibroblast at the injury site [111].

Numerous studies have suggested a significant crosstalk between the β -catenin and TGF- β 1-SMAD-3 signaling pathways to regulate the organ fibrogenesis [10, 108–110, 112–114] (Table 4). Recently, Blyszczuk et al [115] demonstrated the crucial role of β -catenin and TGF- β 1 signaling crosstalk in the progression of myocardial fibrosis and myofibroblast transformation. Inhibition of Wnt signaling by secreted Frizzled-related protein 2 (sFRP2) or Wnt-C59 prevented TGF- β 1-induced transformation of cardiac fibroblasts into myofibroblasts. Furthermore, TGF- β 1 treatment was sufficient to trigger the Wnt secretion and activation of the Wnt pathway in isolated CFs. Blyszczuk et al [115] employed several pharmacological agents (JW55, FH-535 and ICG-001, all suppresses β -catenin-mediated transcription) to test the hypothesis that activation of Wnt signaling is required for the TGF- β 1 mediated fibrotic response. Inhibition of β -catenin was sufficient to blunt the TGF- β 1-induced myofibroblasts transformation. These critical findings with multiple

pharmacological agents were further confirmed by employing genetic loss of function model of β -catenin deficient adult CFs. Indeed, deletion of β -catenin in CFs was sufficient to attenuate the TGF- β 1-induced myofibroblast transformation. Furthermore, Blyszczuk et al [115] demonstrated that co-activation of TGF- β 1 and Wnt signaling pathways is required for the CF differentiation to myofibroblast phenotype in diseased hearts. Taken together, in CFs, cross-talk of Wnt and TGF- β 1-SMAD-3 signaling is a critical regulator of myofibroblasts transformation and fibrogenesis.

Spanjer et al [128] demonstrated that a crosstalk of Wnt-TGF- β 1 signaling is crucial for the activation of primary human lung fibroblasts and pulmonary fibrosis. Specifically, Wnt-5B functions as a ligand for Wnt receptor Frizzled-8 (FZD8) to mediate the effects of TGF- β on fibroblast activation. β -catenin and TGF- β 1-SMAD-3 signaling also cooperate to regulate the mesenchymal phenotype and endothelial-mesenchymal transition EMT [129].

Simultaneous inhibition of both β -catenin and TGF- β 1-SMAD-3 pathways reverted mesenchymal cells to polarized epithelial phenotype, whereas inhibition of a single pathway caused only partial rescue of epithelial features, indicating that both TGF- β 1 and β -catenin pathways cooperate in epithelial differentiation during EMT [129]. Cheon et al [117] further demonstrated the crosstalk of β -catenin and TGF- β 1-SMAD-3 pathways in the cutaneous injury and wound healing model. By employing several mouse models with conditional loss and gain of function approaches, it was demonstrated that β -catenin is required for the TGF- β 1 induced hypertrophic wound phenotype, cell proliferation of dermal fibroblasts, and for the regulation of MMP3 and MMP14 expression [117]. The β -catenin and SMAD pathways also cooperate to regulate myofibroblast formation and pulmonary fibrosis in an integrin α 3 β 1 dependent manner [118]. Thus, interactions between TGF- β 1 and β -catenin signaling pathways are crucial for the regulation of myofibroblast transformation and profibrotic signaling in multiple settings. Zhou et al [122] investigated β -catenin and TGF- β 1 cross-talk in pulmonary alveolar epithelial cells (AEC) in the context of EMT and pulmonary fibrosis. Inhibition or knockdown of SMAD-3 and β -catenin abolished the TGF- β 1 induced myofibroblast transformation phenotype, indicating a requirement for β -catenin in these SMAD-3-dependent effects [122].

Activation of the β -catenin pathway is also required for TGF- β 1 mediated dermal fibrosis. [110] TGF- β activates Wnt signaling in a p38-dependent manner by decreasing the expression of the Wnt antagonist Dickkopf-1. Consistently, enhanced expression of Wnt proteins was observed in tissue samples from human fibrotic diseases. In a gain-of-function approach, activation of the Wnt pathway leads to fibroblast activation *in vitro* and fibrosis *in vivo*. Conversely, transgenic overexpression of Wnt antagonist Dickkopf-1 ameliorates skin fibrosis in multiple models of TGF- β 1 induced organ fibrosis. Thus, activation of the Wnt signaling is necessary for TGF- β -mediated dermal fibrosis [110].

In aortic valve interstitial cells (VICs), β -catenin mediates TGF- β 1-induced myofibroblast differentiation [121]. TGF- β 1 induced rapid β -catenin nuclear translocation in primary porcine VICs through TGF- β receptor I kinase. Pharmacological inhibition or siRNA-mediated gene knockdown of β -catenin inhibited TGF- β 1-induced myofibroblast differentiation. In the diseased aortic valves from pigs, myofibroblasts co-localized with increased protein expression of Wnt3A, β -catenin, TGF- β 1, and phosphorylated SMAD-2/3

[121]. Thus, myofibroblast differentiation of VICs involves crosstalk between TGF- β 1-SMAD-3 and β -catenin signaling pathways [121]. In chondrocytes, SMAD-3 prevents β -catenin degradation and facilitates its nuclear translocation [130]. Specifically, C-terminal region of SMAD-3 interacted with both the N-terminal region and the middle region of β -catenin protein in a TGF- β 1-dependent manner. Both SMAD-3 and SMAD-4 were required for this interaction, and for the protection of β -catenin from proteasomal degradation. Furthermore, the interaction of SMAD-3-SMAD-4- β -catenin protein complex also mediated β -catenin nuclear translocation [130].

8. SIRT3-GSK-3 β and TGF- β 1 signaling network regulates myocardial fibrosis

Another mechanism of GSK-3 β mediated regulation of tissue fibrosis is via the SIRT3-GSK-3 β -TGF- β 1 signaling network [18]. SIRT3 is a member of the mammalian sirtuin family of proteins. There are seven sirtuin isoforms (SIRT1 to SIRT7) expressed in mammalian cells. SIRT3 is a mitochondrial nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase with varying substrates from metabolic biogenesis-associated proteins to stress-responsive proteins. Emerging evidence suggests a critical role of SIRT3 in cardiac pathophysiology [18, 131–133]. Sundaresan et al [18] demonstrated that SIRT3 activates GSK-3 β via deacetylation at the K15 residue, leading to inhibition of TGF- β 1-SMAD-3 signaling and cardiac fibrosis. Conversely, deletion of SIRT3 induces hyperacetylation of GSK-3 β , resulting in impaired activity of GSK-3 β to phosphorylate its downstream targets. As discussed earlier, loss of GSK-3 β activity leads to excessive activation of the TGF- β 1-SMAD-3 signaling pathway and adverse fibrotic remodeling. Importantly, the lysine acetylation mediated GSK-3 β enzymatic activity is completely independent of the phosphorylation status of GSK-3 β at Ser9. SIRT3 specifically acetylated GSK-3 β at residue Lys15 to negatively regulate its enzymatic activity in a Ser9 phosphorylation-independent manner. SIRT3 directly interacts with GSK-3 β to block the SMAD-3 profibrotic transcription program. SIRT3 mediated regulation of TGF- β 1-SMAD-3 signaling is specific to the GSK-3 β isoform as the K15 residue was not found in GSK-3 α [18]. Transgenic overexpression of mitochondrial SIRT3 was sufficient to decrease GSK-3 β acetylation and blockage of TGF- β 1-SMAD-3 signaling and tissue fibrosis.

SIRT3 activation mitigates organ fibrosis in a model of skin sclerosis and pulmonary fibrosis [134]. Pharmacological activator of SIRT3 (hexafluoro) was sufficient to suppress the TGF- β 1 induced activation of SMAD-3 signaling and expression of the profibrotic gene program (Col1a1, Col1a2, α -SMA and fibronectin EDA). Furthermore, hexafluoro treatment attenuated TGF- β -induced fibroblast contractility and migration [134]. Thus, pharmacological activation of SIRT3 is sufficient to potently inhibit SMAD-3 signaling, the profibrotic gene program, myofibroblast differentiation, and fibroblast contraction and migration [134]. Chen et al [132] demonstrated that activation of SIRT3 by resveratrol ameliorates cardiac fibrosis and improves cardiac function via regulation of the TGF- β /SMAD-3 pathway. Resveratrol activated SIRT3 in cardiac fibroblasts, leading to ameliorated collagen deposition and improved cardiac function. In isolated cardiac fibroblasts, pretreatment with resveratrol suppressed the myofibroblast transformation by limiting the

activation of TGF- β 1-SMAD-3 signaling. Thus, activation of SIRT3 by resveratrol could ameliorate cardiac fibrosis and improve cardiac function via the TGF- β 1-SMAD-3 signaling. However, in these later studies, the requirement of GSK-3 β activity was not explored. Thus, further work is required to examine the relevance of GSK-3 acetylation or phosphorylation in these settings.

9. Conclusion

Although a great deal of work is still needed to fully understand the mechanisms of fibrogenesis, a substantial amount of progress has been made over the past few years. *In vivo* studies employing fibroblast-specific gene targeting and lineage tracing are started to emerge and new molecular fibrotic pathways have been identified. Work in my own laboratory has focused on the mechanisms regulating myocardial fibrosis in ischemic hearts. This work has identified a novel mechanism in which crosstalk between GSK-3 β and the classical TGF- β 1-SMAD-3 cascade is critical for the regulation of myocardial fibrosis in diseased hearts. Several other labs have identified the requirement of β -catenin accumulation and nuclear translocation as a critical step for this profibrotic signaling. Thus, the signaling network involving GSK-3 β , β -catenin, and TGF- β 1/SMAD-3, has emerged as a strong regulator of myocardial fibrosis. However, the precise mechanism of this signaling network is less clear and needs further investigation. For instance, in many of these studies, the links between different molecules of the signaling network are missing and therefore remain to be established. A better understanding of this signaling network may provide a promising new therapeutic target for the management of myocardial fibrosis in diseased hearts.

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Abbreviations

AngII	angiotensin II
BAC	bacterial artificial chromosome
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
CBP	CREB-binding protein
CF	cardiac fibroblast
Co-SMAD	co-mediator SMAD
CREB	cAMP-response element-binding protein
ECM	extracellular matrix

EMT	endothelial-mesenchymal transition
FSP1	fibroblast-specific protein 1
GRK2	G-protein coupled receptor kinase 2
GSK-3	glycogen synthase kinase 3
HF	heart failure
I/R	ischemia-reperfusion
I-SMAD	inhibitory SMAD
MEF	mouse embryonic fibroblast
MI	myocardial infarction
NF-κB	nuclear factor-kappa B
PI3K	phosphoinositide 3-kinase
PMCA4	plasma membrane calcium ATPase 4
R-SMAD	receptor-regulated SMAD
SIRT	Silent information regulator
SIS3	SMAD-3 inhibitor 3
SMAD	contraction of Sma and Mad (Mothers against decapentaplegic)
TAK1	TGF- β -activated kinase 1
TCF21	transcription factor 21
TGF-β1	transforming growth factor beta 1
TNF	tumor necrosis factor
VICs	aortic valve interstitial cells
α-SMA	alpha-smooth muscle actin

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Highlights

- Every form of the cardiac disease is associated with myocardial fibrosis.
- Fibrosis leads to myocardial stiffness, dysfunction, and heart failure.
- Activated fibroblasts (myofibroblasts) are the primary effector cells in cardiac fibrosis.
- The TGF- β 1-SMAD2/3 signaling pathway is highly implicated as a mediator of fibrosis.
- Recent work has identified a signaling network of GSK-3 β , β -catenin, and TGF- β -1 is critical for myocardial fibrosis.

Figure 1. Recent explosion in the interest of cardiac fibroblast research

Until recently, most of the heart failure studies were only focused to cardiomyocyte, the recent availability of CF-specific models led to an explosion in the interest of cardiac fibroblast biology and fibrosis research. Recent studies have demonstrated a driving role of cardiac fibroblast in the myocardial disease process.

Figure 2. The Signaling network of GSK-3 β , β -catenin and TGF- β 1/SMAD-3 pathways
 In the classical TGF- β 1-SMAD-3 pathway, binding of ligands to constitutively active type II receptor phosphorylates the type I receptor, which in turn propagates the signal by phosphorylating the R-SMADs (SMAD-2 and SMAD-3). Receptor-mediated phosphorylation of R-SMADs promote association with Co-SMAD (SMAD-4), resulting in nuclear translocation to modulates the target gene expression. We identified that GSK-3 β exerts a critical brake on the classical TGF- β 1-SMAD-3 pathway through direct interactions and also through β -catenin signaling. Specifically, GSK-3 β interacts with, and thereby maintains, the low-level activity of SMAD-3, in which deletion or inhibition of GSK-3 β , leads to aberrant hyperactivation of TGF- β 1-SMAD-3 signaling and myocardial fibrosis. Furthermore, it well established that GSK-3 β is required for phosphorylation and proteasomal ubiquitination of β -catenin. Inhibition of GSK-3 leads to accumulation and nuclear translocation of β -catenin, another important modulator of TGF- β 1-SMAD-3 signaling. Thus, GSK-3 exerts dual control on TGF- β 1-SMAD-3 signaling, by direct interaction and indirectly by modulating the β -catenin signaling. Dvl, disheveled; PDK1, 3-phosphoinositide-dependent protein kinase 1; T β R, TGF- β 1 receptor; RTK, receptor tyrosine kinase; APC, adenomatous polyposis coli.

Table 1

Cardiac studies with CF-specific genetically manipulated mouse models

Target (gene/pathway)	Cre driver promoter	Type	Reference	Phenotype
Kruppel Like Factor 5 (Klf5)	Periostin	Injury inducible deletion	[8]	CF-specific <i>Klf5</i> deletion ameliorates TAC-induced cardiac hypertrophy and fibrosis. By contrast, CM-specific <i>Klf5</i> deletion did not alter the hypertrophic responses.
β -catenin	Col1a2	Tamoxifen-inducible deletion	[10]	CF-specific deletion of β -catenin impairs wound healing and decreases cardiac performance.
p53	Col1a2	Tamoxifen-inducible deletion	[11]	Loss of p53 in cardiac fibroblasts severely decreases the formation of fibroblast-derived endothelial cells, reduces post-infarct vascular density and worsens cardiac function
GSK-3 β	Periostin	Injury inducible deletion	[7]	Fibroblast-specific deletion of GSK-3 β leads to hyperactivation of SMAD-3 resulting in excessive fibrotic remodeling and cardiac dysfunction after myocardial infarction.
GSK-3 β	Col1a2	Tamoxifen-inducible deletion	[7]	FB-specific deletion of GSK-3 β leads to hyperactivation of SMAD-3 resulting in excessive fibrotic remodeling and cardiac dysfunction after myocardial infarction.
Muscleblind-like protein 1 (MBNL1)	Tcf21 ^{MCM} and Postn ^{MCM}	Tamoxifen-inducible overexpression and deletion	[17]	MBNL1 overexpression promotes the transformation of fibroblasts into myofibroblasts. Conversely, deletion of MBNL1 abrogates transformation and impairs the fibrotic phase of wound healing in mouse models of myocardial infarction and dermal injury.
Plasma membrane calcium pump (PMCA4)	Periostin	Injury inducible deletion	[14]	Specific deletion of <i>Pmca4</i> in CFs reduces TAC-induced hypertrophy and cardiac dysfunction. Importantly, these phenotypes were not observed in CM-specific PMCA4 KOs.
Activated fibroblast	Periostin	Inducible targeting cell killing	[9]	Ablation of activated CFs (periostin expressing) results in significantly reduced cardiac fibrosis and improved cardiac function in response to Ang II and myocardial infarction.
Activated fibroblast	Periostin	Inducible targeting cell killing	[12]	Deletion of periostin ⁽⁺⁾ activated fibroblasts (myofibroblasts) reduces collagen production and scar formation after MI.
G protein-coupled receptor kinases (GRK2)	Col1a2	Tamoxifen-inducible deletion	[13]	Cardiac fibroblast GRK2 deletion enhances contractility and remodeling following ischemia/reperfusion injury.
p38	Tcf21 ^{MCM} and Postn ^{MCM}	Tamoxifen-inducible overexpression and deletion	[15]	CF-specific deletion of p38 attenuates myofibroblasts differentiation and fibrosis. This phenotype was also reproduced in a dermal wound healing model.

Target (gene/pathway)	Cre driver promoter	Type	Reference	Phenotype
				Conversely, transgenic mice with CF-specific activation of p38 develops interstitial and perivascular fibrosis in multiple organs.

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Table 2

Advantages and limitations of published fibroblast markers

Marker	Class/function	Expression in CF	Expression in other cells	References
α -SMA	Cytoskeletal, contractile apparatus	Subset of activated fibroblasts	Pericytes/vascular smooth muscle cells and epicardium	[6, 12, 20, 24, 25]
CD90 (Thy-1)	Cell surface protein, critical for cell-cell interactions	Subset of fibroblasts	Pericytes/vascular smooth muscle cells, immune cells, and endothelial cells	[8, 24, 26–29]
Collagen I, III	ECM protein	Majority of fibroblasts	Pericytes/vascular smooth muscle cell, endothelial cells, and cardiomyocytes,	[6, 7, 10, 11, 16, 24, 26, 30–32]
Discoidin domain-containing receptor 2 (DDR2)	Receptor for several ECM proteins	Resident fibroblasts	Epicardium	[6, 30, 33–35]
Fibronectin	ECM protein	Subset of resident and activated fibroblasts	Endothelial cells	[30, 36–38]
fibroblast-specific protein-1 (FSP1)	Calcium-binding protein	Subset of resident and activated fibroblasts	Pericytes/vascular smooth muscle cells, endothelial cells, and immune cells	[6, 12, 23, 30, 39–41]
Platelet-derived growth factor receptor α (PDGFR α)	Tyrosine kinase receptor	Majority of fibroblasts	Cardiac progenitor cells	[6, 12, 24, 30, 42–44]
Periostin	Extracellular matrix protein	At developmental stage and in a subset of activated fibroblast	Epicardium	[6–9, 12, 14, 30, 39, 40, 45, 46]
Stem cells antigen-1 (Sca1)	Cell surface protein and common marker of hematopoietic stem cells	Subset of fibroblast	Cardiac progenitor cells	[30, 47–49]
TCF21	Transcription factor	Majority of resident fibroblasts; however, downregulated in injured heart	Epicardium	[12, 16, 17, 26, 30, 40]
Vimentin	Cytoskeleton	Resident and activated fibroblasts	Endothelial cells and pericytes/vascular smooth muscle cells	[6, 30, 50–53]

Table 3

Molecular players of the TGF β superfamily

Pathways	Ligands	Type I receptor	Type II receptor	Type III receptor	R-SMADs	Co-SMAD	I-SMADs
TGFβ1 athway	TGF β 1, TGF β 2, TGF β 3	T β R1 (ALK5), ALK1 (ACVRL1 or SKR3)	T β R2	T β R3 (betaglycan), endoglin, CRIPTO3 (TDGF1P3)	SMAD2 SMAD3	SMAD4	SMAD7
Activin/Inhibin/Nodal pathway	Activin A, Activin B, Inhibin A, Inhibin B, Nodal	ALK4 (ACVRL1 or ACTRI1B), ALK7 (ACVRL1 or ACTRI1C)	ACTRI1A, ACTRI1B	T β R3 (betaglycan), CRIPTO1 (TDGF1), CRIPTO3 (TDGF1P3)	SMAD2 SMAD3	SMAD4	SMAD7
BMP Pathway	BMP2, BMP4, BMP5, BMP6, BMP7, BMP8A, BMP8B, BMP9, BMP10,	ALK1 (ACVRL1, SKR3), ALK2 (ACVRL1, ACTRI1), ALK3 (BMPRI1A), ALK6 (BMPRI1B)	BMPR2, ACRI1A, ACTRI1B	RGMA, RGMB (DRAGON), RGMC (HJV or HFE2), ENDOGLIN	SMAD1 SMAD5 SMAD8	SMAD4	SMAD6 SMAD7

Table 4

Studies demonstrating critical role of GSK-3 β , Wnt/ β -catenin and TGF- β 1 signaling network in fibrotic diseases

Nature of Crosstalk	Fibrosis/EMT model	Summary of findings	Reference
β -catenin-TGF- β	EMT, renal fibrosis	Crosstalk of β -catenin and TGF- β is critical for EMT in renal fibrosis.	[116]
GSK-3 β - β -catenin-SMAD-3	Dermal fibroblasts, Wound size and cutaneous healing	GSK-3 β - β -catenin-regulates wound size and mediates the effect of TGF- β 1 in cutaneous healing.	[117]
β -catenin- SMAD-3	Pulmonary fibrosis	α 3 β 1 integrin links β -catenin and SMAD signaling to promote myofibroblast formation and pulmonary fibrosis.	[118]
GSK-3-SMAD-3	EMT	GSK-3 β negatively regulates TGF- β 1 and AngII-mediated cellular activity through interaction with SMAD-3.	[119]
GSK-3 β - β -catenin-SMAD-3	EMT	A crosstalk of GSK-3 β - β -catenin and SMAD-3 regulates the activity and stability of myocardin-related transcription factor (MRTF) during the epithelial-myofibroblast transition.	[120]
β -catenin-SMAD-3	Aortic valve fibrosis, EMT	β -catenin mediates mechanically regulated, TGF- β 1-induced myofibroblast differentiation of aortic valve interstitial cells.	[121]
GSK-3 β - β -catenin	Dermal fibrosis	Inhibition of GSK-3 β induces dermal fibrosis by accumulation of the β -catenin.	[96]
β -catenin-TGF- β	Pulmonary alveolar epithelial cells, EMT and pulmonary fibrosis	Interactions between β -catenin and TGF- β signaling pathways mediate EMT in a transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP) dependent manner.	[122]
β -catenin-SMAD-3	Systemic sclerosis	β -catenin is hyper-activated in systemic sclerosis and induces SMAD-dependent fibrotic responses in mesenchymal cells.	[123]
GSK-3-SMAD-3	Cardiac fibroblasts, myocardial fibrosis	GSK-3 β inhibits pro-fibrotic TGF- β 1-SMAD-3 signaling via direct interaction with SMAD-3.	[7]
β -catenin-TGF- β	Human orbital fibroblasts	MiR-29 Mediates TGF- β 1-induced ECM synthesis through activation of the β -catenin pathway in human orbital fibroblasts.	[124]
GSK-3-SMAD-3	Myocardial fibrosis, pulmonary fibrosis	SIRT3 deacetylates and activates GSK-3 β resulting in attenuation of TGF- β 1 signaling and tissue fibrosis in multiple organs.	[18]
β -catenin-TGF- β	Myocardial fibrosis, autoimmune myocarditis	Crosstalk of β -catenin signaling with TGF pathway controls myofibroblast formation and myocardial fibrosis progression in experimental autoimmune myocarditis.	[125]
β -catenin-TGF- β	Pulmonary fibrosis	Inhibition of β -catenin signaling suppresses bleomycin-induced pulmonary fibrosis by attenuating the expression of TGF- β 1.	[126]
β -catenin-TGF- β	Renal tubular epithelial cells	Hydrogen sulfide Inhibits TGF- β 1-induced EMT via β -catenin pathway.	[127]
β -catenin-TGF- β	Cardiac fibroblasts, myocardial fibrosis	Inhibition of Wnt signaling by secreted Frizzled-related protein 2 (sFRP2) or Wnt-C59 prevented TGF- β 1-induced cardiac fibrosis and myofibroblasts transformation.	[115]