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The role of Smad signaling cascades in cardiac fibrosis

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Abstract

Most myocardial pathologic conditions are associated with cardiac fibrosis, the expansion of the cardiac interstitium through deposition of extracellular matrix (ECM) proteins. Although replacement fibrosis plays a reparative role after myocardial infarction, excessive, unrestrained or dysregulated myocardial ECM deposition is associated with ventricular dysfunction, dysrhythmias and adverse prognosis in patients with heart failure. The members of the Transforming Growth Factor (TGF)-β superfamily are critical regulators of cardiac repair, remodeling and fibrosis. TGF-βs are released and activated in injured tissues, bind to their receptors and transduce signals in part through activation of cascades involving a family of intracellular effectors the receptor-activated Smads (R-Smads). This review manuscript summarizes our knowledge on the role of Smad signaling cascades in cardiac fibrosis. Smad3, the best-characterized member of the family plays a critical role in activation of a myofibroblast phenotype, stimulation of ECM synthesis, integrin expression and secretion of proteases and anti-proteases. In vivo, fibroblast Smad3 signaling is critically involved in scar organization and exerts matrix-preserving actions. Although Smad2 also regulates fibroblast function in vitro, its in vivo role in rodent models of cardiac fibrosis seems more limited. Very limited information is available on the potential involvement of the Smad1/5/8 cascade in cardiac fibrosis. Dissection of the cellular actions of Smads in cardiac fibrosis, and identification of patient subsets with overactive or dysregulated myocardial Smad-dependent fibrogenic responses are critical for design of successful therapeutic strategies in patients with fibrosis-associated heart failure.

1. Introduction:

The term “cardiac fibrosis” describes the expansion of the cardiac interstitium due to net accumulation of extracellular matrix (ECM) proteins [1],[2]. Cardiac fibrosis is not a single disease entity, but rather a pathologic response that can be appropriate or inappropriate, depending on the context. For example, following myocardial infarction, massive loss of cardiomyocytes overwhelms the negligible regenerative capacity of the adult mammalian

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heart, and activates a fibrogenic response that ultimately results in scar formation. Despite the absence of contractile function, the collagen-based scar plays an important protective role, maintaining the structural integrity of the ventricle and preventing catastrophic mechanical complications, such as cardiac rupture [3],[4],[5],[6],[7]. Formation of scar following myocardial infarction is part of a reparative response and results in “replacement fibrosis”. In many other myocardial pathologic conditions, such as hypertensive heart disease, aortic stenosis, or diabetic cardiomyopathy, cardiac fibrosis develops insidiously in the absence of significant loss of cardiomyocytes and involves predominantly the interstitial and perivascular areas. These changes may contribute to the pathogenesis of heart failure by promoting both systolic and diastolic dysfunction and may be critically implicated in the development of heart failure with preserved ejection fraction (HFpEF) [8],[9].

The members of the TGF-β superfamily play a critical role in regulation of cardiac fibrotic responses [10],[11]. In humans, the TGF-β superfamily is composed of 33 members. that can be subclassified into several subfamilies. The three TGF-β isoforms, TGF-β1, β2 and β3 are the best studied members of the family in myocardial diseases [12]. The superfamily also includes the Growth differentiation factors (GDFs), the activins, the bone morphogenetic proteins (BMPs), the inhibins, the nodal and anti-Mullerian hormone proteins. Following myocardial injury, several members of the TGF-β superfamily are induced and/or activated and modulate the phenotype of both cardiomyocytes and non-cardiomyocytes [13],[14],[15], [16],[17]. The best characterized intracellular effectors of the TGF-β superfamily proteins are the Smads, a group of 8 structurally related proteins in humans with homologues that have been identified in both vertebrates and invertebrates. The founding member of the family is the product of the Drosophila gene *Mothers against decapentaplegic* (*Mad*), which was found to mediate signaling through the BMP homologue *decapentaplegic* (*Dpp*) [18]. In parallel, the Sma proteins were identified as Mad homologues in nematodes. Thus, the term “Smad”, (combining Sma and Mad) was coined to name the vertebrate members of this family [19]. From a functional perspective, the Smads are classified into 3 groups: a) the Receptor Activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8), responsible for TGF-β superfamily signaling, b) the common Smad (Co-Smad), Smad4, which binds to the R-Smads forming the signaling complex and c) the inhibitory Smads (I-Smads: Smad6 and Smad7), which are involved in negative regulation of R-Smad-mediated cascades [20].

Fibroblasts, immune cells, vascular cells and cardiomyocytes, the main cellular effectors of cardiac fibrosis are highly responsive to TGF-βs and activate Smad-dependent signaling cascades that play a critical role in regulation of the fibrogenic transcriptional program. This review manuscript discusses the role of Smad-dependent signaling cascades in cardiac fibrotic conditions.

2. The TGF-β signaling cascade: from the cell surface to the nucleus

The heart contains latent stores of TGF-β [21] that can be rapidly activated following injury [22] through interactions of the latent TGF-β complexes with proteases [23],[24], specialized matrix proteins (such as ED-A fibronectin and thrombospondin-1) [25],[26],[27] and cell surface integrins [28]. Moreover, in many fibrosis-associated myocardial conditions,

infiltration with platelets capable of releasing large amounts of TGF-β from their granules [29], and de novo synthesis of TGF-β isoforms in cardiomyocytes, fibroblasts, immune cells and vascular cells [30],[17],[16], further increase the amounts of activatable TGF-βs in the site of injury. Several other members of the TGF-β superfamily are also upregulated in the injured and remodeling myocardium, including activins [31], BMPs [15] and GDFs [13].

All TGF-β superfamily members transduce signals through binding to characteristic combinations of type I and type II TGF-β receptors (TβRs) [32]. Humans have 7 type I TβRs, (also known as activin-like receptor kinase (ALK)1–7), and 5 type II TβRs (TβRII, ActRII, ActRIIB, AMHRII and BMPRII) [33]. The three TGF-β isoforms (TGF-β1, -β2, and -β3) act through a single type II receptor (TβRII) that may interact with ALK5 [34], or ALK1 (or both) depending on the cell type and the context [35],[36],[37],[38]. Some studies have suggested that ALK2 and ALK3 may also mediate certain TGF-β-induced actions [39]. Other members of the superfamily use different TGF-β receptor combinations. Activins signal through ALK4, or ALK7 after binding to the ActRII or ActRIIB type II receptors. On the other hand, the members of the BMP family signal through a range of combinations, including one of the type I receptors ALK1 ALK2, ALK3 and ALK6 and one of the ActRII, ActRIIB and BMPRII type II receptors [40] [41].

Binding of a TGF-β superfamily member to its receptors triggers formation of a heterotetrameric complex, composed of two type I and two type II receptor molecules (Figure 1). Subsequently, the phosphorylated type I receptor interacts with and phosphorylates members of the R-Smad family at the carboxyterminal Ser-Ser-X-Ser (SSXS) motif, activating a Smad-dependent (canonical) signaling cascade. Type I receptors have preferred Smad partners: ALK5, ALK4 and ALK7 phosphorylate Smad2 and Smad3, whereas ALK1, ALK2, ALK3 and ALK6 phosphorylate Smad1, Smad5 and Smad8 [42]. After phosphorylation by the type I receptor, the R-Smads dissociate from the receptor and form trimeric complexes with the common Smad, Smad4. These complexes can be either homomeric or heteromeric: one Smad4 molecule can bind to two molecules of the same R-Smad, or may form a mixed complex, composed of two different R-Smads (Smad2 and Smad3, or even Smad1 and Smad3). Subsequently, the R-Smad/Smad4 complex translocates to the nucleus, where it binds to Smad-binding elements or GC-rich sequences in the promoter regions of target genes, regulating their transcription [43].

The Smad cascades are modulated through the effects of accessory receptors (such as betaglycan and endoglin) [42], interactions with cytoplasmic proteins, and negative feedback pathways. Endoglin has been suggested to regulate the balance between Smad1/5 and Smad2/3 cascades in response to TGF-βs. It is predominantly expressed in endothelial cells and in activated fibroblasts, and negatively regulated ALK5-Smad2/3 responses, while enhancing ALK1-Smad1/5/8 signaling [44],[45],[46]. Betaglycan, on the other hand can either activate or inhibit TGF-β signaling responses, depending on its expression levels, and on contextual factors [47], [48]. Several other transmembrane molecules (including CD44 and neuropilin-1) [49],[50] have been reported to act as non-selective co-receptors that may modulate TGF-β responses, while interacting with other growth factors and bioactive mediators.

Negative feedback mechanisms act to restrain overactive Smad signaling responses. At the receptor level, TGF-β-induced overexpression of the cell surface pseudo-receptor BAMBI (BMP and activin membrane-bound inhibitor) competes with TβRI for ligand binding [51], [52] and may suppress TGF-β/R-Smad responses[53]. The I-Smads Smad6 and Smad7 are induced following TGF-β stimulation and compete with R-smads for their binding to activated TβRI or Smad4, while also mediating TβRI degradation by recruitment of Smurf ubiquitin ligases [54]. Moreover, the nuclear co-repressors Ski and SnoN can bind to the translocated R-Smad/Smad4 complex promoting its degradation via Smurf2, or directly preventing transcription of Smad-target genes by recruiting histone deacetylases [55].

It should be emphasized that, in addition to Smad signaling cascades, TGF-βs also activate non-canonical signaling pathways, such as mitogen-activated protein kinase (MAPK), TGF-β-activated kinase 1 (TAK1), Rho GTPase, phosphatidylinositol3-kinase/AKT and focal adhesion kinases (FAK) [56] [57] [58]. These non-canonical pathways extensively interact with the Smad-mediated cascades and add additional layers of complexity to the biology of the TGF–β response [59],[60],[61], Moreover, because these non-Smad cascades are also common downstream effector pathways for a wide range of mediators, dissection of their relative role in mediating effects of TGF-β superfamily members is challenging.

3. Can Smads be activated through mechanisms independent of TGF-β superfamily members?

Although the members of the TGF-β superfamily are considered the main activators of Smad signaling, a growing body of evidence suggests that TGF-β-independent mechanisms may also contribute to Smad activation in certain cell types. In epithelial cell lines, viral infection has been suggested to activate Smads in a TGF-β-independent manner [62]. In macrophages, phagocytosis rapidly and consistently activated Smad3 in the absence of TGF-β secretion [30], In NK cells, effects of Smad4 were found to be TGF-β-independent [63]. Experiments in renal mesangial cells suggested that advanced glycation end-products may activate R-Smads through a TβRII-independent mechanism [64]. The significance of TGF-β-independent Smad signaling in cardiac fibrosis has not been documented. Although the fibrogenic actions of angiotensin II are associated with marked activation of Smad2 and Smad3, these effects are likely indirect and involve activation and induction of TGF-β [65], [66]. Thus, Smad activation in fibrotic cardiac conditions should be viewed as the result of induction and/or activation of TGF-β superfamily members.

4. The cellular basis of cardiac fibrosis

As the main matrix-producing cells, activated fibroblasts are the key cellular effectors of myocardial fibrosis, regardless of underlying etiology [67]. In many cardiac fibrotic conditions, fibroblasts convert to secretory myofibroblasts, expressing contractile proteins, such as α-smooth muscle actin (α-SMA), and producing large amounts of structural matrix proteins and fibrogenic matricellular macromolecules [68],[69],[70],[71]. Myofibroblasts contribute to the fibrotic response, not only by secreting matrix proteins, but also by producing proteases, such as matrix metalloproteinases (MMPs), and their inhibitors [72], [73], thus regulating matrix metabolism. Emerging evidence suggests that myofibroblast

conversion is not required for fibrogenic activation. In a mouse model of type 2 diabetes, increased interstitial and perivascular collagen deposition was not associated with myofibroblast conversion [74]. Moreover, single cell transcriptomic analysis suggests that in remodeling hearts, high matrix gene synthesis in fibroblast subpopulations may occur in the absence of myofibroblast transdifferentiation [75],[76]. Thus, alternative pathways of fibroblast activation may significantly contribute to cardiac fibrotic responses. The expansion and activation of fibroblasts and myofibroblasts in remodeling hearts may also involve several other cell types, including immune cells, cardiomyocytes and vascular cells. Macrophages and lymphocytes have been implicated in fibroblast activation through secretion of fibrogenic growth factors, cytokines and matricellular proteins [77],[78],[79], [80]. Mast cells may also contribute to fibrotic cardiac remodeling by releasing their fibrogenic granular contents [81],[82]. Under conditions of ischemic, mechanical or metabolic stress, cardiomyocytes are also capable of producing fibrogenic mediators and may contribute to fibroblast activation [83],[84]. Vascular endothelial cells can also produce bioactive mediators that stimulate fibroblasts. Several studies have suggested that endothelial cells may undergo endothelial-to-mesenchymal transition (EndMT) [85],[86],[87] thus directly participating in the myocardial fibrotic response. However, several publications using robust lineage tracing strategies in models of myocardial infarction and pressure overload have challenged the contribution of EndMT in cardiac fibrotic conditions [88],[89], [90].

The bulk of the experimental evidence suggests a major contribution of fibroblast Smad3 signaling in the cellular responses leading to myocardial fibrosis. In contrast, evidence supporting the role of other Smad proteins in fibroblast activation, and data on the potential involvement of Smad cascades in fibrogenic activation of other cell types are limited.

5. Smad signaling cascades in fibroblasts

Activation of Smad2 and Smad3 has been extensively demonstrated in fibroblasts infiltrating fibrotic and remodeling hearts; in contrast, much less evidence is available on Smad1/5/8 activation [91],[17],[92]. In isolated cardiac fibroblasts TGF-β isoforms, but not BMPs, rapidly activate Smad2 and Smad3 signaling [91],[93]. In vitro, Smad3 is a central activating signal for cardiac fibroblasts that regulates several different functions (Table 1, Figure 2) [10]. First, Smad3 induces myofibroblast conversion, increasing α-SMA expression and promoting its incorporation into stress fibers, the hallmark of myofibroblast transition [94], [95]. Smad3-mediated myofibroblast conversion involves direct effects on α-SMA transcription [94], [96], and may be accentuated by indirect actions, such as upregulation of fibronectin [7], a specialized matrix protein that stimulates acquisition of a myofibroblast phenotype [97] or downregulation of the transcription factor Forkhead box protein O3a (FoxO3a) [98], a signal that inhibits myofibroblast conversion [98, 99]. Second, Smad3 stimulates the transcription of structural and matricellular extracellular matrix proteins, including type I and type III collagens, fibronectin, periostin and tenascin-C [7, 94], [100], [101], and induces synthesis of matrix-crosslinking enzymes, such as lysyl-oxidase [102] and tissue transglutaminase [103]. Third, Smad3 promotes a matrix-preserving phenotype characterized by suppressed synthesis and reduced activity of matrix metalloproteinase (MMP)-3 and MMP-8 and induction of anti-proteases, such as Tissue Inhibitor of

Metalloproteinases (TIMP)-1 [93],[91]. Fourth, Smad3 stimulates fibroblast expression of α2, α5, β3and α11 integrins, facilitating interactions between the fibroblasts and the extracellular matrix; these interactions may be important for formation of an organized scar [7] [104], Fifth, Smad3 may promote fibroblast migration and may enhance the capacity of fibroblasts to contract free-floating collagen lattices [94] (which is an indicator of fibroblast activation and is independent on myofibroblast conversion) [105]. Sixth, some studies have suggested that Smad3 may increase fibroblast viability under conditions of stress by exerting anti-apoptotic actions [106]. Finally, in addition to all these activating actions, Smad3 inhibits fibroblast proliferation, suggesting that Smad3-dependent myofibroblast activation is accompanied by tight regulation of the density of cardiac fibroblasts. The effects of Smad3 on cardiac fibroblasts are modulated through interactions with Smad-independent pathways [102],[7],[107], or through synergistic interactions with other fibrogenic transcription factors, such as scleraxis [108].

In vitro studies using isolated cardiac fibroblasts also suggest a significant role for Smad2 in activation of a fibrogenic transcriptional program (Figure 2), albeit with less consistent effects on functional activity. siRNA knockdown experiments suggested that Smad2 enhances fibronectin, periostin and versican expression by unstimulated cardiac myofibroblasts [109]. Smad2 deletion experiments using Cre-expressing adenovirus transfection in Smad2 fl/fl cardiac fibroblasts suggested broad effects of Smad2 in TGF-β-induced extracellular matrix gene synthesis. Moreover, Smad2 knockdown was found to inhibit incorporation of α-SMA into myofibroblast stress fibers [92]. However, in contrast to the critical effects of Smad3, Smad2 did not mediate integrin synthesis in cardiac fibroblasts [92] and Smad2 loss did not affect fibroblast-mediated contraction of collagen lattices [96]. The distinct effects of Smad2 and Smad3 on fibroblast gene transcription and function may be due to distinct patterns of activation and nuclear translocation, or to different interactions with co-repressors and co-activators that may affect their transcriptional targets.

Very limited information is available regarding the role of the Smad1/5/8 pathway in cardiac fibroblast phenotype and function. Findings from studies examining the role of Smad1 signaling in fibroblast responses in other organs have produced conflicting results. In lung fibroblasts, Smad1 activation was found to be enhanced by the accessory receptor betaglycan and antagonized the stimulatory actions of ALK5/Smad3 signaling [110]. In contrast, in a mouse model of fibrosis due to forced expression of ALK5 that recapitulates features of scleroderma, activation of a fibrogenic program was surprisingly found to be dependent on Smad1 [111], and was attributed to a ALK5/ALK1/Smad1 axis. In cardiac fibroblasts, inhibition of matrix gene expression by BMP7 was attributed to activation of Smad1 signaling [112]. The significance of these observations in regulation of cardiac fibrotic responses in vivo is not known.

6. The inhibitory Smads as negative regulators of fibroblast function

The I-Smads, Smad6 and Smad7 lack the carboxyterminal SSXS motif and cannot be phosphorylated upon binding to type 1 receptors, but act to inhibit signals transduced by TGF-β superfamily ligands through interactions with TβRs or R-Smads [113]. Smad6 preferentially inhibits BMP responses, mediated through ALK3 and ALK6, whereas Smad7

inhibits both TGF-β and BMP-induced cascades [114]. Several molecular mechanisms have been proposed to explain the inhibitory effects of I-Smads on TGF-β superfamily signaling. First, I-Smads may directly associate with TGF-β receptors, inhibiting TβRI kinase activity, or simply interfering with R-Smad:TβR binding [115],[116],[117]. Second, I-Smads may form a complex with the transmembrane pseudoreceptor BAMBI, inhibiting TβR-driven R-Smad activation [52]. Third, I-Smads may interact with the Smurf1 and Smurf2 type E3 ligases promoting degradation of R-Smads [118],[119]. Fourth, I-Smads may interfere with formation of the R-Smad:Smad4 complex [120].

Whether Smad6 is involved in regulation of fibrosis has not been investigated. Smad7 on the other hand has been suggested to act as a negative regulatory signal that may inhibit myocardial fibrosis. In vitro, Smad7 synthesis is induced upon TGF-β stimulation and Smad7 overexpression in cardiac fibroblasts suppresses collagen synthesis [121],[122]. The in vivo role of Smad7 in regulation of cardiac fibrotic responses is supported predominantly through associative data. In models of ventricular remodeling, Smad7 induction may serve as a TGF-β-induced endogenous inhibitory signal that restrains fibrogenic activation [121], [123]. In contrast, in other pathologic conditions, such as atrial fibrillation, atrial fibrosis has been attributed to reduced Smad7 levels in response to neurohumoral activation [124]. Direct in vivo evidence documenting the role of endogenous Smad7 in regulation of fibroblast responses is lacking. Two published studies demonstrated increased cardiac fibrosis in a hypomorphic Smad7 mutant line in which exon 1 of the Smad7 gene was deleted ($Smad7^{\Delta ex1}$)[125],[126]. Unfortunately, the significance of these findings is unclear, as these animals exhibit preserved Smad7 functions due to the presence of an intact MH2 domain, the key effector domain involved in Smad7 interactions with TβRs and R-Smads [117], [127].

7. Smad activation in immune cells and endothelial cells may trigger fibrogenic responses.

In addition to their effects on fibroblasts, Smad signaling may regulate phenotype and function of other cell types, thus contributing to their fibrogenic actions. In macrophages Smad3 plays an important role in activation of a phagocytic program, but also mediates the transition of macrophages to an anti-inflammatory phenotype in response to phagocytosis [30]. Phagocytic Smad3 null macrophages had impaired capacity to synthesize fibrogenic TGF-βs [30]. Whether this defect has an impact on the fibrogenic potential of macrophages has not been tested. In endothelial cells, Smad3 has been implicated in EndMT in a model of diabetic renal injury [128]. Whether Smad-dependent EndMT contributes to cardiac fibrosis has not been tested. However, this mechanism is unlikely considering the strong evidence from lineage tracing studies suggesting that the majority of activated fibroblasts in cardiac fibrotic conditions are not derived from endothelial cells [88],[89],[90],[129].

8. The role of Smad signaling cascades in homeostasis of the cardiac ECM network.

Smad cascades are critically involved in cardiac development [130]; however, their role in homeostasis of the adult heart is poorly understood. The adult mouse myocardium has high levels of constitutive Smad2 and Smad3 expression; however, baseline Smad2/3 phosphorylation is low [109]. In vivo studies showed that fibroblast-specific Smad3 (but not Smad2) loss modestly but significantly attenuated collagen levels in young adult mouse hearts, without affecting cardiac geometry or function [109]. These findings support the notion that fibroblast Smad3 signaling may play a role in maintaining the basal levels of collagen in the myocardium; however, this effect is not critical for preservation of function. Whether other Smad cascades are involved in homeostasis of the cardiac ECM network has not been investigated.

9. Smad signaling cascades in conditions associated with cardiac fibrosis (Table 2).

9.1. The role of Smad cascades in reparative fibrosis of the infarcted heart.

Myocardial infarction results in sudden loss of up to a billion cardiomyocytes, activating an inflammatory reaction that clears the infarct from dead cells and matrix debris and sets the stage for fibroblast-driven repair [131],[132]. Formation of a scar is critical for preservation of the structural integrity of the infarcted ventricle; however, excessive, unrestrained or expanded fibrosis may cause adverse remodeling, contributing to systolic and diastolic dysfunction and to the pathogenesis of heart failure. Induction and activation of TGF-βs in the infarcted myocardium is associated with activation of Smad2 and Smad3 in all cell types involved in cardiac repair, including border zone cardiomyocytes, infarct fibroblasts and macrophages [91],[92],[30]. The relative contribution of various members of the TGF-β superfamily in Smad2/3 activation remains unclear. The 3 TGF-β isoforms, the major activators of Smad2 and Smad3 cascades exhibit distinct patterns of induction following myocardial infarction with early upregulation of TGF-β1 and TGF-β2 and delayed increase in TGF-β3 levels [16],[30]. Early studies using mice with global germline loss of Smad3 provided insights into the role of the pathway in fibrosis of the infarcted heart. Complete absence of Smad3 attenuated collagen deposition in the infarcted and remodeling myocardium, despite increased infiltration of the infarct with myofibroblasts [91],[94]. Based on in vitro studies, the effects of global Smad3 loss were attributed to alterations in fibroblast phenotype and function, leading to attenuated expression of structural collagens, tenascin-C and fibronectin, and reducing secretion of Connective Tissue Growth Factor (CTGF)/CCN2, a downstream mediator of TGF-β-induced fibrogenesis [94]. However, considering the broad effects of Smad3 on all cell types involved in cardiac repair and remodeling, and the effects of Smad3 signaling on baseline homeostasis [133], dissection of cellular mechanisms using the global loss-of-function model is challenging.

Investigations using cell-specific loss-of-function models have significantly enhanced our understanding of the role of Smad cascades in myocardial infarction. (Table 2). Studies using cardiomyocyte-specific knockouts suggested that cardiomyocyte Smad3 signaling is

not implicated in cardiac homeostasis, but contributes to the pathogenesis of adverse remodeling and dysfunction following reperfused myocardial infarction [7]. The detrimental actions of cardiomyocyte Smad3 in post-infarction ventricular dysfunction were attributed to pro-apoptotic effects that may involve activation of oxidative pathways and to induction and activation of MMP2 that may accentuate matrix degradation, causing adverse dilative remodeling [7].

On the other hand, Smad3 loss in infarct myofibroblasts perturbed repair of the infarcted heart, increasing the incidence of catastrophic late rupture in the model of non-reperfused infarction, and accentuating adverse remodeling in the model of reperfused infarction. Impaired repair in the absence of myofibroblast Smad3 signaling was related to perturbed fibroblast:extracellular matrix interactions that resulted in formation of a disorganized scar [7]. Smad3 plays an important role in induction of fibroblast integrins, the cell surface proteins that link the cells to the extracellular matrix [7]. Smad3-mediated integrin synthesis in fibroblasts is critically involved in activation of an oxidative response, that promotes a reparative program. Moreover, integrin synthesis may be important for scar organization, contributing to myofibroblast alignment in the healing infarct [7], a process that may involve expression of polarity genes [134]. In contrast to the critical role of myofibroblast Smad3 in cardiac repair, Smad2 activation in myofibroblasts did not play a significant role in post-infarction repair [92]. Although both Smad2 and Smad3 are activated in infarct myofibroblasts, only Smad3 is involved in integrin upregulation [92].

The role of the Smad1/5/8 cascade in the pathogenesis of post-infarction fibrosis remains unknown. Smad1 activation in the infarcted ventricle may involve induction of both BMPs and TGF-βs. Cardiomyocyte Smad1 has been suggested to play a protective role following ischemic injury through effects that may involve activation of anti-apoptotic signals [135]. Whether these effects result in reduced fibrosis has not been tested. Moreover, direct effects of Smad1 signaling in fibroblast activation have not been explored.

9.2. Smad signaling in fibrotic remodeling of the pressure-overloaded heart

Myocardial activation of the Smad2/3 pathway has been reported in both adult and pediatric patients with heart failure [136]. Adult patients with dilated cardiomyopathy exhibited higher levels of myocardial Smad2/3 activity; this was associated with a more prominent fibrotic response [136]. Left ventricular pressure overload is the predominant pathophysiologic perturbation responsible for cardiac remodeling and fibrosis in patients with chronic hypertension or aortic stenosis. Studies in animal models of pressure overload induced through transverse aortic constriction or through neurohumoral activation showed robust myocardial activation of Smad2 Smad3 [137],[138] and Smad1 [139]. The molecular links between mechanical stress and activation of Smad signaling cascades remain poorly understood, but may involve angiotensin II-mediated actions and subsequent activation and induction of TGF-βs in the cardiac interstitium. Early studies using global loss-of-function approaches suggested that Smad3 mediates fibrosis and ventricular dysfunction in a model of angiotensin II infusion [140], and may promote fibrosis following pressure overload [141]. Moreover, ALK5 inhibition attenuated dysfunction and inhibited fibrosis in rodent models of left ventricular pressure overload; these protective actions were attributed to

inhibition of Smad2/3 signaling [142],[143],[144]. However, considering the broad effects of Smad3 on all cells involved in cardiac remodeling and fibrosis (including cardiomyocytes, vascular cells, fibroblasts and immune cells), dissection of cell biological mechanisms responsible for the effects of R-Smads requires cell-specific interventions.

Experiments in fibroblast and myofibroblast-specific knockout mice have suggested an important role for Smad3 signaling in activation of fibroblasts in failing and remodeling hearts. These actions appear to have a major impact on cardiac function. Deletion of Smad3 (but not Smad2) from cardiac fibroblasts attenuated the cardiac fibrotic response to pressure overload. The fibrogenic actions of Smad3 were attributed to increased transcription of extracellular matrix genes, including type I and type III collagens, periostin, fibronectin, tenascin-C, and to upregulation of integrins [96]. In an independent study focusing on the role of fibroblast Smad3 in pressure overload-induced cardiac remodeling, early activation of Smad3 in myofibroblasts protected from systolic dysfunction by preserving the extracellular matrix through downmodulation of collagenases. The findings suggested that Smad3 signaling in activated myofibroblasts plays a crucial role in TGF-β-mediated suppression of the collagenases MMP3 and MMP8 and in upregulation of TIMP1, inhibiting fragmentation of the matrix under conditions of mechanical stress. In the absence of Smad3 in fibroblasts, increased MMP8-mediated proteolytic activity was associated with collagen denaturation and generation of pro-inflammatory matrix fragments that enhance inflammation, promote cardiomyocyte apoptosis and cause dysfunction. [93]. Thus, activated myofibroblasts may play an important protective role in the early stages of cardiac remodeling.

9.3. Smad signaling in fibrosis of the diabetic heart.

Diabetes, obesity and metabolic dysfunction are associated with an increased incidence of fibrosis that involves not only the myocardium [145],[146],[74] but also other organs, such as the liver and kidney [147]. Cardiac fibrosis may contribute to the pathogenesis of diastolic dysfunction and to the development of Heart Failure with Preserved Ejection Fraction (HFpEF), a common form of heart failure in diabetic and obese subjects [148]. Myocardial induction of TGF-β superfamily members and activation of Smad2 and Smad3 cascades have been consistently reported in animal models of type 1 and type 2 diabetes [149],[150], [133],[151]. In contrast, evidence of activation of the Smad1/5/8 cascade in the diabetic heart is lacking; however, Smad1 activation in the diabetic kidney has been implicated in mesangial expansion [152]. Activation of the TGF-β/Smad2/3 axis in the diabetic myocardium may reflect, at least in part, the effects of hyperglycemia on expression of TGF-β receptors [153]. Moreover, diabetes-associated activation of the renin-angiotensin-aldosterone system (RAAS), oxidative stress, chronic stimulation of inflammatory cytokines and protein kinase C (PKC) activation may induce de novo synthesis of TGF-β, or activate latent stores, leading to stimulation of Smad2 and Smad3 signaling cascades [154],[155], [156].

Our knowledge on the role of Smad3 in diabetic cardiac fibrosis is derived from experiments using a global haploinsufficiency model. In the db/db mouse model of obesity-associated type 2 diabetes, Smad3 heterozygotes had attenuated fibrosis and improved diastolic

function, associated with reduced collagen deposition and increased MMP activity. The reduction in interstitial collagen in animals with partial loss of Smad3 was associated with mild ventricular dilatation [133]. The findings are consistent with an important role for Smad3 in mediating fibrosis in diabetic hearts. However, considering the broad effects of Smad3 in all myocardial cells, whether these findings reflect Smad-dependent actions on fibroblast activity remains unknown. Moreover, the role of other R-Smads in diabetes-associated fibrosis is not known.

9.4. Smad signaling in aging-associated fibrosis.

Senescence of the heart is associated with structural and morphological changes as hypertrophy and fibrosis that may lead to increased ventricular stiffness and impaired diastolic function [157]. Loss of one TGF-β1 allele in TGF-β1 heterozygous mice ameliorated age-associated myocardial fibrosis and reduced myocardial stiffness [158]. Whether aging-associated fibrosis is due to activation of Smad signaling pathways in fibroblasts remains unknown. Smad signaling in cardiomyocytes plays an important role in cardiac homeostasis in aging hearts [159]. Mice with cardiomyocyte-specific Smad4 loss exhibited increased hypertrophy associated with reduced cardiomyocyte survival and accentuated fibrosis as they aged [160],[159]. Fibrotic remodeling in the absence of cardiomyocyte Smad4 may be due to cell death and subsequent activation of a reparative program, or may reflect paracrine effects of stressed, or injured cardiomyocytes on interstitial fibroblasts.

Moreover, perturbed Smad signaling may explain the impaired reparative response of the senescent heart to injury. 24-month old mice exhibited increased post-infarction ventricular dilation, associated with markedly reduced deposition of collagen in the infarct zone [161]. The pathologic alterations noted in senescent mouse infarcts resembled the pathology of healing infarcts in mice with myofibroblast-specific Smad3 loss [7]. Moreover, fibroblasts harvested from senescent mouse hearts exhibit blunted Smad2 activation in response to TGF-β stimulation [161]. These observations support the intriguing hypothesis that the aging-associated impairment in repair following myocardial infarction may be related to attenuated TGF-β/Smad2/3 activation [162]. Perturbed Smad3 signaling in senescent fibroblasts may reflect a reduced reparative reserve that may be responsible for formation of a disorganized scar, composed of a fragmented matrix network. These age-associated alterations may reduce the tensile strength of the infarct, accentuating adverse remodeling and promoting post-infarction heart failure.

9.5. Smad signaling in myocarditis-induced cardiac fibrosis

Myocardial fibrosis is an important cellular mechanism responsible for development of dysfunction in patients with myocarditis who develop chronic dysfunction and cardiomyopathy. TGF-βs have been implicated in the pathogenesis of myofibroblast activation and fibrosis in models of autoimmune [163] and viral myocarditis[164]. However, evidence supporting the involvement of Smad signaling cascades in myocarditis-induced fibrosis is scarce. In a mouse model of chronic myocarditis due to Chagas disease, ALK5 inhibition was found to attenuate fibrosis[165]. The protective actions were attributed to inhibition of Smad2/3 activity.

9.6. Smad signaling in valve fibrosis

Patients with mitral valve prolapse exhibit myxomatous degeneration of the valve, associated with activation of valve interstitial cells, matrix remodeling and valve fibrosis. In patients with mitral valve prolapse who underwent repair for severe mitral regurgitation, Smad2/3 activation was noted in fibrotic valve tissue and was associated with extracellular matrix deposition [166],[167]. Smad2/3 signaling was implicated in activation of a fibrogenic phenotype in valve interstitial cells [167]. In addition to fibrotic involvement of the mitral valve, patients with mitral valve prolapse also exhibit myocardial interstitial remodeling that is independent of the volume overload associated with severe mitral regurgitation [168]. These myocardial fibrotic changes may explain the high incidence of arrhythmias in patients with severe mitral valve prolapse. It is tempting to hypothesize that a common Smad-dependent mechanism may be involved in the pathogenesis of valvular and ventricular fibrosis in patients with malignant mitral valve prolapse. However, evidence supporting this notion is lacking.

10. Targeting Smads in cardiac fibrosis

TGF-β signaling pathways are critically involved in cardiac fibrosis, remodeling and dysfunction and represent promising therapeutic targets [12],[169],[170]. Targeting R-Smad signaling is feasible; however, the cell-specific and context-dependent actions of Smad cascades and our limited knowledge on the effects of key members of the Smad family hamper therapeutic translation, posing several major challenges. First, Smad3 activation plays distinct roles in various cell types that greatly affect outcome following myocardial injury. In the infarcted heart, fibroblast and macrophage Smad3 activation serves a reparative role, whereas cardiomyocyte Smad3 activation promotes dysfunction [7],[30]. Thus, development of effective therapeutics targeting the Smad3 cascade would require cell-specific interventions. Second, human patients with heart failure or myocardial infarction exhibit remarkable pathophysiologic heterogeneity. Following myocardial infarction, some patients may have excessive pro-inflammatory activation and defective matrix deposition, thus developing dilative ventricular remodeling and systolic dysfunction, while others have accentuated fibrotic responses and may develop diastolic dysfunction. Accentuated fibrosis may involve overactive Smad3 signaling; however effective therapeutic strategies would require identification of fibrosis-prone patients using biomarkers or imaging strategies[171]. Third, the effects of Smad3 inhibition in patients with heart failure may be dependent on the severity of fibrotic remodeling and the phenotype of fibrotic lesions. In chronic hypertensive heart failure, sustained or overactive Smad3 activation in interstitial cells is likely to be detrimental, promoting matrix deposition and accentuating diastolic dysfunction. However, in the early stages of hypertensive cardiac remodeling, Smad3-dependent activation of myofibroblasts may exert protective matrix-preserving actions that preserve the matrix that surrounds the cardiomyocytes and prevent release of pro-inflammatory matrikines [93]. Third, our current knowledge on the role of Smad cascades in myocardial disease is limited to evidence on the role of Smad3 in cardiomyocytes and fibroblasts. We currently have very limited information regarding the role of Smad3 in regulation of lymphocyte, dendritic cell and vascular cell function in the remodeling heart, and we know very little regarding the potential role of endogenous Smad2 and Smad1/5/8 cascades in myocardial disease. Fourth,

considering the role of Smad signaling pathways in cardiac and vascular homeostasis and in tissue repair, chronic therapy to inhibit Smad3 or Smad4 may carry significant risks [133], [159].

11. Conclusions:

Smad signaling cascades play a critical role in cardiac fibrotic responses; however, our current understanding of their actions is not sufficient to design therapeutic interventions. Moreover, the context-dependent and cell-specific actions of receptor-activated Smads pose major challenges in therapeutic implementation. Extensive experimental work is needed to study the patterns and mechanisms of Smad activation in myocardial diseases and the role of specific members of the family in regulating phenotype and function of the cells involved in cardiac remodeling. Moreover, clinical investigations are needed to identify heart failure patient subpopulations with perturbed or overactive Smad responses in order to tailor therapeutic interventions.

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HIGHLIGHTS

1. TGF-β superfamily members signal through Smad-dependent and non-Smad pathways.
2. Smad3 signaling plays a crucial role in fibroblast activation following myocardial injury.
3. Myofibroblast-specific Smad3 activation protects the infarcted heart from rupture and adverse remodeling.
4. Smad2 does not play a crucial role in activation of cardiac fibroblasts in myocardial injury models.

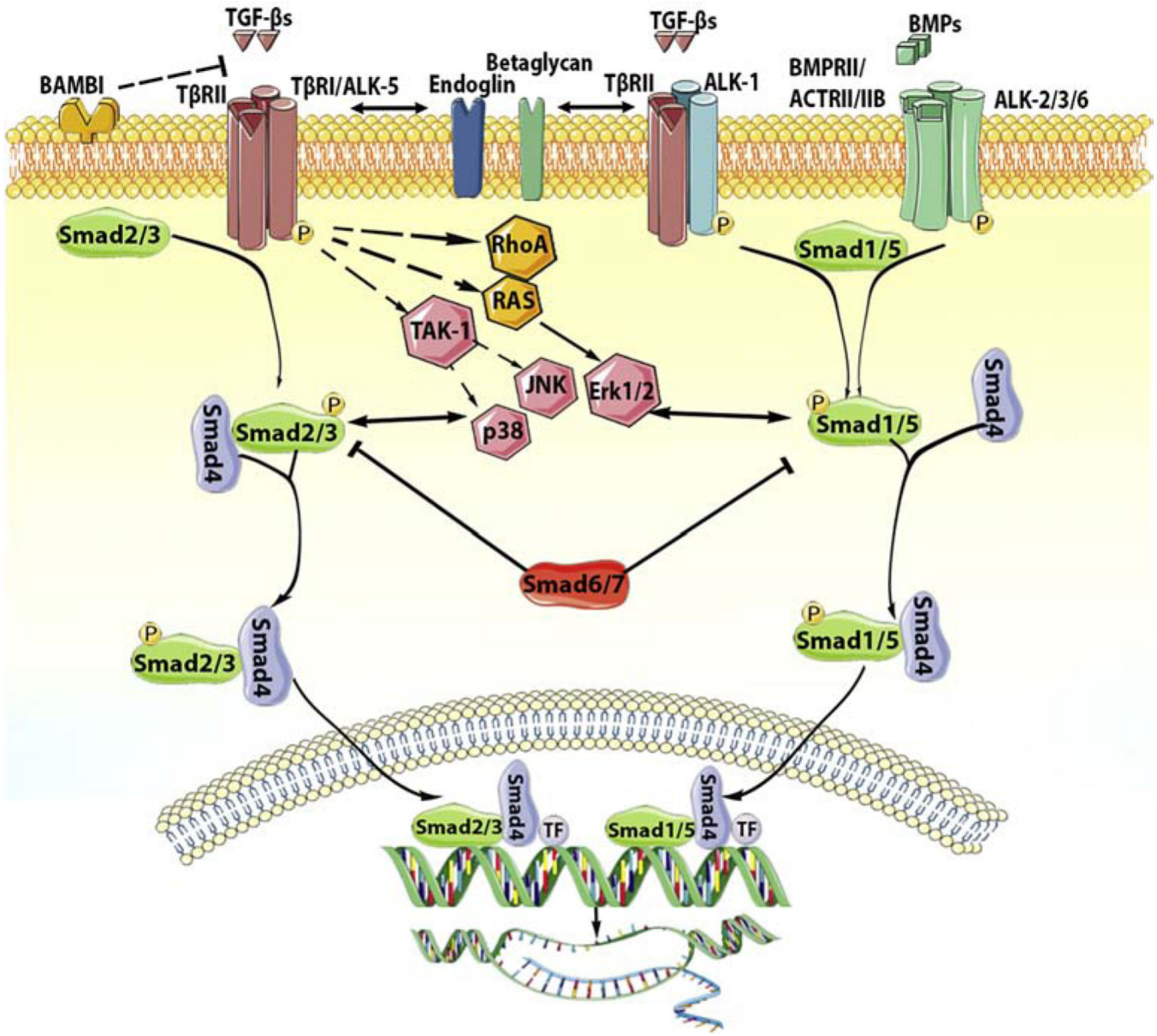

Figure 1: Signaling cascades activated by the TGF-β superfamily.

TGF-β superfamily ligands signal by binding to distinct combinations of two type II, and two type I receptors. TGF-βs bind TβRII inducing transphosphorylation of the type I receptor ALK5. Activated ALK5 phosphorylates the receptor-activated Smads (R-Smads) Smad2 and Smad3, which then form a heterotrimeric complex with the common Smad, Smad4, promoting the translocation of the Smad complex to the nucleus. Interactions between the Smad complex and transcriptional activators or repressors regulate transcription of target genes. In some cell types, TGF-βs may also act through another type I receptor, ALK1, stimulating Smad1/5/8 signaling. BMPs also bind to their specific type II receptors, subsequently activating type I receptors (ALK2, ALK3, ALK6) and phosphorylating Smad1/5. Smad1 and Smad5 then bind to Smad4 and translocate to the nucleus regulating transcription. In addition to the Smad-dependent signaling, TGF-β superfamily members

may also signal through non-canonical Smad-independent cascades. The inhibitory Smads (Smad6 and Smad7) negatively regulate TGF-β superfamily cascades. Accessory receptors, such as endoglin and betaglycan, modulate TGF-β signaling. The cartoon was designed using Servier Medical Art (https://smart.servier.com/).

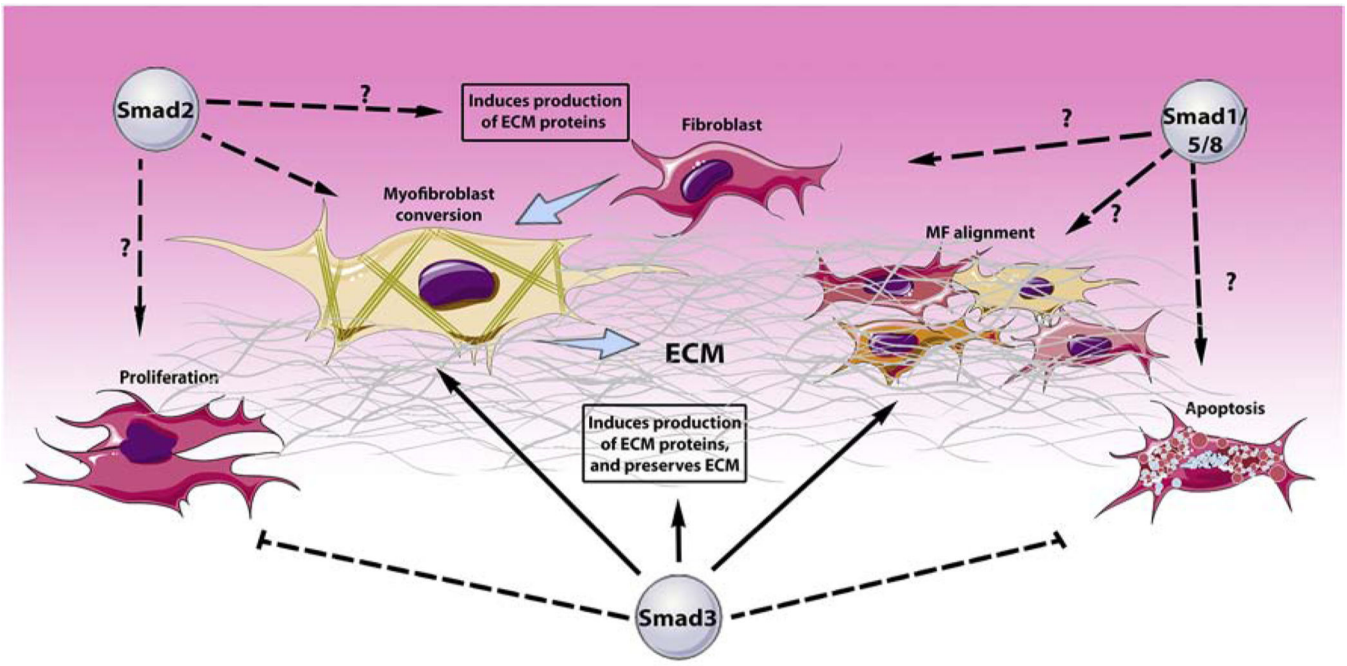

Figure 2: Smad-mediated actions in cardiac fibroblasts.

The Smad proteins are key intracellular effectors of cascades involving TGF-β superfamily signaling. In cardiac fibroblasts, receptor-activated Smads regulate viability, proliferation, migration, transcriptional profile, phenotype and function. Smad3 activates fibroblast synthesis of extracellular matrix (ECM) proteins, while preserving the ECM by reducing protease-mediated matrix degradation. Smad3 also promotes the conversion of cardiac fibroblasts to myofibroblasts, and regulates myofibroblast alignment and formation of an organized scar by regulating integrin expression. Moreover, Smad3 tightly regulates cardiac fibroblast numbers by regulating proliferation and apoptosis. Smad2, has been reported to modulate fibroblast gene expression in vitro; however, its' in vivo effects are more limited. The role of Smad1/5/8 in regulation of cardiac fibroblast phenotype is not known. The cartoon was designed using Servier Medical Art (https://smart.servier.com/).

Table 1:

In vitro effects of Smad signaling cascades in regulation of cardiac fibroblast phenotype

Species	Culture Condition	Smads manipulation	Role of Smad	Reference
Human	Collagen pads	Pharmacologic Smad3 inhibition with SIS3.	Smad3 mediates TGF-β1-induced α-SMA and α11 integrin expression.	[104]
Rat	Culture plates	Pharmacologic Smad3 inhibition with SIS3.	Smad3 mediates TGF-β1-induced LOX expression.	[102]
Rat (neonatal)	Culture plates	Pharmacologic Smad3 inhibition with SIS3.	Smad3 mediates TGFβ1-induced cytoprotective and antiapoptotic effects in fibroblasts exposed to simulated ischemia/reperfusion	[106]
Rat (neonatal)	Culture plates	Pharmacologic Smad3 inhibition with SIS3.	Smad3 promotes cardiac myofibroblast conversion by mediating TGF-β1-induced downregulation of FoxO3a.	[98]
Mouse	Culture plates	Smad2/Smad3 siRNA knockdown.	• Smad3 mediates baseline collagen I, collagen IV, fibronectin and TSP1 synthesis. • Smad2 mediates baseline collagen V, fibronectin, periostin and versican.	[109]
Mouse	Collagen pads/ Culture plates.	Smad2/Smad3 siRNA knockdown.	• Smad3, but not Smad2, mediates α2 and α5 integrin expression. • Both Smad 2 and Smad3 stimulate α-SMA incorporation into stress fibers	[92]
Mouse	Collagen pads	Fibroblasts from global Smad3 null mice	Smad3 mediates TGF-β1-induced transcription of tissue transglutaminase.	[103]
Mouse	Culture plates	Fibroblasts from global Smad3 null mice	Smad3 mediates TGFβ–induced collagen III and tenascin-C synthesis.	[100]
Mouse	Collagen pads	Fibroblasts from global Smad3 null mice	Smad3 mediates TGF-β-induced TIMP1 upregulation and MMP3/MMP8 suppression, promoting a matrix-preserving phenotype.	[93]
Mouse	Collagen pads/ Culture plates	Fibroblasts from global Smad3 null mice.	• Smad3 accentuates contraction of fibroblast-populated lattices. • Smad3 mediates expression of Integrins α2, α5 and β3 in pad fibroblasts. • Smad3 mediates α-SMA synthesis only in plated fibroblasts.	[7]
Mouse	Collagen pads/ Culture plates	Fibroblasts from global Smad3 null mice.	• Smad3 mediates anti-proliferative and promigratory effects of TGF-β. • Smad3 mediates contraction of fibroblast-populated lattices. • Smad3 stimulates CTGF and extracellular matrix gene synthesis.	[94]
Mouse	Culture plates	Fibroblasts from global Smad3 null mice.	Smad3 mediates angiotensin II-induced upregulation of Collagen I, α-SMA, TNF-α, IL-1β, and MCP-1.	[140]
Mouse	Collagen pads/ Culture plates	Cre overexpression in fibroblasts from Smad2, Smad3 and Smad2/3 fl/fl mice to delete loxP-targeted Smads.	• Smad3 (but not Smad2) mediates TGF-β1-induced fibroblast-populated pad contraction. • Both Smad2 and Smad3 stimulate expression of extracellular matrix genes (*Col1a1, Col1a2, Col3a1, Fn1, Fbln1, Tnc*), matrix regulators (*Loxl2, Adam12, Adam30, Sparc*) and cell adhesion genes (*Itga2, Itga8, Itgb3*).	[101]

Col, collagen; CTGF, connective tissue growth factor; Fbln, fibulin; Fn, fibronectin; IL, interleukin; Itg, integrin; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; LOX, lysyl oxidase; SMA, smooth muscle actin; TIMP, Tissue inhibitor of metalloproteinases; Tnc, tenascin; TNF, tumor necrosis factor; TGF, transforming growth factor.

Table 2:

In vivo Effects of Smad Signaling in Cardiac Fibrosis

Model	Intervention	Role of Smad	Proposed Mechanism	Reference
Age-associated changes	Cardiomyocyte- specific Smad4 knockout mouse	Cardiomyocyte Smad4 protected the heart from age-associated hypertrophy and fibrosis.	Smad4-mediated protection of cardiomyocytes from injury, or suppression of fibrogenic signals.	[160]
Non-reperfused MI	Myofibroblast-specific Smad2 or Smad3 loss.	Myofibroblast Smad3 a. Regulates organization of myofibroblast arrays. b. Mediates alignment of structural collagen fibers in the infarct Myofibroblast Smad2 does not play a major role in infarct healing.	Smad3-mediated integrin α2 and α5 activation.	[92]
Reperfused MI	Global Smad3 null mice	Smad3 mediates cardiac collagen deposition in the infarct, the border zone and the remote remodeling.	Smad3-mediated synthesis of extracellular matrix genes and TIMPs.	[100]
Reperfused MI	Global Smad3 null mice	Smad3 mediates deposition in the infarct, promotes myofibroblast conversion, but reduces myofibroblast proliferation.	Smad3-mediated anti-proliferative actions, α-SMA expression, induction of ECM proteins and CTGF.	[94]
Reperfused and non-reperfused MI	Myofibroblast-specific Smad3 loss	Myofibroblast Smad3: a. Prevents late cardiac rupture. b. Mediates formation and organization of well-aligned arrays of myofibroblasts.	Smad3-mediated activation of α5 integrin-NOX2 axis.	[7]
Pressure overload (TAC)	Myofibroblast-specific Smad3 KO mice	Myofibroblast Smad3 preserves the extracellular matrix and attenuates collagen degradation	Smad3-mediated inhibition of collagenase (MMP3/8)-driven matrix degradation,	[93]
Pressure overload (TAC) Overexpression of a cardiomyocyte-specific, latency-resistant TGF-β mutant transgene.	Conditional Fibroblast and Myofibroblast-specific TβR1, TβR2, Smad2, Smad3 and Smad2/3 null mice.	Fibroblast Smad3, but not Smad2, mediates the fibrotic response in the pressure-overloaded heart and in a genetic model of cardiac TGF-β overactivation.	Smad3-mediated transcription of matrix genes.	[101]
Pressure overload (TAC).	Global Smad3 null mice	Smad3 mediates myocardial fibrosis	Smad3-mediated collagen synthesis.	[141]
Pressure overload	ALK5 inhibition using a small molecule inhibitor (SM16)	ALK5 mediates fibrosis and diastolic dysfunction.	Smad2/3-mediated expression of pro-fibrotic genes.	[142]
Angiotensin II infusion	Global Smad3 null mice	Smad 3 mediates angiotensin II-induced cardiac fibrosis.	Smad 3-mediated induction of TGFβ1, CTGF, collagen I/III and α-SMA.	[140]
db/db mouse	Obese diabetic db/db mice with partial or complete Smad3 loss.	Smad3 promotes fibrosis in obese diabetic mice.	Smad3-mediated suppression of MMP activity.	[133]
T. Cruzi infection	Pharmacological ALK5 inhibition with GW788388.	ALK5 mediates fibrosis.	Smad2/3-mediated matrix synthesis.	[165]

TAC, transverse aortic constriction; CTGF, connective tissue growth factor; ECM, extracellular matrix; MI, myocardial infarction; MMP, matrix metalloproteinase; NOX, NADPH Oxidase; SMA, smooth muscle actin; TIMP, Tissue inhibitor of metalloproteinases.