NOVEL THERAPEUTIC TARGETS AND EMERGING TREATMENTS FOR FIBROSIS

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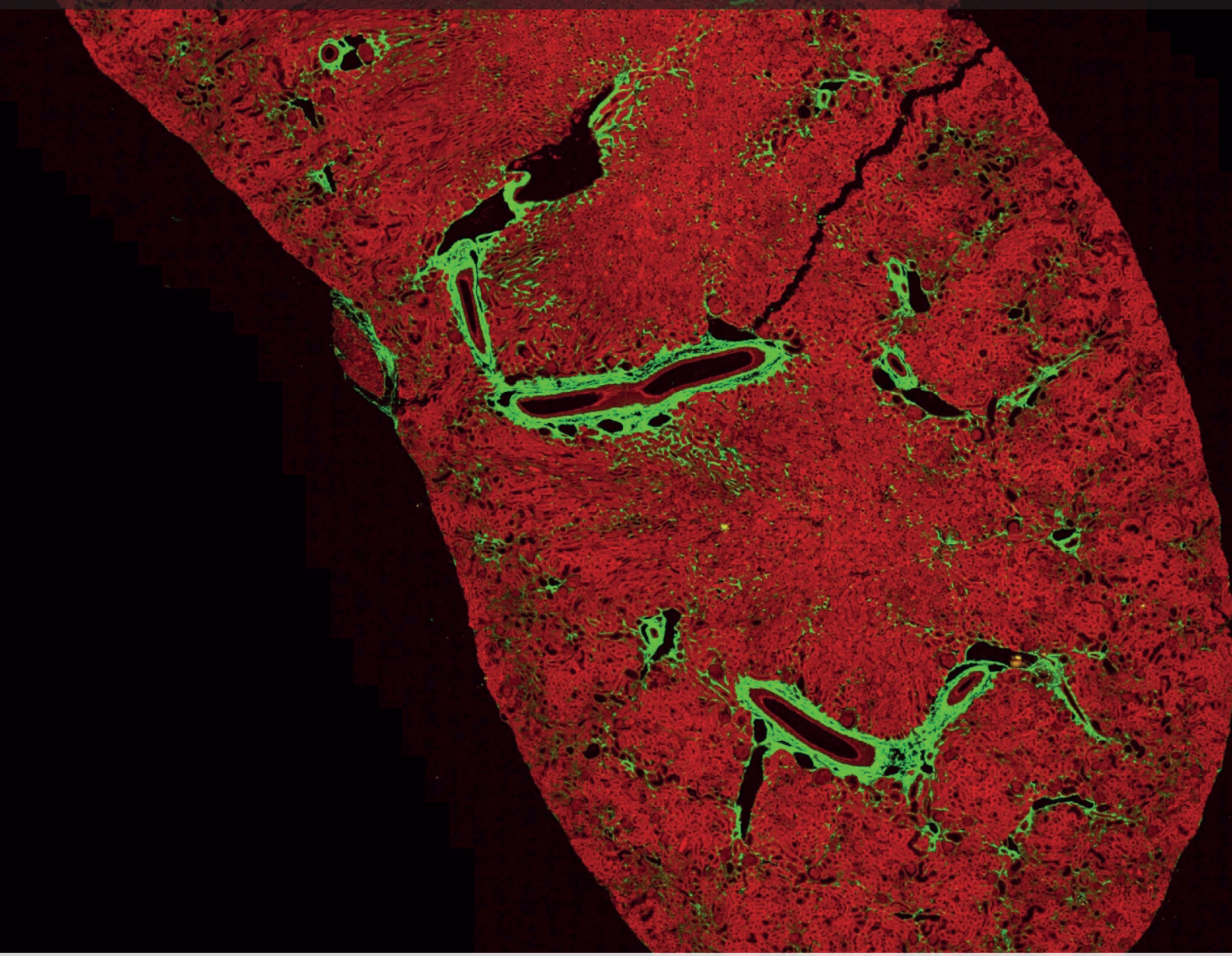

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NOVEL THERAPEUTIC TARGETS AND EMERGING TREATMENTS FOR FIBROSIS

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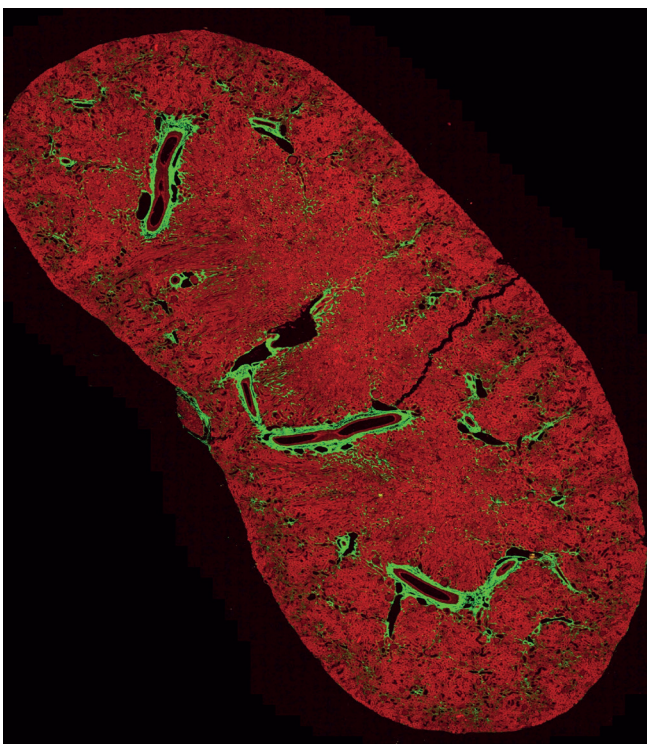

Stain-free multiphoton imaging (Histoindex Genesis 200) of a kidney section from a uni-nephrectomised mouse with deoxycorticosterone acetate (DOCA)/salt-induced hypertension. Green shows the presence of vascular, peri-vascular and interstitial fibrillary collagens that constitute fibrosis.

Image by Chrishan S. Samuel.

For decades we have known that the overgrowth, hardening and scarring of tissues (so-called fibrosis) represents the final common pathway and best histological predictor of disease progression in most organs. Fibrosis is the culmination of both excess extracellular matrix deposition due to ongoing or severe injury, and a failure to regenerate. An inadequate wound repair process ultimately results in organ failure through a loss of function, and is therefore a major cause of morbidity and mortality in disease affecting both multiple and individual organs.

Whilst the pathology of fibrosis and its significance are well understood, until recently we have known little about its molecular regulation. Current therapies are often indirect and non-specific, and only slow progression by a matter of months. The recent identification of novel therapeutic targets, and the development of new treatment strategies based on them, offers the exciting prospect of more efficacious therapies to treat this debilitating disorder.

This Research Topic therefore compromises several up-to-date mini-reviews on currently known and emerging therapeutic targets for fibrosis including: the Transforming Growth Factor (TGF)-family; epigenetic factors; Angiotensin II type 2 (AT_2) receptors; mineralocorticoid receptors; adenosine receptors; caveolins; and the sphingosine kinase/sphingosine 1-phosphate and notch signaling pathways. In each case, mechanistic insights into how each of these factors contribute to regulating fibrosis progression are described, along with how they can be targeted (by existing drugs, small molecules or other mimetics) to prevent and/or reverse fibrosis and its contribution to tissue dysfunction and failure. Two additional reviews will discuss various anti-fibrotic therapies that have demonstrated efficacy at the experimental level, but are not yet clinically approved; and the therapeutic potential vs limitations of stem cell-based therapies for reducing fibrosis while facilitating tissue repair. Finally, this Research Topic concludes with a clinical perspective of various anti-fibrotic therapies for cardiovascular disease (CVD), outlining limitations of currently used therapies, the pipeline of anti-fibrotics for CVD and why so many anti-fibrotic drugs have failed at the clinical level.

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EDITORIAL
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Editorial: Novel Therapeutic Targets and Emerging Treatments for Fibrosis

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Keywords: fibrosis, fibrogenesis, collagen, pharmacology, therapeutics

Editorial on the Research Topic

Novel Therapeutic Targets and Emerging Treatments for Fibrosis

During development and wound healing, physiological fibrogenesis maintains connective tissue integrity and structure through synthesis of extracellular matrix (ECM). However, although many organs have a capacity to repair and regenerate after acute or mild injury, severe or ongoing stress results in fibrosis, which refers to the overgrowth, hardening, and scarring of tissues and ultimately causes a progressive loss of organ function (Wynn, 2007). Fibrosis results from an excess accumulation of scleroproteins, mainly collagen, which replaces parenchyma and stiffens tissues. This is in turn a consequence of both pathological fibrogenesis and the physical properties of scleroproteins, namely their aqueous insolubility, tendency to aggregate, and resistance to proteolytic digestion. Unfortunately, current therapeutic strategies for fibrosis are often indirect and non-specific, and only slow progression by a matter of months.

Whilst the pathology of fibrosis and its functional significance are well-understood, until recently we have known little about its molecular regulation. These mechanisms and their subsequent therapeutic targeting is the subject of this Research Topic. This themed issue therefore comprises several up-to-date mini-reviews and original studies on currently known and emerging therapeutic targets for preventing fibrosis and enhancing repair. In each case, mechanistic insights into how each of these factors contributes to regulating fibrosis progression is described, along with how they can be targeted by existing drugs, new mimetics, and cell-based therapies.

PROGRESSION

While fibrosis represents a final common pathway to chronic and severe injury, we also know that the trajectory to organ failure can be highly variable (Li et al., 2012). The first article in this Research Topic therefore discusses the role of intrinsic and extrinsic risk factors in determining both susceptibility to injury and rate of progression, using chronic kidney disease as an example (Hewitson et al.). The role of epigenetic mechanisms in this process is emphasized here and in a study of histone marks in experimental kidney fibrosis (Hewitson et al.)

HYPOXIA AND ISCHEMIA

Local tissue hypoxia is a feature of fibrotic disorders in many organs although it is often unclear whether hypoxia is a consequence or cause of fibrosis (Darby and Hewitson, 2016). Destruction of capillaries through accumulation of ECM results in a loss of oxygen delivery, local tissue hypoxia and upregulated hypoxia-inducible factor (HIF)-1α signaling. These aberrant processes in turn further upregulate ECM production in a vicious cycle. Xiong and Liu address the relationship between vascularization and

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fibrosis in disease, focusing on targeting HIF-1α. Related to this Nistri et al. explore the significance of Notch1 signaling in cardiac ischemia.

LIGAND-RECEPTOR RELATIONSHIPS AND MOLECULAR MECHANISMS

Central to our understanding of fibrosis has been the recognition that transforming growth factor beta (TGF-β1) is key to both the fibrogenesis and the perpetual activation of collagen-producing cells that occur in chronic conditions (Meng et al., 2016; Yue et al., 2017). Much of the focus in this Research Topic is therefore on the role of TGF-β1 signal transduction in fibrogenesis. Accordingly, Walton et al. discuss the contribution of TGF-β1 ligands to the pathogenesis of fibrosis. The significance of local TGF-β1 storage (Walton et al.) and its autocrine actions (Hewitson et al.) are likewise discussed. Caveolin has emerged as a crucial regulator of TGF-β1 signal transduction through TGF-β1 receptor internalization and degradation. This specific role and the more general significance of Calveolin-1 is reviewed in detail by Shihata et al.

For many years we have also realized that various members of the circulating and local renin-angiotensin-aldosterone system are directly and indirectly pro-fibrotic. Foremost are the fibrogenic actions of angiotensin II (Ang II) and aldosterone via binding to the angiotensin II type1 receptor (AT_1R) and mineralocorticoid receptor (MR), respectively. Indeed, in many organs, Ang II and aldosterone inhibition and blockade are currently the best available anti-fibrotic therapies (Fang et al.). However, we now also realize that these ligand receptor families are more complex than first thought. While activation of AT_1R by Ang II mediates vasconstriction, inflammation, and fibrogenesis, activation of the Ang II type 2 receptor (AT_2R) has counter-regulatory vasodilatory, anti-inflammatory, and anti-proliferative effects. Wang et al. therefore examine the significance of the AT_2R in fibrosis.

Cardiovascular and renal diseases are also associated with dysregulation of MR signaling, which has direct fibrotic effects, only some of which are related to aldosterone. Consistent with this Tesch and Young provide an overview of MR activation and signaling.

The bioactive lipid sphingosine 1-phosphate (S1p) intriguingly acts as both an intracellular signal transduction mediator, and when exported, a ligand for G protein-coupled receptors. In their mini-review Vestri et al. discuss the biology of S1p, its relevance to fibrogenesis, and effects of its modulation, including off target actions.

The pleiotropic properties of adenosine are also directly relevant to fibrosis. Again, however, ligand-receptor interactions are complex and context specific with pro-fibrotic and anti-fibrotic actions possible. Vecchio et al. explore the current understanding of the role of the most abundant adenosine receptor, $A_{2B}AR$, in cardiac fibrosis.

THERAPEUTIC STRATEGIES

In many cases authors go on to discuss possible therapeutic approaches based on the above mechanisms. In their review of novel anti-fibrotic therapies, McVicker and Bennett provide several examples including microRNA, peroxisome proliferator-activated receptors, and strategies that directly target TGF-β1 signaling, including the endogenous inhibitors relaxin and bone morphogenic protein-7. The last of these is also discussed by Walton et al. Activation of the Notch1 pathway limits the extent of ischemic damage, promotes coronary angiogenesis and revascularization of the ischemic myocardium (Nistri et al.). In their mini review Wang et al. focus on pre-clinical testing of Compound 21, the most widely studied AT_2R agonist in chronic disease, while Tesch and Young explore the therapeutic potential of MR receptor antagonism. Specific application of the S1p receptor agonist Fingolimod is examined by Ahmed et al.

However, despite the significance of fibrosis, restoration of organ function is more complex than simply removing or preventing fibrosis. Amelioration of fibrosis needs to be accompanied by tissue repair. Consistent with this stem cell therapies offer an exciting opportunity to enhance tissue repair in chronic organ disease (Lim et al.). The specific application of stem cell-derived exosomes is also demonstrated here experimentally in a rodent model of hepatic fibrosis (Alhomrani et al.).

Finally, this Research Topic provides a clinical perspective of various anti-fibrotic therapies for cardiovascular disease (CVD), outlining limitations of currently used therapies, the pipeline of anti-fibrotics for CVD, and why so many anti-fibrotic drugs have failed at the clinical level (Fang et al.).

CONCLUSIONS

In conclusion, the recent identification of novel therapeutic targets, and the development of new treatment strategies based on them, offers the exciting prospect of more efficacious therapies to treat fibrosis. We sincerely thank the authors for their contributions and hope that each article in this Research Topic will both inform and generate further interest in this debilitating disorder.

AUTHOR CONTRIBUTIONS

CS and TH wrote the Editorial and gave final approval for publication.

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MINI REVIEW
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Progression of Tubulointerstitial Fibrosis and the Chronic Kidney Disease Phenotype – Role of Risk Factors and Epigenetics

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Although the kidney has capacity to repair after mild injury, ongoing or severe damage results in scarring (fibrosis) and an associated progressive loss of kidney function. However, despite its universal significance, evidence highlights a population based heterogeneity in the trajectory of chronic kidney disease (CKD) in these patients. To explain the heterogeneity of the CKD phenotype requires an understanding of the relevant risk factors for fibrosis. These factors include both the extrinsic nature of injury, and intrinsic factors such as age, gender, genetics, and perpetual activation of fibroblasts through priming. In many cases an additional level of regulation is provided by epigenetic mechanisms which integrate the various pro-fibrotic and anti-fibrotic triggers in fibrogenesis. In this review we therefore examine the various molecular and structural changes of fibrosis, and how they are influenced by extrinsic and intrinsic factors. Our aim is to provide a unifying hypothesis to help explain the transition from acute to CKD.

Keywords: kidney disease, fibrosis, fibrogenesis, epigenetics, autocrine, TGF-β1

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THE PATHOLOGY OF ACUTE KIDNEY DISEASE

When a mild or an acute kidney injury (AKI) occurs, tissue repair mechanisms are usually able to restore function. This wound repair consists of consecutive but overlapping events; inflammation, extracellular matrix (ECM) synthesis (fibrogenesis), resolution, regeneration, and remodeling (Hewitson, 2009). While there are subtle differences in pathogenesis, in general, injury results in an acute neutrophil and monocyte infiltration, which is over time replaced by macrophages. Re-epithelialisation is predicated upon resolution of inflammation, which includes amongst other signals a switch from a pro-inflammatory macrophage phenotype (M1) to a pro-repair one (M2). In each nephron segment the regenerative capacity depends on distinct epithelial lineages, and requires stabilizing scaffolds to guide reconstitution (Suarez-Alvarez et al., 2016). An important point therefore is that the underpinnings of fibrosis begin as a necessary and well-organized attempt to stabilize tissue through maintaining basement membranes and structural integrity for repair and regeneration.

EXAGGERATED FIBROSIS ACCOMPANIES CHRONIC KIDNEY DISEASE

If repair mechanisms are disrupted, or the injury-causing stimulus persists, AKI can progress into a chronic disorder, characterized by non-recoverable organ remodeling and scarring (fibrosis)

(Hewitson, 2009). Histologically this process presents itself as glomerulosclerosis, vascular sclerosis and tubulointerstitial fibrosis, with the last being the best predictor of deteriorating renal function, regardless of etiology (Hewitson, 2009). This transition has led many to conclude that there is a 'point of no return,' a stage from which recovery and repair is no longer possible (Ito et al., 2004). Fibrosis exacerbates progression through capillary rarefaction and subsequent tissue hypoxia (Darby and Hewitson, 2016) while hypoxia itself in turn directly stimulates further fibrogenesis (Nangaku, 2006). Renal parenchymal fibrosis is therefore a failure of repair, and is a final common pathway in all progressive renal disease.

CELLULAR BASIS OF TUBULOINTERSTITIAL FIBROSIS

In this review we focus on the pathogenesis of tubulointerstitial fibrosis due to its universal significance, and resemblance to fibrosis in general.

Experimental models and patient renal biopsy studies of tubulointerstitial fibrosis have repeatedly shown that activation of tubule epithelial cells and interstitial fibroblasts is responsible for excess ECM production, in particular collagen, which constitutes scar tissue. Injured epithelial cells synthesize collagen, which manifests itself as both basement membrane thickening (collagen IV) and interstitial fibrosis (collagens IV, I). Damaged tubules also release cytokines and pro-fibrotic signals that activate adjacent fibroblasts (Frank et al., 1993). The other key event in this process is an exponential increase in the number of fibroblasts after injury (Hewitson et al., 1995). These cells originate from not only resident fibroblasts, but also from renal tubules through epithelial mesenchymal transition, pericytes, circulating progenitors (reviewed in Hewitson, 2009), and even macrophages (Wang et al., 2017). The "fibroblasts" that accumulate during kidney disease are therefore a heterogeneous population of cells, which have been difficult to characterize cyto-chemically. Although a number of putative fibroblast markers have been identified, most investigators have associated *de novo* expression of alpha-smooth muscle actin (αSMA) with an activated phenotype. This so-called myofibroblast is characteristically hyperproliferative, contractile and fibrogenic. Similar phenotypic transitions occur in tubular epithelial cells (Darby and Hewitson, 2007) and glomerular mesangial cells (Johnson et al., 1991), highlighting the universal applicability of this process.

FIBROGENESIS IS CYTOKINE DRIVEN

At the molecular level, fibrogenenic cell activation is a predominantly cytokine driven process. Signals can be specific to the injury or derived from the uremic milieu systemically. Regardless, in each case the AP1 transcription factor c-Jun seems to be a central molecular mediator of fibroblast activity in multiple organs (Wernig et al., 2017).

Since the landmark studies of Border and colleagues demonstrating a role for transforming growth factor beta 1 (TGF-β1) in glomerulosclerosis (Border and Ruoslahti, 1990), a multiplicity of evidence has implicated TGF-β1 as the pre-eminent fibrogenic cytokine. This has been supported by demonstrating both direct fibrogenic action and benefits from targeting TGF-β1 pathways pharmacologically (reviewed in Meng et al., 2016). Despite the established significance of TGF-β1 in fibrogenesis, controversies continue to exist. Translation to clinically useful therapies based on targeting this molecule have been uniformly disappointing (Voelker et al., 2017), thought to be due to the other pleotropic properties of TGF-β1 (Meng et al., 2016). Ablation of the TGF-β receptor ameliorates fibrosis in some studies (LeBleu et al., 2013), but not others (Neelisetty et al., 2015). While these discordant, and sometimes unexpected results, need to be viewed in context of the overall evidence, they have led us to reappraise the significance of TGF-β1 and how it acts. Foremost is the recognition that TGF-β1 is an autocrine factor acting on the resident and infiltrating inflammatory cells that produce it. TGF-β1 is secreted in a latent form in complex with latency associated peptide (LAP). LAP is itself disulfide linked to a further protein, latent TGF-beta binding protein (LTBP), which targets latent TGF-β1 to the matrix after secretion. A number of pathological features including proteases, oxidative stress, integrins (Annes et al., 2003) and changes in ionic strength (Lawrence et al., 1985) can release active TGF-β1 through cleavage and conformational changes in bonding. Paracrine actions are therefore severely limited by formation of the latent TGF-β1 complex immediately adjacent to each cell, and which cannot readily traverse the basement membrane (Venkatachalam and Weinberg, 2015) (**Figure 1**).

Additionally, other cytokines and growth factors, such as platelet derived growth factor (PDGF) (Johnson et al., 1998; Buhl et al., 2016) and angiotensin II (Ruster and Wolf, 2006), are also pro-fibrotic in a paracrine fashion (**Figure 1**). Recent findings also suggest that the paracrine fibroblast growth factor 23 (FGF23) signaling may be relevant to fibrogenesis. FGF23 was originally described as a bone-derived member of the endocrine FGF family known to regulate mineral handling. Circulating levels of this hormone rise early in chronic kidney disease (CKD) and are predictive of disease progression. Whilst the factors driving this increase from bone are partially understood, *de novo* expression in the kidney has also been noted following injury, leading to speculation about its functional significance in this context (Smith, 2014). Trying to isolate a fibrogenic role for FGF23 has been problematic due to the systemic changes in its synthesis and other mineral factors that occur in CKD. In this regard unilateral ureteric obstruction (UUO) has been particularly useful, as fibrogenesis in this model does not involve changes in bone-mineral parameters, and the presence of an intact contralateral kidney means that the animals are also not uremic. Using this model we have recently identified temporal and spatial increases in distal tubular FGF23 expression in early renal fibrosis, and demonstrated that FGF23 increases myofibroblast differentiation and fibrogenesis in a dose related fashion *in vitro* (Smith et al., 2017).

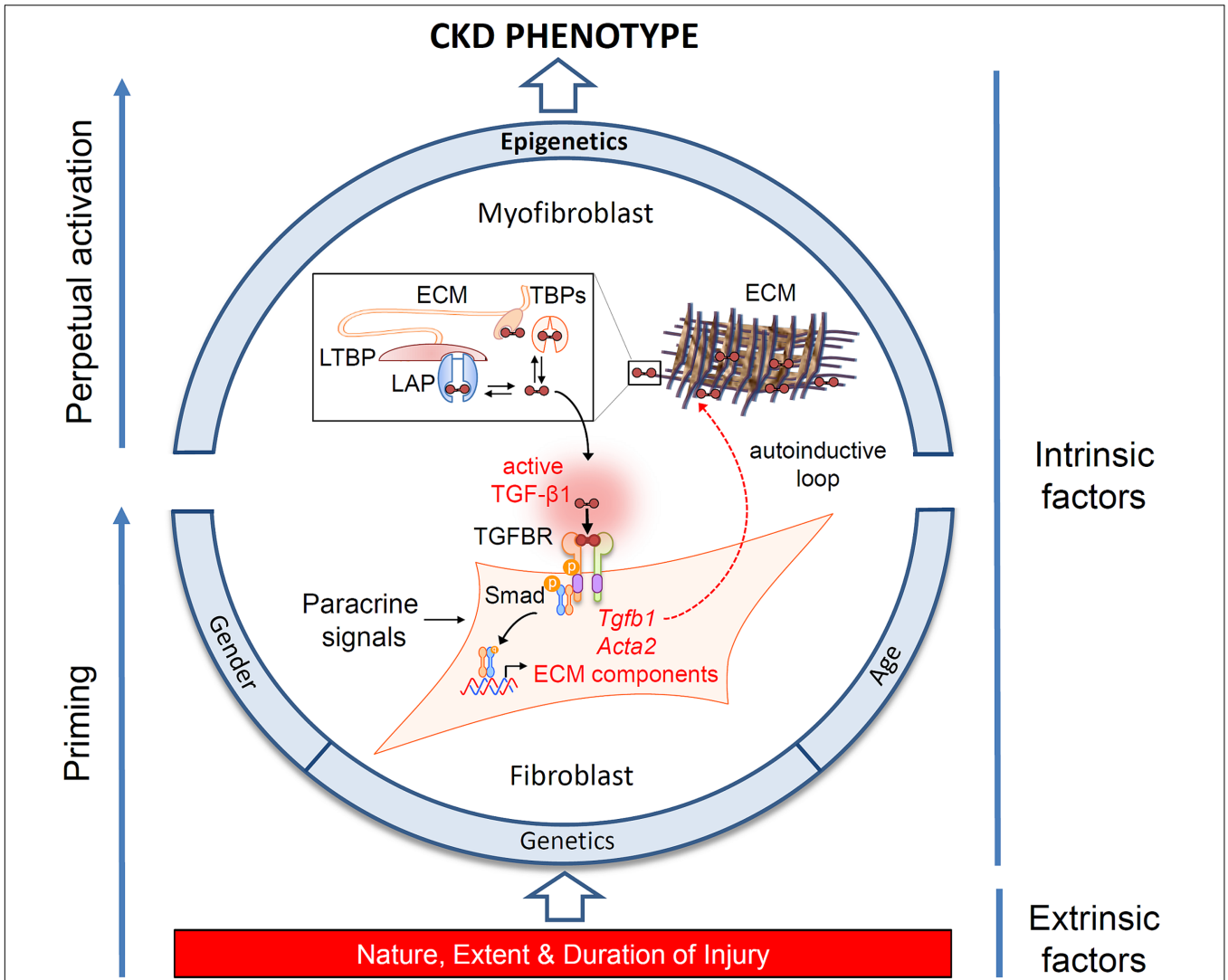

FIGURE 1 | Determinants of the chronic kidney disease (CKD) phenotype. Schematic representation of the interplay of extrinsic and intrinsic risk factors in the progression of CKD, and their effects on myofibroblast differentiation. Fibroblast recruitment and activation is under control of paracrine and autocrine signals released in response to injury. The schema highlights the significance of an autocrine amplification of transforming growth factor beta1 (TGF-β1) signaling in injury-primed cells and their perpetual activation through epigenetic mechanisms. TGF-β1 is secreted as an inactive dimeric complex with latency associated peptide (LAP), bound in turn to latent TGF-β binding protein (LTBP). These complexes are subsequently incorporated into the extracellular matrix (ECM) and must be cleaved to release active TGF-β1. TGF-β binding proteins (TBPs) abundant in the extracellular fluid (e.g., decorin, betaglycan, fucoidan, heparin) also sequester TGF-β1, limiting activation at remote sites. Structurally, diffusion of TGF-β1 is also restricted by tubule basement membranes (not shown). Activation is therefore constrained to the fibroblast cell surface, where multi-step proteolysis releases TGF-β1 and allows binding to its cognate receptor complex (TGFBR) in an exclusively autocrine fashion. Canonical signaling via Smad phosphorylation drives the expression of intracellular α-smooth muscle actin expression (*Acta2*), ECM synthesis and further TGF-β1 gene expression generating an autoinductive feed-forward loop.

FACTORS AFFECTING THE PROGRESSION OF FIBROSIS

The importance of several modifiable and unmodifiable risk factors (**Figure 1**) is borne out in epidemiological studies which show that the likelihood of progression in humans can be modeled by variables including age, gender and baseline renal function (Tangri et al., 2016). Fortunately our understanding of the cellular and molecular role of these factors has been aided by the fact that the final endpoint of fibrosis is remarkably similar in different species and etiologies (**Figure 2**), meaning that we have robust and reproducible experimental models to investigate specific risks for progression of fibrosis.

Nature of Injury

The causes of kidney disease are diverse and include immunological, genetic, infectious, metabolic, physical and hemodynamic stresses amongst others. In each case the degree of fibrosis is determined in part by the extent of the damage, and the duration of injury, with severity and frequency of tubule

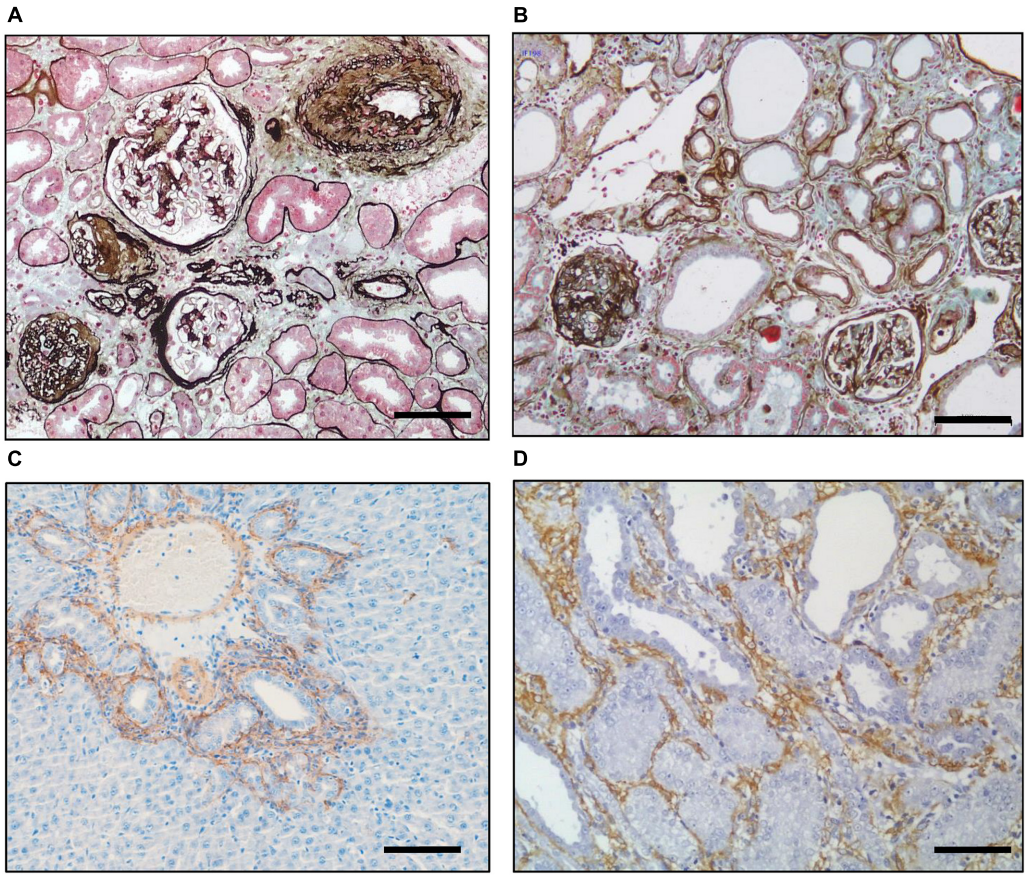

FIGURE 2 | Inherent similarities between fibrosis in different species and organs. Silver–Masson trichrome staining of a diabetic **(A)** human and **(B)** rat kidney showing the underlying similarity of fibrosis in the two organs. Immunohistochemical staining for αSMA (brown) showing myofibroblast recruitment in the rat after **(C)** bile duct ligation in the liver and **(D)** unilateral ureteric ligation in the kidney. Scale bar = 100 μm. [Derived with modification from Hewitson (2012)].

injury being shown to determine prognosis (Takaori et al., 2016). Consistent with this, severity of AKI predicts progression to CKD (Ishani et al., 2009). The loss of basement membrane in severe injury is a major impediment to repair as it represents a loss of a scaffold for regeneration. The failure of injured epithelium to regenerate and repair results in glomerulosclerosis and tubular atrophy (Hewitson, 2009). We also know that the loss of regenerative capacity in tubular epithelial cells after injury corresponds to arrest in the G2/M phase of the cell cycle (Yang et al., 2010). Prolonged G2/M arrest triggers a pro-fibrotic phenotype in cultured tubule epithelial cells with a corresponding increase in TGF-β1, connective tissue growth factor (CTGF) and collagen IV mRNA transcripts (Yang et al., 2010).

Perpetual Activation of Fibroblasts

It has long been recognized that fibroblasts cultured from human kidneys with interstitial fibrosis grow at a faster rate and produce more collagen than those derived from normal kidneys, and that these changes are passed on from passage to passage (Rodemann and Muller, 1990). A key feature in parenchymal scarring therefore lies in the perpetual activation of myofibroblasts. This has parallels in the skin where extracellular signal-regulated kinase (ERK) is found to be constitutively activated in dermal fibroblasts isolated from patients with scleroderma (Asano et al., 2005). In this case an upregulated expression of $\alpha_v\beta3$ integrin in fibroblasts from scleroderma compared to normal dermal fibroblasts provides an autocrine loop through its actions as a receptor for adjacent latent TGF-β1 (Asano et al., 2005). Our observations in rat renal fibroblasts are also consistent with a priming of cells during injury, making them more susceptible to fibrogenic signals, a difference that is maintained across generations in culture. In our recent study, we found that while FGF23 enhances TGF-β1 signaling in fibroblasts from kidneys with UUO, it failed to activate fibrogenic pathways in those derived from normal kidneys (Smith et al., 2017). Further analysis revealed greater FGF and TGF receptor density on fibrotic fibroblasts compared to their normal counterparts, and a feed forward induction of TGF-β1 expression and activity by FGF23 (Smith et al., 2017).

Age

A progressive decline in renal function is common with aging (Weir, 1997), albeit with wide variability. Both hemodynamic

and structural changes occur (Zhou et al., 2008) and aging rats can be shown to have impaired redox homeostasis (Aydin et al., 2012) and angiogenesis (Kang et al., 2001). Replicative senescence through telomere shortening is seen (Zhou et al., 2008), although the phenotype of telomere deficient mice confirm that it is not the only relevant factor (Schildhorn et al., 2015). Taken together these changes predispose older kidneys to new acute organ injury (Liu et al., 2017), as well as exacerbating progression of CKD. Likewise, there is evidence to suggest that the kidneys of elderly people are more sensitive to primary and secondary renal disease (Razzaque, 2007; Ishani et al., 2009).

Genetics

Approximately 25% of incident dialysis patients have close relatives with CKD (Freedman et al., 2005), and the distinct susceptibilities of different rodent strains to experimental CKD strongly suggests that genetic variations impact renal fibrogenesis (Kokeny et al., 2009). Likewise, familial clustering and disparities in prevalence of CKD across race suggest a strong genetic component to progression (Uwaezuoke et al., 2016). However, identifying relevant polymorphisms in human kidney disease has been somewhat disappointing to date (Tampe and Zeisberg, 2014). Even though there are strong associations between single nucleotide polymorphisms and incident CKD, the association with end stage kidney disease, and therefore progression, is poor (Boger et al., 2011).

Gender

Many studies have also shown a gender basis to progression of senescence and CKD, with epidemiological studies showing that females have a lower prevalence and slower rate of progression than males (Yu et al., 2010). Consistent with this, bioactive estrogen metabolites both prevent renal collagen synthesis *in vitro* (Lei et al., 1998) and reduce glomerulosclerosis and interstitial fibrosis *in vivo* (Maric et al., 2004). Nevertheless other investigations suggest that the male predominance is due to detrimental effects of testosterone (Baylis, 1994; Hewitson et al., 2016), rather than the protective effects of estrogen.

EPIGENETIC REGULATION OF FIBROSIS AND PROGRESSION

While risk estimates based on the above factors allow accurate discrimination of those who are likely to progress over a 5-year period (Tangri et al., 2016), they do not explain the marked heterogeneity in the trajectory to renal failure frequently encountered (Li et al., 2012; O'Hare et al., 2012). Moreover, it is uncertain whether these factors capture risk over longer time horizons and developments in therapeutic options targeting these factors have been limited. This has led to the search for additional pathways that might modulate a person's risk. A major shift in our understanding of fibrosis has been the recognition that epigenetic mechanisms operate at an additional level to integrate the various intrinsic and extrinsic pro-fibrotic triggers and fibrogenesis (**Figure 1**).

Epigenetics refers to stable changes in gene activity that are heritable in cell division, but do not involve changes in the DNA sequence (Reddy and Natarajan, 2015). Epigenetic modifications and consequential changes in protein expression have been described in diverse forms of renal disease including renal cancer, transplantation, autoimmune disease, and diabetes (reviewed in Chmielewski et al., 2011). Under normal conditions epigenetic modifications are balanced and reversible, but this may be disrupted in disease.

Often referred to as a second genetic code, epigenetic regulation of renal gene expression involves multiple mechanisms including post-replicative DNA methylation (Bechtel et al., 2010), RNA interference (McClelland et al., 2015; Gomez et al., 2016) and post-translational histone modifications (Wing et al., 2013).

Epigenetic regulation can occur via direct modification of genomic DNA. In this case DNA is methylated by the attachment of a methyl group to the 5′ position of cytosine residues in specific regions of DNA where cytosine and guanosine are separated by a single phosphate group (CpG sites). CpG methylation generally leads to transcriptional repression of genes (Mann and Mann, 2013). Interestingly, Bechtel et al. (2010) have identified several DNA methylations unique to fibroblasts derived from fibrotic kidneys. Epigenetic silencing of RASL1, a suppressor of the proto-oncogene Ras, results in persistent activation of fibroblasts. Importantly their time course studies also suggested a physiological reversible activation with short-term exposure to TGF-β1, and an irreversible methylation of the RASL1 promoter with long-term TGF-β1 exposure, providing a mechanistic rationale for priming.

Epigenetic silencing also occurs via non-coding micro RNAs (miR) which are small (19–25 nucleotides) RNA molecules that cleave or translationally repress targeted mRNA, and are well-described in kidney disease (Gomez et al., 2016) and transplantation (Jelencsics and Oberbauer, 2015). Various miR have a specific role in regulating ECM production, with both anti-fibrotic and pro-fibrotic actions through aberrant TGF-β1 signaling (Meng et al., 2016). Specific examples include pro-fibrotic miR-21 (Denby et al., 2011; Kolling et al., 2017) and miRNA-130 (Bai et al., 2016), and anti-fibrotic miR-29 (Liu et al., 2010). Actions may also be pleiotropic and context specific (Jelencsics and Oberbauer, 2015) with miR-192 being both anti-fibrotic and pro-fibrotic depending on local circumstances (Chung et al., 2010). miR-132 is of particular interest as it has recently been shown to counteract progression of renal fibrosis *in vivo* by selectively inhibiting myofibroblast proliferation (Bijkerk et al., 2016).

Recent attention has focused on the significance of post-translational histone modifications (marks) in the kidney. Nucleosomes consist of chromosomal DNA wrapped around core histone subunits (H2A, H2B, H3 and H4). These histones also have a flexible N-terminus tail of amino acids that extends from the nucleosome and can be post-translationally modified by phosphorylation, sumoylation, ubiquitination, acetylation (Ac), and methylation (Me). Modifications to the amino acid Lysine (K) on H3 and H4 histone tails have proven to be particularly relevant in fibrogenesis. Acetyl groups are added by

the actions of various acetyl transferases (HAT) and removed by histone deacetylases (HDAC), with net enrichment a balance between the activities of these enzymes. Likewise, methylation is under the control of various methyl transferases (MHT) and their corresponding demethylases (HDM). Unlike acetylation however, more than one methyl group can be added meaning that histones can be mono- di- or tri- methylated. These histone modifications are docking sites for various proteins including co-activators, co-repressors, chromatin remodeling proteins. Their interaction with specific gene promoters form a pattern that regulates transcription (Allis and Jenuwein, 2016). Enrichment of H3KAc is often, but not exclusively, associated with relaxed chromatin and active gene expression, while H3K methylation can either activate or repress transcription depending on the Lysine involved, and the degree of methylation (Allis and Jenuwein, 2016).

Global histone methylation and acetylation are consistent features in fibrosis. TGF-β1 induces time dependent increases in methylation of K9 on H3 in renal fibroblasts *in vivo* (Sun et al., 2010). Histone marks regulate fibroblast differentiation in a variety of fibrotic pathologies (Guo et al., 2009; Mann et al., 2010; Zhou et al., 2010; Cho et al., 2012; Perugorria et al., 2012) including the kidney (Irifuku et al., 2016). More specifically, TGF-β1 exposure can both enrich active histone marks, and decrease repressive marks, at various pro-fibrotic gene promoters in renal mesangial cells (Sun et al., 2010; Yuan et al., 2013), epithelial cells (Sasaki et al., 2016), and fibroblasts (Irifuku et al., 2016; Sasaki et al., 2016). Histone phosphorylation at H3Ser10 seems to contribute to the G2/M arrest in tubule epithelia, although its target gene remains unknown (Yang et al., 2010).

Despite their potential significance, defining the role of histone marks will not be easy for a number of reasons. Rather than a code *per se*, it is probably the combined pattern of various histone marks that is relevant. Furthermore, our own studies show that even when there is no overall quantitative change in the prevalence of a mark, there are nuclear re-distributions of individual marks in response to TGF-β1, indicative of the dynamic state of histone modifications (Hewitson et al., 2017). We therefore need to better delineate the interaction of the various acetylation/methylation enzymes and their corresponding deacetylation/demethylation counterparts to better understand the basis of metabolic memory in fibrosis.

CONCLUSION

While the kidney can recover from acute and limited damage, critical illness or a persistent injury leads to a complex chain of direct and indirect sequelae resulting in chronic progressive renal impairment without repair. Severe and ongoing injury, influenced by susceptibility and epigenetics, leads to priming, feed-forward induction, and perpetual activation of fibroblasts, and a failure to return to tissue homeostasis through repair. This model provides a unifying hypothesis helping explain the transition from AKI to CKD (**Figure 1**). A better understanding of specific modifiable risk factors will excitingly provide new targets for therapeutic intervention.

AUTHOR CONTRIBUTIONS

TH, SH, and ES contributed to writing of manuscript, gave final approval and are responsible for the work.

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MINI REVIEW
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Targeting Hypoxia Inducible Factors-1α As a Novel Therapy in Fibrosis

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Fibrosis, characterized by increased extracellular matrix (ECM) deposition, and widespread vasculopathy, has the prominent trait of chronic hypoxia. Hypoxia inducible factors-1α (HIF-1α), a key transcriptional factor in response to this chronic hypoxia, is involved in fibrotic disease, such as Systemic sclerosis (SSc). The implicated function of HIF-1α in fibrosis include stimulation of excessive ECM, vascular remodeling, and futile angiogenesis with further exacerbation of chronic hypoxia and deteriorate pathofibrogenesis. This review will focus on the molecular biological behavior of HIF-1α in regulating progressive fibrosis. Better understanding of the role for HIF-1α-regulated pathways in fibrotic disease will accelerate development of novel therapeutic strategies that target HIF-1α. Such new therapeutic strategies may be particularly effective for treatment of the prototypic, multisystem fibrotic, autoimmune disease SSc.

Keywords: fibrosis, chronic hypoxia, hypoxia inducible factor, targeted therapy, systemic sclerosis

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INTRODUCTION

Fibrotic disease is a kind of chronic hypoxia related disease with pathogenesis that includes increased extracellular matrix (ECM) deposition, and widespread vasculopathy (Gabrielli et al., 2009). Fibrosis is increasingly seen as the result of deregulated tissue repair in response to chronic hypoxia that results in the excessive accumulation of ECM. Severe chronic hypoxia is overt in involved tissues of fibrotic disease patients (Distler et al., 2004). There are a variety of mechanisms leading to persistent chronic hypoxia. First, continuous and extensive microangiopathy caused by inflammation (Kahaleh, 2004; Cheung et al., 2017) or metabolic stress (Petersen et al., 2017; Wang et al., 2017) is regarded as an early and possibly the earliest pathogenic event in the fibrotic disease (Kahaleh et al., 1979) that leads to chronic hypoxia. Chronic hypoxia in turn induces vascular remodeling ultimately giving rise to progressive luminal narrowing and blockage (Flavahan et al., 2003) resulting in progressive exacerbation of the chronic hypoxic state. Moreover, excessive deposition of ECM, the hallmark of fibrosis (Bhattacharyya et al., 2012), further worsens hypoxia by increasing diffusion distances between blood vessels and tissue cells and increased tissue pressure. Extensive microangiopathy, vascular remodeling, and ECM deposition leads to vascular rarefaction and chronic hypoxia that directly contributes to progressive amplification of fibrosis. Increasing evidence has demonstrated that chronic hypoxia is actively involved in the pathogenesis of fibrosis (Ho et al., 2014) by stimulating the production of ECM including fibronectin-1, IGF-binding protein 3 (Distler et al., 2007), collagens, and collagen-modifying enzymes such as COL4A1, COL4A2, COL5A1, COL9A1, COL18A1, procollagen prolyl hydroxylases (P4HA1 and P4HA2), and lysyl hydroxylases (procollagen lysyl hydroxylase and procollagen lysyl hydroxylase 2)

(Manalo et al., 2005). Hence, persistent and extensive chronic hypoxia is a distinctive feature of fibrotic disease that definitely aggravates tissue fibrosis.

Hypoxia inducible factors (HIFs) are regarded as the "master regulators" (Imtiyaz and Simon, 2010) in response to the hypoxic environment and are essential for mediating adaptive reactions to hypoxia (Appelhoff et al., 2004; Farahani et al., 2012). HIFs are in a family of basic–helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) DNA binding transcription factors (Greer et al., 2012) and are heterodimers composed of two different subunits: HIF-α, that is oxygen regulated, and HIF-β, that is expressed constitutively in the nucleus (Wang et al., 1995; Semenza, 2003). There are at least three α subunits-HIF-1α, HIF-2α, and HIF-3α, that accumulate in the cytoplasm and translocate into the nucleus to form heterodimers with a β subunit. After translocating to the nucleus, the HIF heterodimers associate with co-activators and bind to hypoxia response elements (HREs) in gene promoters to initiate gene transcription (Kaelin and Ratcliffe, 2008; Semenza, 2009). Hypoxia induces stabilization and nuclear translocation of HIF-α subunits and their transcriptional activity (Kaelin and Ratcliffe, 2008) by inhibiting the activity of both prolyl hydroxylases and factor-inhibiting HIF1. Hypoxia increases the half-life of HIF-1α from 5 min to approximately 60 min (Huang et al., 1998).

Compelling evidence indicates that HIF-1α plays a key role in vascular remodeling under hypoxic conditions (Yu et al., 1999). The extensive and cumulative vascular remodeling in arterioles that accompanies chronic hypoxia results in multiple internal organ fibrosis and pulmonary hypertension (PH). Of note, PH associated with pulmonary fibrosis is the major cause of mortality in individuals suffering from fibrotic disease, such as SSc, and accumulating evidence has revealed that HIF-1α is implicated in producing excessive ECM which was the underlying cause of fibrosis (Distler et al., 2007; Higgins et al., 2007; Halberg et al., 2009; Ueno et al., 2009; Zhou et al., 2009; Gilkes et al., 2013; Nayak et al., 2016). Fibrosis is typically characterized by prolonged and/or exaggerated activation of fibroblasts (Ho et al., 2014). Strong and stable expression of HIF-1α was found in fibrotic dermal fibroblasts cultured under hypoxic conditions, 1% oxygen, equivalent to a PO_2 value of 7 mmHg, which is close to the 10th percentile measured in involved dermal areas of fibrotic disease patients (Hong et al., 2006; Distler et al., 2007). Furthermore, increased expression of HIF-1α occurred in subcutaneous fibroblasts from healthy skin (Modarressi et al., 2010) and fibrotic skin (Hattori et al., 2015) exposed to hypoxic conditions *in vitro*. Fibroblasts isolated from human arteries also exhibited a remarkable up-regulation of HIF-1α under hypoxic conditions (Krick et al., 2005). In a more detailed study, HIF-1α completely translocated from the cytosol into the nucleus (Wenger, 2002) in dermal fibroblasts from fibrotic disease patients after hypoxic exposure (Distler et al., 2004). HIF-1α expression is elevated in a number of fibrotic diseases (Fine et al., 1998; Baan et al., 2003; Zhang et al., 2003, 2004) and overt up-regulation of HIF-1α in the skin of naïve SSc patients was observed compared with normal skin (Ioannou et al., 2013) further suggesting that HIF1α is involved in the pathogenesis of fibrotic disease, particularly in SSc (Wipff et al., 2009). In addition, HIF-1α is particularly related to subgroups of SSc patients with prominent vascular manifestations (Wipff et al., 2009). Inhibition of HIF-1α is therefore a rational strategy for novel therapeutic development since effective therapies are not yet available for fibrotic disease, such as SSc.

HIF-1α AND ECM (Figure 1A)

Fibrosis is characterized by excessive deposition of ECM in organs or tissues including different kinds of collagens, hyaluronic acid, fibronectin, and proteoglycans (Ho et al., 2014). HIF-1α contributed to the up-regulated gene expression for several ECM and non-ECM in fibroblast cultures *in vitro*.

Increased expression of pro α2 (I) collagen (COL1A2), thrombospondin (TSP) 1, and transforming growth factor β–induced protein (TGF βi) were observed in both mouse embryonic and human dermal fibroblasts under hypoxic conditions (Distler et al., 2007). Bentovim et al. (2012) demonstrated that HIF-1α induced collagen hydroxylation and normal collagen secretion in the hypoxic milieu by directly activating transcription of the collagen prolyl 4-hydroxylase enzyme (P4H) and pyruvate dehydrogenase kinase 1 (Pdk1). HIF-1α deficiency resulted in impaired collagen secretion in the presence of hypoxia. Similarly, HIF-1α mediates ECM accumulation through NADPH oxidase (NOX) *in vitro* in cultured renal mesangial cells (Nayak et al., 2016). Microarray genome expression profiling from skin biopsies of fibrotic disease patients revealed that a prominent alteration in gene expression underlying fibrosis is within the transforming growth factor β (TGF-β) pathway (Whitfield et al., 2003), and TGF-β was closely involved in the induction of ECM (Falanga et al., 1987). However, HIF-1α is upstream of TGF-β production, and hypoxia-induced TGF-β production requires HIF-1α (Zhou et al., 2009). Qian et al. (2015) demonstrated that inhibition of HIF-1α reduced TGF-β expression *in vivo* as well.

Epithelial-to-mesenchymal transition (EMT) can be characterized by acquisition of mesenchymal markers such as α-smooth muscle actin (α-SMA). EMT results in the production of more ECM including α-SMA and vimentin (Strutz et al., 1995; Zeisberg and Kalluri, 2004) and requires HIF-1α expression (Zhou et al., 2009). Higgins et al. (2007) demonstrated that increased HIF-1α expression may promote fibrogenesis by facilitating EMT. Plasminogen activator inhibitor-1 (PAI-1), found in the ECM (Podor and Loskutoff, 1992) and a key inhibitor of fibrinolysis (Collen and Lijnen, 1991), inhibited proteolytic processes that were linked with fibrosis (Eddy et al., 1995). HIF-1α heterodimers with HIF-1β induced by hypoxia *in vivo* bind HRE in the PAI-1 promoter and induce PAI-1 expression (Kietzmann et al., 1999). Lysyl oxidase (LOX) is important for normal synthesis of collagen and elastin (Giampuzzi et al., 2000; Oleggini et al., 2007). LOX is a transcriptional target for HIF-1α-HIF-1β heterodimers (Halberg et al., 2009) that translocate into the nuclear compartment of fibrogenic cells (Li et al., 1997) and is up-regulated during fibrogenesis. Higgins et al. (2007), Halberg et al. (2009) showed

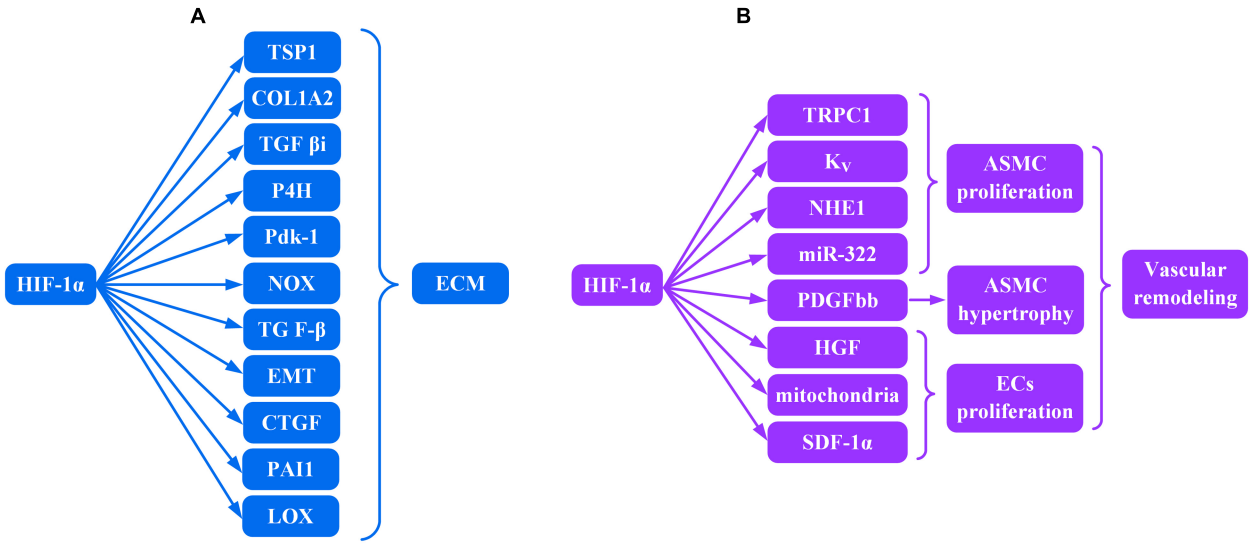

FIGURE 1 | Role of HIF-Iα transcription factor in the production of **(A)** ECM and **(B)** vascular remodeling.

that HIF-1α could up-regulate the expression of LOX *in vivo* and *in vitro*, leading to the accumulation of collagen and other components involved in establishing and remodeling the ECM. Halberg et al. (2009) pinpointed LOX as a key player in HIF-1α mediated deposition of ECM. Furthermore, connective tissue growth factor (CTGF) has been reported to enhance cell proliferation and ECM production in fibroblasts (Frazier et al., 1996). Mounting evidence has demonstrated that expression of CTGF is upregulated during fibrotic disorders (Igarashi et al., 1996; Ito et al., 1998; Leask et al., 2002; Higgins et al., 2003), and in hypoxia, the induction of CTGF is directly mediated by HIF-1α-HIF-1β heterodimer binding to the CTGF associated HRE (Higgins et al., 2004). In summary, HIF-1α is ubiquitous in many different tissues (Wang et al., 1995) and in fibrotic disease contributes to persistent pathofibrogenesis in multiple organs by stimulating production of excessive ECM.

HIF-1α AND VASCULAR REMODELING (Figure 1B)

Vascular remodeling is primarily composed of dysregulated proliferation of endothelial cells (ECs) and an increase in the number (hyperplasia) and volume (hypertrophy) of arterial smooth muscle cells (ASMC) resulting in progressive vascular occlusion and chronic hypoxia. High expression of HIF-1α within endothelial plexiform lesions (Tuder et al., 2001) and ASMC (Bonnet et al., 2006) suggests a strong correlation between HIF-1α and proliferative vasculopathy.

Arterial smooth muscle cells hyperproliferation in the media of the artery was suggested to be the key event in vascular remodeling (Cheng et al., 2017). Transient receptor potential channel (TRPC) 1, a non-selective cation channel, is permeable to Ca^{2+} ions. Increase in levels of TRPC1 mediated by bone morphogenetic protein4 (BMP4) (Wang et al., 2015) was HIF-1α dependent in ASMC (Wang et al., 2006). Reduction in voltage-gated K^+ currents, resulting in membrane depolarization and activation of voltage-dependent Ca^{2+} channels and subsequently increasing Ca^{2+} influx, was regulated by HIF-1α as well (Shimoda et al., 2001). Both voltage-gated K^+ (Kv) channels and TRPC1, mediated by HIF-1α, contributed to an increase in cytosolic free Ca^{2+} which was a major trigger for ASMC proliferation (Veith et al., 2016). ASMC proliferation may be a consequence of up-regulated aquaporin 1 as a result of the increased cytosolic free Ca^{2+} (Yun et al., 2015). Furthermore, both TRPC1 silencing by small interfering RNA (siRNA) and TRPC1 knockout impaired hypoxia-induced ASMC proliferation *in vitro*, and $TRPC1^{-/-}$ mice had less vascular muscularization compared with wild type mice (Malczyk et al., 2013). In addition, hypoxic induction of the Na^+/H^+ exchanger isoform 1 (NHE1) expression and alkalinization of intracellular pH were regulated by HIF-1α (Shimoda et al., 2006). Both activation of the Na^+/H^+ exchanger and alkalinization of intracellular pH were necessary for ASMC proliferation (Quinn et al., 1996). Zeng et al. (2015) demonstrated that HIF-1α transcriptionally upregulated the expression of miR-322 in hypoxia, which led to proliferative responses of ASMC due to direct targeting of BMPR1a and smad5. Similarly, Platelet derived growth factor bb (PDGFbb) can induce proliferation of ASMC *in vitro* and *in vivo* (Schermuly et al., 2005). PDGFbb-induced signaling gave rise to the hypertrophy of ASMC both *in vitro* and *in vivo* (Ke et al., 2016) via excessive deposition of hyaluronic acid (HA) in smooth muscle cells (Pullen et al., 2001). The possible mechanism is through tyrosine 31 (Y31) and 118 (Y118) phosphorylation of paxillin, which was attenuated by HIF-1α knockdown (Veith et al., 2014).

Similarly, HIF-1α is also involved in the proliferation of ECs. Abnormally proliferating ECs are characterized by low numbers of mitochondria (Xu et al., 2007). Knockdown

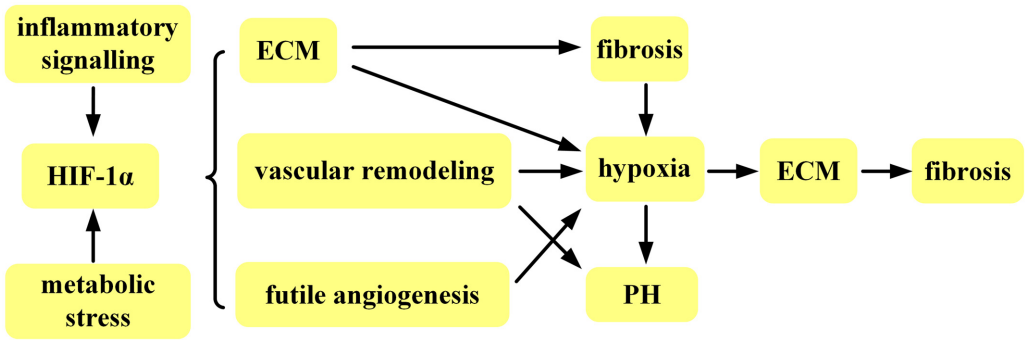

FIGURE 2 | Simplified overview of HIF-1α implicated in fibrotic disease.

of HIF-1α increased the numbers of mitochondria in ECs *in vitro* (Fijalkowska et al., 2010) and suggests that the reduced mitochondria number in abnormally proliferating ECs may be a consequence, at least in part, of increased HIF-1α expression. HIF -1α inducible factors include hepatocyte growth factor (HGF) (Kitajima et al., 2008) and stromal-derived factor-1a (SDF-1a) (Ceradini et al., 2004). A special kind of hematopoietic endothelial stem cell, $CD34^+CD133^+$hemangioblast, may promote angioproliferative vascular remodeling (Asosingh et al., 2008). Local production of chemoattractants, such as SDF-1α and HGF, by diseased endothelium can recruit substantial numbers of $CD34^+CD133^+$hemangioblasts to sites of angioproliferative vascular remodeling (Farha et al., 2011). Both signal transducers and activators of transcription (STAT) 3 (Xu and Erzurum, 2011) and chloride intracellular channel 4 (CLIC4) (Wojciak-Stothard et al., 2014) contribute to the hyperproliferative pathology of ECs invoking another important role for HIF-1α in vascular fibrosis.

TARGETING HIF-1α IN FIBROSIS

Studies to date indicate that HIF-1α is intimately involved in persistent pathofibrogenesis, vascular remodeling, and PH in fibrotic disease. Severe, multiple organ fibrosis associated with the continuous accumulation of HIF-1α, caused by chronic or prolonged hypoxia in fibrotic disease, suggests that HIF-1α maybe a promising target for novel fibrotic disease treatments, such as SSc.

Recently, hypoxic prodrugs, projecting to be specifically activated in the low O_2 milieu, deliver the active agent to hypoxic tissues through reduction of the prodrug by cellular reductases (Phillips, 2016). These hypoxic prodrug agents may significantly alleviate off-target effects of the biological therapy by limiting active drug to hypoxic tissue and only inhibiting HIF-1α in hypoxic tissues. Gene therapy targeting HIF-1α may also be effective for therapy in hypoxia-related diseases as well (Tal et al., 2008; Wang et al., 2008; del Rey et al., 2009; Chen et al., 2016). In addition, the therapeutic benefits of HIF-1α inhibitors would be maximized in the presence of delivery carriers that eliminate pharmacokinetic and stability problems and minimize potential systemic toxicity. For example, liposomes and nanoscale-based drug delivery systems may be applied as a delivery assistant for HIF-1α gene therapy (Wang et al., 2008; Chen et al., 2016). The most successful example of a successful liposomal drug delivery system may be that for Amphotericin B, which has been widely applied in the clinic for treating invasive fungal infections. Amphotericin B is a highly effective drug but with potential severe toxic side effects (Barratt and Bretagne, 2007; Wasko et al., 2012). Amphotericin B encapsulated in liposome has significantly reduced toxicity as well as increased therapeutic benefit when administered systemically encapsulated within liposomes (Torchilin, 2005; Allen and Cullis, 2013). Antisense oligonucleotides targeted to HIF-1α mRNA combined with doxorubicin were successfully delivered to oncocytes by poly (ethylene glycol) polymer (PEGylated) liposomes as drug carriers (Wang et al., 2008). Furthermore, YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole], a HIF-1α inhibitor, reduced ECM accumulation *in vivo* (Nayak et al., 2016). Trichostatin A, identified indirectly to down-regulate HIF-1α, has been applied in clinical trials in patients with cancers (Kim et al., 2001) and has been shown to reduce the release of collagen from fibrotic dermal fibroblasts *in vitro* (Huber et al., 2005). In conclusion, a viable therapy option for fibrotic disease may include agents that target and inhibit HIF-1α since delivery vehicles may help reduce off-target effects and enhance therapeutic efficiency (Sercombe et al., 2015).

On the other hand, HIF-1α has been repeatedly observed to assist wound healing through inflammation, angiogenesis, vasculargenesis, and fibroplasia in acute injury (Semenza, 1998, 1999). The most prominent contraindication for systemic administration of HIF-1α inhibitors, therefore, is trauma. An analogy for adverse effects that may accompany biological therapy to inhibit HIF-1α may be tumor necrosis factor-α (TNF-α) inhibitors in rheumatic diseases. TNF-α inhibitors inhibit inflammation that is necessary for tissue repair. HIF-1α is required for repair in acute injury as well (Darby and Hewitson, 2016). In particular, we need to be vigilant about physiological repair events such as menstruation within the context of HIF-1α inhibitor administration.

Persistent and remarkable up-regulation of vascular endothelial growth factor (VEGF) has been observed in all stages of fibrotic disease (Distler et al., 2004)

and actively proliferating ECs of plexiform lesions (Tuder et al., 2001). VEGF is the predominant proangiogenic factor regulated by HIF-1α in other hypoxia related diseases, but VEGF up-regulation and consequent massive and extensive microangiopathy in fibrotic disease is HIF-1α independent, even with hypoxia (Distler et al., 2004). HIF-1α expression did not correlate with up-regulated VEGF in affected tissues from patients suffering from fibrotic disease (Distler et al., 2004). Since HIF-1α is critical for VEGF up-regulation in other hypoxia related diseases, consideration must be given to the question of whether deceased VEGF after systemic HIF-1α inhibition may severely decrease and impair neoangiogenesis. The worry may be unnecessary for fibrotic disease and its associated massive and extensive microangiopathy in affected tissues and organs. Up-regulated expression of VEGF is also driven by interleukin-1β, PDGF, and TGF-β, all of which are up-regulated in fibrotic disease and can stimulate the expression of VEGF (Pertovaara et al., 1994; Kissin and Korn, 2003). Moreover, the role played by HIF-2α and HIF-3α in the over-expression of VEGF has not yet been extensively investigated. Above all, sufficient tissue vascularization depends on strict regulation of VEGF expression rather than on persistent up-regulated expression of VEGF (Distler et al., 2004). The formation of chaotic vessels, feathered with glomeruloid and haemangioma-like morphology, was partly due to chronic and uncontrolled over-expression of VEGF (Drake and Little, 1995; Sundberg et al., 2001). Dor et al. (2002) designed an animal model system in which a source of VEGF could be specifically induced and steadily maintained for a desired duration and then subsequently switched off. Time-dependent regulation of VEGF expression was necessary for adequate and normal vascularization (Dor et al., 2002). Persistent, uninterrupted exposure to VEGF led to formation of irregularly shaped, sac-like vessels resulting in decreased blood flow compared to normal, mature, functional blood vessel formation after short-term over-expression of VEGF (Dor et al., 2002). Irregularly shaped, sac-like vessels observed in nailfold, a prominent character of the prototypic fibrotic disease-SSc (LeRoy, 1996), may also suggest that persistent up-regulated expression of VEGF is involved in fibrotic disease, and is harmful rather than beneficial, regardless of whether VEGF is HIF-1α independent in fibrosis (Distler et al., 2004). Other angiogenic factors contribute less to neovascularization and have no effect on irregular and sac-like vessels in the presence of persistent and remarkable up-regulation of VEGF. The US FDA approved FTY720 inhibits HIF-1α accumulation by inhibiting the S1P signaling pathway. FTY720 transformed a chaotic vascular network to vascular normalization while simultaneously and subsequently redressing hypoxia *in vivo* and *in vitro* (Gstalder et al., 2016). That result gives further credence to the suggestion that HIF-1α is implicated in chaotic angiogenesis. This result also suggests that targeting HIF-1α would be a viable strategy for fibrotic disease, such as SSc, without impairing normal angiogenesis.

CONCLUSION

HIF-1α *per se* is helpful in repairing injury and correcting hypoxia via multiple mechanisms, however, prolonged exposure to HIF-1α is harmful and contributes to persistent pathofibrogenesis in fibrotic disease (**Figure 2**). Furthermore, fibrosis in organs resulting in organ failure accounts for much of the morbidity and mortality associated with fibrotic disease. SSc is prototypic multisystem fibrotic disease and present immunosuppressive therapy exhibits intolerable side effects without selectively targeting the immunopathogenic mechanisms responsible for SSc. In addition, fibrosis in SSc is not restricted to a single organ, but rather involves multiple internal organs and skin. Biotherapy targeting HIF-1α, therefore, is a promising therapeutic alternative that is more likely to confer therapeutic benefits specific to fibrotic disease, particularly to SSc, by attenuating fibrosis and terminating or delaying vascular remodeling.

AUTHOR CONTRIBUTIONS

AX wrote and assembled the manuscript. YL prepared the figures and revised the manuscript.

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MINI REVIEW
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Notch Signaling in Ischemic Damage and Fibrosis: Evidence and Clues from the Heart

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Notch signaling is a major intercellular coordination mechanism highly conserved throughout evolution. In vertebrates, Notch signaling is physiologically involved in embryo development, including mesenchymal cell commitment, formation of heart tissues and angiogenesis. In post-natal life, Notch signaling is maintained as a key mechanism of cell–cell communication and its dysregulations have been found in pathological conditions such as ischemic and fibrotic diseases. In the heart, Notch takes part in the protective response to ischemia, being involved in pre- and post-conditioning, reduction of reperfusion-induced oxidative stress and myocardial damage, and cardiomyogenesis. Conceivably, the cardioprotective effects of Notch may depend on neo-angiogenesis, thus blunting lethal myocardial ischemia, as well as on direct stimulation of cardiac cells to increase their resistance to injury. Another post-developmental adaptation of Notch signaling is fibrosis: being involved in the orientation of mesenchymal cell fate, Notch can modulate the differentiation of pro-fibrotic myofibroblasts, e.g., by reducing the effects of the profibrotic cytokine TGF-β. In conclusion, Notch can regulate the interactions between heart muscle and stromal cells and switch cardiac repair from a pro-fibrotic default pathway to a pro-cardiogenic one. These features make Notch signaling a suitable target for new cardiotropic therapies.

Keywords: Notch, heart, fibrosis, ischemia-reperfusion, cardiac preconditioning

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INTRODUCTION

The Notch pathway is a major intercellular short-range coordination mechanism highly conserved throughout evolution and similar in all multicellular organisms from invertebrates to mammals (Kopan and Ilagan, 2009). Notch designates a trans-membrane receptor encoded by a gene originally identified as that responsible for the appearance of a 'notch' in the wings of *Drosophila melanogaster*. In mammals, four Notch receptors, 1–4, and five canonical ligands, Jagged 1–2 and Delta-like (DLL) 1,3 and 4, have been identified as membrane-spanning proteins (Guruharsha et al., 2012).

The mechanism of Notch signaling does not exploits the classical signal transduction pathways of most surface receptors: upon ligand binding, the surface metalloprotease ADAM10 clips the Notch extracellular domain just outside the plasma membrane and releases an extracellular Notch fragment which remains bound to its ligand and is then endocytosed by the ligand-bearing cell, which in turn undergoes signaling. Then, the inner membrane protease γ–secretase cleaves

the Notch intracellular domain (NICD), the active form of Notch, which is released in the cytoplasm, migrates to the nucleus and binds to CSL transcription factors (also known as RBP-Jκ) regulating Notch target gene expression (Guruharsha et al., 2012). The encoded proteins regulate further expression of many downstream genes, some of which can either maintain the cell in an uncommitted state or induce differentiation, while others regulate cell proliferation and apoptosis (Miele et al., 2006). The increasing interest in Notch pathway ad a key regulator of cell function and differentiation has been paralleled by the development of appropriate methods and tools for its investigation in cellular and animal models, as exhaustively reported in a recent review (Zacharioudaki and Bray, 2014).

In Vertebrates, Notch signaling is physiologically involved in embryo development and morphogenesis which exploit its ability to mediate intercellular communication. In fact, the formation of distinct organs and tissues requires adhesion mechanisms which promote and maintain the sorting of different cell populations. In post-natal life, Notch signaling is maintained as a key mechanism of cell–cell communication and its dysregulations are implicated in tumor development and metastasis and in non-neoplastic pathological conditions such as ischemic and fibrotic diseases, sometimes playing a dual role as pathogenic mechanism or adaptive/compensatory response (Harper et al., 2003; Hori et al., 2013). In this context the heart, whose complex assembly requires the precise coordination of diverse cells, represents an appropriate paradigm to understand the roles of the Notch pathway in health and disease.

NOTCH PATHWAY IN THE DEVELOPING AND DISEASED HEART

Notch signaling is a key mechanism of normal heart morphogenesis, being required for the formation of the atrioventricular canal and valves, outflow tract and coronary vessels, and for growth and differentiation of the endocardium, myocardium and epicardium (High and Epstein, 2008; Luxán et al., 2016). Among the cellular mechanisms operating during cardiac morphogenesis, Notch signaling has been shown to mediate epithelial-mesenchymal transition (EMT) of endocardial precursor cells: in particular, Notch signaling down-regulates surface cadherin expression and disables intercellular adhesion among these cells, allowing them to move, reach the atrio-ventricular and outflow tract regions, and pattern the cardiac valves (Timmerman et al., 2004). Moreover, during growth and three-dimensional organization of the ventricular myocardium, Notch signaling is required to sustain cardiomyocyte precursor proliferation and differentiation as well as compaction of the primitive trabecular myocardium (High and Epstein, 2008; Luxán et al., 2016). Finally, the Notch pathway is crucial for coronary vasculogenesis, namely the formation of primary vascular rudiments from the epicardial mesenchyme, and angiogenesis, namely the sprouting of new vessels from pre-existing ones. Both phenomena are regulated by vascular endothelial growth factor (VEGF), whose downstream pathway involves Notch/Jagged up-regulation by endothelial cells (Ferrara et al., 2003). Notch signaling appears a homeostatic regulator of the endothelium, since it can mediate either proliferation and resistance to apoptosis during active angiogenesis or contact inhibition and cell cycle arrest during blood vessel stabilization. In this latter phase, Notch also favors the recruitment of pericytes from the mesenchyme and stimulates growth, migration and resistance to apoptosis of vascular smooth muscle cells, thereby promoting the build-up of functional blood vessels (Sainson and Harris, 2008).

The pivotal role of the Notch pathway in heart morphogenesis emerges from both studies on Notch or Jagged1 knock-out mice and clinical reports, showing that defective Notch signaling is correlated with cardiac malformations such as Tetralogy of Fallot in Alagille syndrome, aberrant bicuspid aortic valve and left ventricular non-compaction cardiomyopathy (High and Epstein, 2008; Luxán et al., 2016). In the adult heart, Notch signaling between mature cells is absent under physiological conditions but can be roused to take part in the protective response to injury, as later discussed.

Another morphogenetic effect of the Notch pathway with major repercussions on the adult diseased heart is the regulation of stromal cell differentiation and extracellular matrix (ECM) production (Hu and Phan, 2016). In the embryo, Notch signaling promotes EMT and generates mesenchymal cells: in turn, these cells differentiate into different stromal cell lineages, including the cardiac valves and coronary endothelium. Although in adult tissues EMT is suspected to play some role in the generation of new fibroblasts and myofibroblasts, the main pro-fibrotic cells, during the development of organ fibrosis (Hu and Phan, 2016), the existing evidence suggests that Notch signaling can rather exert anti-fibrotic effects on the diseased heart, for instance by counteracting the pro-fibrotic cytokine transforming growth factor (TGF)-β and reducing myofibroblast proliferation (Nemir et al., 2014).

NOTCH PATHWAY IN THE ISCHEMIC HEART

Myocardial infarction (MI), one of the leading causes of death worldwide, mainly depends on coronary artery occlusion and ischemia followed by reperfusion (I/R), in which blood flow restoration is accompanied by oxidative stress exacerbating myocardial damage. Noteworthy, the adult myocardium can re-express fetal genes as an adaptive response to injury: in this context, increased Notch1 signaling was demonstrated in surviving cardiomyocytes of the MI border zone (Gude et al., 2008). Several studies have shown that Notch signaling protects the heart from I/R-induced myocardial injury: activation of Notch1 pathway limits the extent of ischemic damage, promotes coronary neo-angiogenesis and revascularization of the ischemic myocardium, reduces myocardial fibrosis and improves heart function (Gude et al., 2008; Kratsios et al., 2010; Li et al., 2011; Ferrari and Rizzo, 2014). Conversely, in systemic Notch1 deficient mice, I/R leads to the development of a larger myocardial infarct area and worsening of heart function than wild-type controls (Li et al., 2011).

Notch1 plays an important role in the protection of ischemic myocardium during pre- (IPC) and post-conditioning (IPost), well-known adaptive responses of the heart to increase its resistance to I/R injury (Zhao et al., 2003). Notch1 pathway is activated during myocardial IPC and IPost and leads to reduction of cardiomyocyte apoptosis, infarct size and contractile impairment. Conversely, inhibition of Notch1 signaling by N1ICD knockdown abrogates IPC- and IPost-induced cardioprotection (Zhou et al., 2013).

The mechanisms underlying Notch-mediated cardio-protection are complex and involve an interplay between mature and immature cardiomyocytes, cardiac progenitors cells (CPCs) and bone marrow (BM)-derived cells (**Figure 1**). Notch1 prevents cardiomyocyte apoptosis by activation of PI3K/AKT pro-survival signaling and regulation of apoptotic genes (Kratsios et al., 2010; Pei et al., 2013; Zhou et al., 2013). Moreover, Notch signaling induces cell cycle re-entry of immature cardiomyocytes (Campa et al., 2008; Sassoli et al., 2011), promotes proliferation and myogenic differentiation of CPCs (Boni et al., 2008), decreases oxidative/nitrosative stress (Pei et al., 2013) and prevents cardiac fibrosis (Fan et al., 2011).

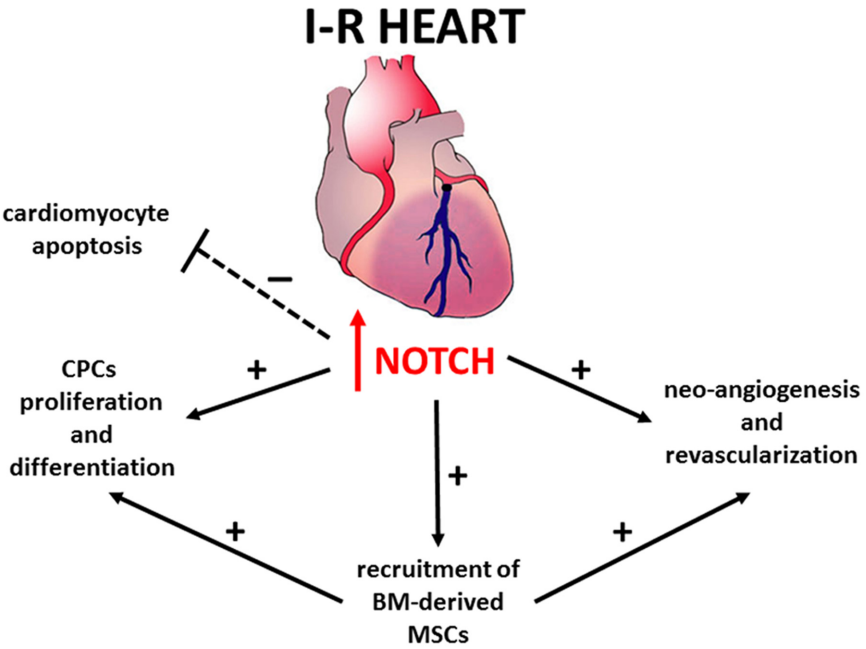

FIGURE 1 | Notch signaling in repair and regeneration of the ischemic and reperfused heart.

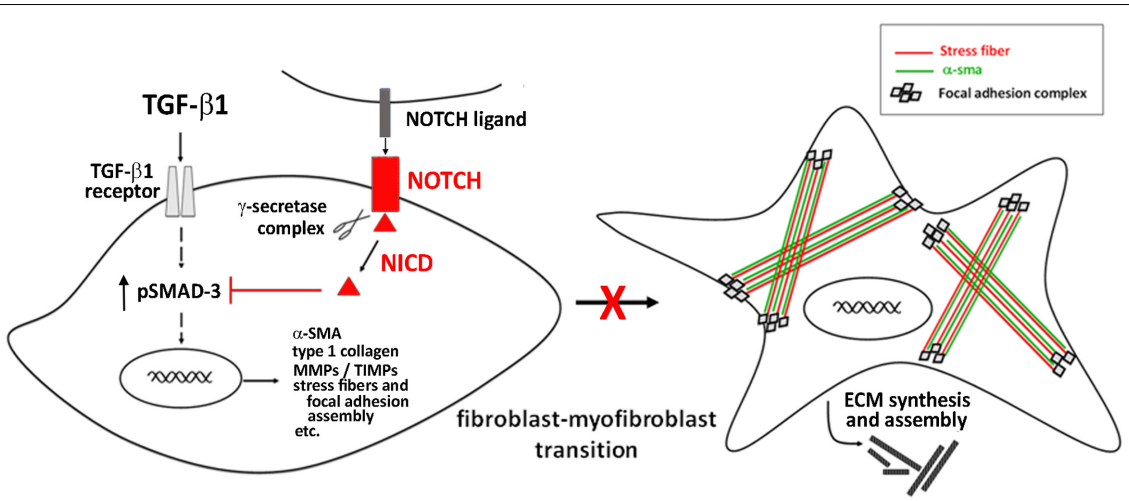

FIGURE 2 | Notch signaling in the regulation of cardiac fibrosis.

Recent studies indicate that Notch1 is ineffective in promoting cardiac regeneration in adults due to permanent epigenetic modifications at the Notch-responsive promoters which render their transcriptional repression irreversible (Felician et al., 2014). Consistently, in cardiac-specific Notch1 deficient mice, the loss of Notch1 in post-natal cardiomyocytes did not affect the severity of myocardial injury (Li et al., 2011). Hence, the beneficial effects of Notch1 re-activation previously observed in transgenic animals (Kratsios et al., 2010) could be mediated *via* other cell types, such as CPCs and BM-derived cells. Accordingly, Notch1 recruits BM-derived mesenchymal stem cells (MSCs) in the infarction border zone, promoting proliferation and preventing apoptosis (Li et al., 2011). Moreover, transplantation of N1ICD-overexpressing MSCs reduces – while that of Notch1-deficient MSCs increases – both infarct size and contractile impairment (Li et al., 2011). Overall, these findings suggest that the maintenance or reactivation of Notch signaling in cardiac cells can be a therapeutic target to protect against myocardial damage.

NOTCH PATHWAY IN CARDIAC FIBROSIS

Cardiac fibrosis, a late complication of many heart diseases, can occur as myocardial replacement fibrosis to prevent cardiac rupture, for instance after MI, or as interstitial fibrosis without cardiomyocyte loss, an adaptation to chronic injury by functional overload, ischemia and cardiomyopathies. Started as compensatory to organ damage, cardiac fibrosis becomes maladaptive and dysfunctional in the long term (Rockey et al., 2015; Travers et al., 2016). In general, fibrosis results from an imbalance between ECM synthesis and degradation by fibrogenic cells, chiefly myofibroblasts. In response to pro-inflammatory and pro-fibrotic mediators up-regulated in cardiac injury, among which TGF-β1 plays a major role, resident cardiac fibroblasts, CD45+ hemopoietic stromal cells and, perhaps, EMT-derived fibroblasts, vascular pericytes and immune cells are recruited and prompted to differentiate into myofibroblasts, characterized by dual immunophenotypical and ultrastructural features of fibroblasts and smooth muscle cells (Bani and Nistri, 2014; Ivey and Tallquist, 2016; Pinto et al., 2016). Myofibroblast contraction and excess ECM deposition cause the distortion of the normal myocardial architecture. Moreover, myofibroblasts secrete a variety of mediators which stimulate autocrine cell activation and fibrogenesis and exert paracrine effects on the cells nearby, causing chronic inflammation and further cardiomyocyte dysfunction (Travers et al., 2016).

Several studies have shown that Notch signaling is involved in counteracting cardiac fibrosis, primarily via inhibition of myofibroblast differentiation. In particular, the expression of Notch1, 3, and 4 are down-regulated during fibroblast–myofibroblast transition in neonatal hearts, while Notch signaling inhibition promotes myofibroblast formation (Fan et al., 2011). Consistently, in a mouse model of pressure overload, Notch1 controlled the balance between fibrotic and regenerative repair by inhibiting myofibroblast proliferation and promoting mobilization and expansion of cardiac muscle precursor cells (Nemir et al., 2014). Recently, *in vivo* intramyocardial delivery of hydrogels containing the Notch1 ligand Jagged-1 in rats with MI reduced cardiac fibrosis (Boopathy et al., 2015). Moreover, augmentation of Notch3 expression by lentiviral transfection inhibited fibroblast–myofibroblast transition both in TGF-β1-treated cardiac fibroblasts *in vitro* and in mice with MI, minimizing cardiac fibrosis (Zhang et al., 2016). As previously mentioned, Notch signaling can inhibit EMT (Zhou et al., 2015; Hu and Phan, 2016), which also contributes to cardiac fibrosis (von Gise and Pu, 2012). This point, however, remains controversial: indeed, in Notch transgenic mice undergoing MI and pressure overload, Notch induced epicardial cells to undergo EMT and generate a multipotent stromal cell population capable of differentiating into fibroblasts and producing reparative fibrosis (Russell et al., 2011).

The main identified mechanism by which Notch signaling interferes with myofibroblast differentiation consists in its ability to antagonize TGF-β/Smad3 signaling, the key intracellular pathway promoting cell activation and fibrogenesis (Zhang et al., 2016; Travers et al., 2016) (**Figure 2**).

Targeting the Notch pathway may be a meaningful therapeutic strategy for cardiac fibrosis. In this context, the hormone relaxin, known as anti-fibrotic agent and under clinical investigation in heart failure patients (Moin et al., 2016), was shown to inhibit the TGF-β1/Smad3 axis and to counteract TGF-β1-induced transition of neonatal cardiac stromal cells and NIH3T3 fibroblasts to myofibroblasts acting *via* the up-regulation of Notch1 signaling (Sassoli et al., 2013; Squecco et al., 2015; Zhou et al., 2015).

CONCLUSION

The Notch pathway is pivotal in the protection and healing of the ischemic heart because it regulates key mechanisms of myocardial resistance to ischemia and controls the balance between fibrotic and regenerative repair. Targeting Notch signaling, for example by soluble Notch ligands or Notch pathway activating molecules delivered intramyocardially or embedded into suitable biomaterials (Gude et al., 2008; Limana et al., 2013; Boccalini et al., 2015; Boopathy et al., 2015), may be a worthwhile therapeutic approach to cardiovascular disease.

AUTHOR CONTRIBUTIONS

SN conceived the manuscript and wrote the chapter on cardiac ischemia-reperfusion, CS wrote the chapter on cardiac fibrosis, DB wrote the Introduction and the chapter on heart embryology. All authors contributed equally to conceiving and drawing of figures.

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MINI REVIEW
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Targeting TGF-β Mediated SMAD Signaling for the Prevention of Fibrosis

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Fibrosis occurs when there is an imbalance in extracellular matrix (ECM) deposition and degradation. Excessive ECM deposition results in scarring and thickening of the affected tissue, and interferes with tissue and organ homeostasis – mimicking an exaggerated "wound healing" response. Many transforming growth factor-β (TGF-β) ligands are potent drivers of ECM deposition, and additionally, have a natural affinity for the ECM, creating a concentrated pool of pro-fibrotic factors at the site of injury. Consequently, TGF-β ligands are upregulated in many human fibrotic conditions and, as such, are attractive targets for fibrosis therapy. Here, we will discuss the contribution of TGF-β proteins in the pathogenesis of fibrosis, and promising anti-fibrotic approaches that target TGF-β ligands.

Keywords: fibrosis, TGF-β, activin, muscle, skeletal, myostatin, propeptide

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INTRODUCTION

In fibrotic disease, increased deposition of extracellular matrix (ECM) proteins compromises tissue architecture, and interferes with normal organ function. Fibrosis is frequently observed in the heart, liver, lungs, and kidneys, but can arise in any tissue that has suffered chronic insult. Triggers for fibrosis can be either biological (e.g., persistent bacterial/viral infections, genetic disorders or tissue injury) and/or environmental (e.g., pollutant/chemical exposure or allergens) (Macneal and Schwartz, 2012). Fibrosis is primarily driven by inflammatory cytokines including the interleukins (Nikolic-Paterson et al., 1996; O'Reilly et al., 2012), and members of the transforming growth factor-β (TGF-β) superfamily (Roberts et al., 1986; Meng et al., 2016), such as TGF-β1, activin A and activin B. Many of these ligands are expressed by infiltrating inflammatory cells, which migrate toward damage tissues. Of interest, these TGF-β ligands not only promote ECM deposition, but they also become concentrated within the accumulating matrix, thereby accelerating the pro-fibrotic response.

TGF-β LIGANDS IN FIBROSIS

The TGF-β superfamily includes the TGF-β isoforms (TGF-β1, -β2 and -β3), activins and inhibins, growth differentiation factors (GDFs), bone morphogenetic proteins (BMPs), and anti-mullerian hormone (AMH). Although structurally similar, these ligands elicit distinct biological responses, based on their cell/tissue-specific expression, their interactions with inhibitory molecules and the unique combinations of receptors with which they complex. TGF-β ligands form receptor complexes with one of seven type I receptors (also termed activin-like kinase or ALK receptors) in combination with one of five type II receptors (ActRIIA, ActRIIB, TGFBRII, BMPRII,

and AMHRII). For TGF-β1, ligand contact with the type II receptor TGFBRII leads to recruitment and phosphorylation of the type I receptor ALK-5. The BMPs, however, firstly contact their type I receptor (namely ALK3/ALK6), and then induce kinase activity toward the type II receptor BMPRII (reviewed in Miyazawa et al., 2002). In all instances, ligand-receptor complex formation leads to the activation of kinase domains within the receptors, which potentiate phosphorylation cascades involving SMAD transcription factors. The TGF-β isoforms and activins converge to induce intracellular signaling via SMAD-2/3 transcription factors. Activation of SMAD-2/3 regulates to the expression of several profibrotic genes, including collagens [*COL1A1, COL3A1, COL5A2, COL6A1, COL6A3, COL7A1,* (Verrecchia et al., 2001a,b)], plasminogen activator inhibitor-1 (*PAI-1*; Dennler et al., 1998; Hua et al., 1998), various proteoglycans (Schonherr et al., 1991; Romaris et al., 1995; Dadlani et al., 2008), integrins (Margadant and Sonnenberg, 2010), connective tissue growth factor (Chen et al., 2002), and matrix metalloproteases (MMPs; Yuan and Varga, 2001). BMPs via activation of SMAD-1/5/8, are capable of suppressing TGF-β mediated fibrotic gene expression (Wang and Hirschberg, 2003). As such, hyperactivation of activin/TGF-β-mediated SMAD-2/3 signaling promotes fibrosis, whereas increased BMP/SMAD-1/5/8 activity is likely anti-fibrotic. Here, we describe the contribution of TGF-β ligand induced SMAD signaling to the pathogenesis of human fibrosis, and emerging therapeutic strategies that target these ligands.

ACTIVATORS OF THE SMAD-2/3 AXIS AND FIBROSIS

TGF-β isoforms – TGF-β ligands that activate the SMAD-2/3 intracellular pathway have been heavily implicated in fibrosis. In particular, TGF-β1 is considered a major driver of human fibrotic pathologies. Circulating or tissue levels of TGF-β1 are elevated in human hepatic (Nagy et al., 1991), renal (Ketteler et al., 1994), and pulmonary fibrosis (Molina-Molina et al., 2006), as well as during cardiac failure (Khan et al., 2014). Exogenous TGF-β1 in rodents is sufficient to induce fibrosis in the lungs (Sime et al., 1997), and kidneys (Clouthier et al., 1997). Tissue specific pro-fibrotic activities of TGF-β1 are outlined in **Table 1**.

Mechanistically, TGF-β1 promotes fibrosis by driving the differentiation of quiescent fibroblasts into matrix secreting myofibroblasts (Vaughan et al., 2000; Lijnen et al., 2003). Under duress fibroblasts differentiate into a proto-myofibroblast lineage, which in the presence of TGF-β1 (and ED-A fibronectin), become fully differentiated myofibroblasts (Gabbiani, 2003; **Figure 1**). Notably, TGF-β1 drives the production of α-smooth muscle (α-SM) actin, which gives the myofibroblasts their contractility. These cells represent activated fibroblasts, with a high synthetic capacity for ECM proteins (Ignotz and Massague, 1986). In response to heightened TGF-β1 signals, dominating myofibroblasts deposit excessive ECM, which compromises the local tissue architecture. TGF-β1 can further exacerbate this response by acting as a chemoattractant for some inflammatory cells (Wahl, 1992; Ludviksson and Gunnlaugsdottir, 2003), and is abundantly expressed by infiltrating macrophages in fibrotic tissue (Denholm and Rollins, 1993).

TGF-β1 is ubiquitously expressed within the body, and its target receptors (ALK5 and TGFβRII) have been identified on most cell types. As such, elevated levels of TGF-β1, either locally or systemically, can result in widespread manifestations of fibrosis. Acting through the SMAD-2/3 axis, TGF-β1 drives the expression of key ECM genes, including collagens (Verrecchia et al., 2001a), fibronectin (Hocevar et al., 1999), and PAI-1 (Dennler et al., 1998). These proteins are key components of developing fibrotic tissue. Additionally, TGF-β1 has a natural affinity for the ECM, binding directly to fibrillin microfibrils (Taipale et al., 1996). As such, TGF-β1 becomes concentrated in the accumulating ECM, thereby exacerbating the fibrotic response. TGF-β1 activity is kept in-check in healthy tissues by its naturally affiliated propeptide (termed 'latency associated peptide'), which prevents TGF-β1 from complexing with its target receptors (Bottinger et al., 1996). For signaling to be initiated, an activation mechanism is required to break the propetide's hold. For TGF-β1, activators include integrins (namely $\alpha_V\beta_6$), thrombospondin-1 (TSP-1) and plasmin proteases (Lyons et al., 1990; Crawford et al., 1998; Munger et al., 1999; Morris et al., 2003). Significantly, in inflammatory conditions *de novo* expression of some/all of these activators increases (Zhang et al., 1999; Lopez-Dee et al., 2011), leading to activation and potentiation of TGF-β1 signaling (Popov et al., 2008). TGF-β1 activation is also increased in fibrotic tissue via biomechanical tissue stiffness, which causes force-dependant removal of the TGF-β1 propeptide (Wipff et al., 2007).

Although TGF-β1 is the best characterized pro-fibrotic factor within the family, TGF-β2 also displays potent fibrotic activity. TGF-β2 accumulates in the bile ducts in human fibrotic liver disease (Milani et al., 1991), and has been implicated in the fibrotic response associated with glaucoma (Wordinger et al., 2014). Remarkably, TGF-β3 appears to have anti-fibrotic activity in some tissues. TGF-β3 plays a key role in regulating epidermal and dermal cell motility during wound repair, a TGFβ-isoform-specific effect (Occleston et al., 2008). TGF-β3 is expressed at high concentrations during wound repair, and unlike TGF-β1 and -β2 isoforms, can promote wound healing without fibrotic scarring (Ferguson et al., 2009).

Activins – Similar to TGF-β1, activins can trigger a pro-fibrotic response in several tissues via activation of the SMAD-2/3 cascade. Activins promote the proliferation of fibroblasts, their differentiation into myofibroblasts (Ohga et al., 1996; Ota et al., 2003; Yamashita et al., 2004), and the accumulation of ECM (Yamashita et al., 2004; Murakami et al., 2006). Serum concentrations of activin are elevated in patients suffering cystic fibrosis (Hardy et al., 2015), acute respiratory failure (de Kretser et al., 2013), chronic kidney disease (Agapova et al., 2016), and heart failure (Yndestad et al., 2004). Indeed, increased serum activin is a hallmark of many human chronic conditions and can have catastrophic consequences for affected patients. Our studies have shown that activins can drive the multi-organ wasting syndrome, cachexia (Chen et al., 2014), and that high levels of activin can induce a marked fibrotic response in skeletal muscle and liver, characterized

TABLE 1 | Summary of evidence for TGF-β ligands in fibrosis.

LIGAND	Signaling pathway	ANTI/PRO-fibrotic	Liver fibrosis	Kidney fibrosis	Cardiac fibrosis	Muscle fibrosis	Lung fibrosis	Examples of therapeutic approaches
TGF-β1/ TGF-β2	TGF-β1 uses TGF-βRI (ALK5), TGF-βRII, and induces SMAD 2/3 signal. TGF-β2 also uses TGF-βRIII (betaglycan)	PRO	TGF-β1 induces α-SMA and type 1 collagen expression, and promotes migration/invasion of hepatic stellate cells (HSCs), the major producers of collagen in the liver (Presser et al., 2013). TGF-β2 accumulates in the bile ducts in human fibrotic liver disease, encouraging collagen deposition (Milani et al., 1991).	TGF-β1 drives differentiation of renal epithelial cells into α-SMA positive myofibroblasts, which also secrete collagen (Fan et al., 1999). In human glomerular disease, TGF-β1 protein expression is positively correlated with interstitial fibrosis severity and ECM production (Goumenos et al., 2001).	TGF-β1 and ECM production is upregulated following cardiac infarction in rats. Exogenous TGF-β1 can drive myocardial fibrosis *in vivo*. Cardiac fibroblasts differentiate into myofibroblasts in the presence of TGF-β1 (summarized in Lijnen et al., 2000).	High levels of TGF-β1 promote increased ECM deposition and attract inflammatory cells during muscle repair (summarized in Delaney et al., 2017). TGF-β1 expression is upregulated in dystrophic patients (Bernasconi et al., 1999).	TGF-β1 induces severe fibrosis in rat lungs, and is upregulated in patients suffering idiopathic pulmonary fibrosis (IPF) (Sime et al., 1997; Molina-Molina et al., 2006).	Small molecule inhibitor Pirfenidone™ (Azuma, 2012) has been approved for the treatment of IPF. TGF-β1 targeting mAbs have also been tested in human IPF patients (Voelker et al., 2017), and TGF-β2 mAbs have anti-scaring activity in human glaucoma patients (Mead et al., 2003).
TGF-β3	TGF-βRI (ALK5), TGF-βRII, induces SMAD 2/3 signal	ANTI	TGF-β3 can alleviate the degree of hepatic fibrosis and tissue injury via the suppression of type 1 collagen synthesis (Zhang et al., 2010).			TGF-β3 is upregulated in muscle fibrosis, but competes with the pro-fibrotic TGF-β1 activity (Zhao et al., 2010).		Recombinant TGF-β3 (Juvista™) demonstrated to improve wound healing during clinical trials (Ferguson et al., 2009).
Activin A	ActRIIA/ActRIIB, ALK4/7, induces SMAD 2/3 signal	PRO	Activin A is upregulated in rat models of liver fibrosis, and drives collagen production from hepatocytes (Sugiyama et al., 1998). Activin A is produced by activated HSCs *in vitro* and *in vivo*, and its activity appears to be unopposed by follistatin (Patella et al., 2006).	Activin A promotes cell proliferation, induces differentiation into myofibroblasts, and promotes expression of collagen in primary cultured renal interstitial fibroblasts in rats (Yamashita et al., 2004). Activin A expression is upregulated in multiple mouse models of chronic kidney disease (Agapova et al., 2016).	Heart failure patients have elevated activin A serum levels, which correlate with disease severity. Cardiomyocytes are the primary source of activin A production in the heart as it fails (Yndestad et al., 2004).	Activin A hyper-expression promotes muscle fibrosis, as evidenced by an increase in differentiated myofibroblasts and accompanied increase in ECM deposition (Chen et al., 2014).	Activin A expression is increased in cystic fibrosis (CF) patients, and activin inhibition reduces disease progression in a mouse model of CF (Hardy et al., 2015). Serum activin A and B concentrations are elevated in critically ill patients suffering acute respiratory failure (de Kretser et al., 2013).	Follistatin can improve muscle function and reduce fibrosis in two models of muscle disease (Mendell et al., 2015, 2017). Other ligands traps such as sActRII and propeptides work effectively in mouse models of activin-induced fibrosis (Chen et al., 2015; Agapova et al., 2016).

(Continued)

TABLE 1 | Continued

LIGAND	Signaling pathway	ANTI/PRO-fibrotic	Liver fibrosis	Kidney fibrosis	Cardiac fibrosis	Muscle fibrosis	Lung fibrosis	Examples of therapeutic approaches
Myostatin	ActRIIA/ActRIIB, ALK4/5, induces SMAD 2/3 signal	PRO			Exogenous expression of myostatin from cardiomyocytes promotes interstitial fibrosis (Biesemann et al., 2015), and myostatin expression is increased following heart injury (Sharma et al., 1999). Aging hearts in myostatin null mice exhibit reduced fibrosis (Morissette et al., 2009).	Myostatin is a negative regulator of muscle mass and overexpression results in a pro-fibrotic response, with increased myofibroblasts and ECM production (Li et al., 2008).		Follistatin and soluble ActRII blocked myostatin signaling during clinical trials, leading to a reduction in fibrosis (Attie et al., 2013; Mendell et al., 2015; Campbell et al., 2016).
BMP7	BMPRII, BMPRI (ALK3/6), induces SMAD 1/5/8 signal	ANTI	BMP7 demonstrated to inhibit liver fibrosis and suppress activation of HSCs, via downregulation of TGF-β1 and α-SMA (Wang et al., 2014).	BMP7 expression decreases in renal fibrosis, and exogenous BMP-7 is protective in multiple animal models of nephropathies (summarized in Li et al., 2015).	BMP7 activates infiltrating monocytes into anti-inflammatory M2 macrophages, which inhibits apoptosis and fibrosis in prediabetic cardiomyopathy (Urbina and Singla, 2014).		BMP7 significantly reduced the progression of silica-induced fibrosis in rats, via upregulation of the SMAD1/5/8 axis and downregulation of SMAD2/3 signaling (Yang et al., 2013).	A BMP7 mimetic, AA123, demonstrated anti-fibrotic effects in a mouse model of kidney disease (Sugimoto et al., 2012).
BMP9	BMPRII, BMPRI (ALK1/5), induces SMAD 1/5/8 signal	PRO	BMP-9 promotes liver fibrosis via HSC differentiation, and promotes collagen 1 and fibronectin production (Bi and Ge, 2014; Breitkopf-Heinlein et al., 2017). BMP9 appears to mediate these actions through ALK1/5 (Munoz-Felix et al., 2016).					BMP-9 derivatives have been examined as bone regenerative agents (Bergeron et al., 2009, Bergeron et al., 2012). No specific BMP-9 intervention has been trialed to date for fibrosis therapy.

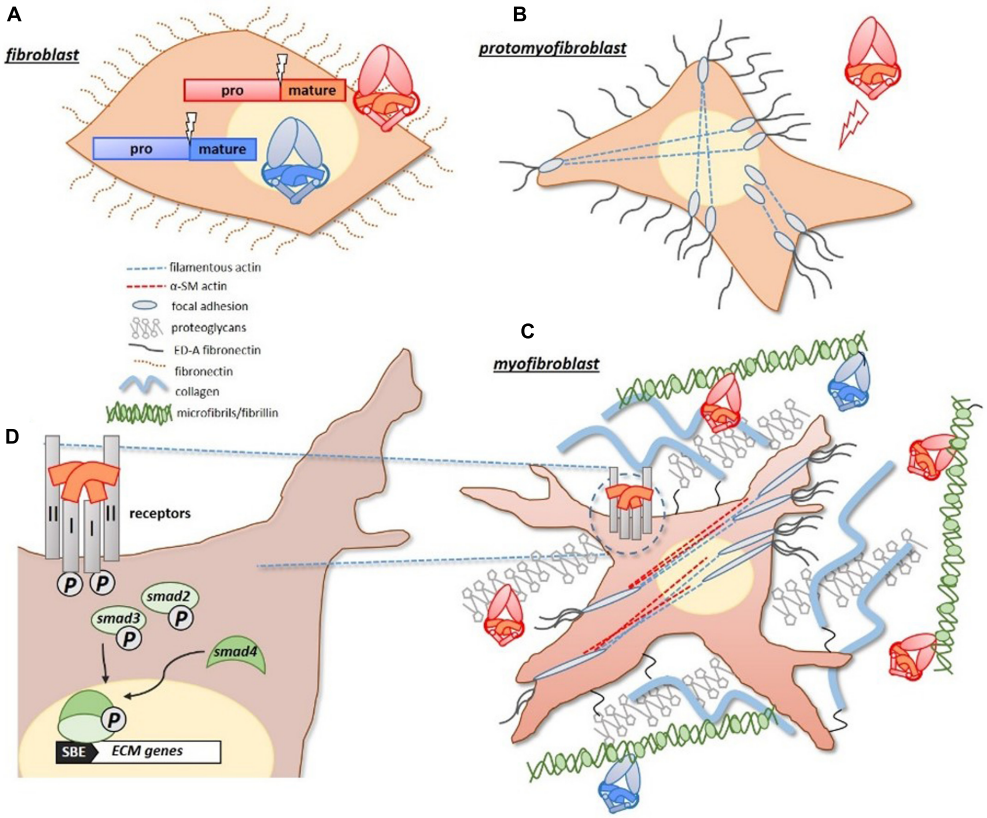

FIGURE 1 | Pro-fibrotic activities of TGF-β proteins. **(A)** Pro-fibrotic TGF-β ligands, including TGF-β1 and activins (red/orange), and anti-fibrotic BMP ligands (blue) are similarly synthesized as large precursor proteins with pro-/mature-domains. **(B)** Under duress, quiescent fibroblasts differentiate into proto-myofibroblasts which express actin filaments that extend from a focal adhesion complex, and express extracellular ED-A fibronectin. **(C)** With increased exposure to TGF-β1, and ED-A fibronectin, proto-myofibroblasts become fully differentiated matrix-secreting myofibroblasts. The identifiable presence of α-smooth muscle actin (α-SMA), gives the myofibroblasts their measurable contractility. Both TGF-β1 and activins have a natural affinity for matrix proteins, such as fibrillin (microfibrils) and proteoglycans, and become concentrated in the expanding matrix of fibrotic tissue. The BMPs can also bind to fibrillin-containing microfibrils. **(D)** The activities of TGF-β1 and activins are initiated at the cell surface, where they form receptor complexes with type I/II receptors. Receptor activation leads to an intracellular phosphorylation cascade involving SMAD-2/3 transcription factors. SMAD-2/3 complex with SMAD-4, and translocate to the nucleus where they drive the expression of target matrix genes (via a Smad Binding Element, or SBE, in the promoter). Figure generated using Gabbiani (2003) as a guide.

by an influx of differentiated myofibroblasts and accompanied ECM deposition. Significantly, we have shown that the fibrotic pathology induced by activins can be fully reversed (Chen et al., 2014, 2015), highlighting the potential of anti-activin therapy to treat muscular dystrophies, in which patients suffer severe muscular fibrosis.

Activin is one of four major TGF-β ligands that signal through the activin type II receptors, ActRIIA/ActRIIB. Activin A, activin B, myostatin (GDF-8), and GDF-11 can all complex with ActRIIA/ActRIIB and initiate SMAD-2/3 intracellular activity. Despite this, all four ligands have non-overlapping bioactivities *in vivo* owing to their cell/tissue specific expression and distinct preferences for type I receptors (ALK4, ALK5 or ALK7). Myostatin is expressed almost exclusively in skeletal muscle, and can mimic the pro-fibrotic response observed under high activin conditions (Li et al., 2008). Myostatin promotes the proliferation of fibroblasts in muscle, and induces the expression of ECM proteins including collagen and fibronectin both *in vitro* and *in vivo* (Li et al., 2008). Given the structural homology of GDF-11 with myostatin (Walker et al., 2017) and shared receptor contacts, it is predicted that at high local concentrations GDF-11 will also exhibit pro-fibrotic activity.

Unlike the TGF-β isoforms, activins are secreted in an 'active' form. To constrain their activity, activins (and myostatin) are regulated extracellularly by follistatin. Follistatin binds directly to activin-related ligands, shielding their receptor contact sites so as to limit their signaling potential. Follistatin has opposing activity to activins/myostatin in fibrosis; attenuating early liver fibrosis (Patella et al., 2006), and lung fibrosis (Aoki et al., 2005) in murine models.

Like the TGF-β isoforms, activins have a natural affinity for the ECM – binding to heparin-sulphated proteoglycans (HSPGs) such as perlecan (Li S. et al., 2010). HSPGs are upregulated in many human fibrotic conditions, including human idiopathic lung fibrosis (Jiang et al., 2010; Westergren-Thorsson et al., 2017), Duchenne's Muscular Dystrophy (Alvarez et al., 2002), liver

disease (Roskams et al., 1996) and kidney fibrosis (Ebefors et al., 2011). Additionally, heparanase, the enzyme that metabolizes the carbohydrate chains on these proteoglycans, is also upregulated in fibrotic pathologies (Lv et al., 2016). Increased heparanase activity drives the release and activation of growth factors like activin and TGF-β (Masola et al., 2014). Consequently, activins affinity for HSPGs ensures an enriched pool of bioactive growth factors in the accumulating matrix.

ACTIVATORS OF THE SMAD-1/5 AXIS AND FIBROSIS

Whilst hyper-activation of the SMAD-2/3 pathway is reported to be pro-fibrotic, signaling through the alternate SMAD-1/5 pathway is anti-fibrotic. Of all the SMAD-1/5 activators, BMP-7 has the most well documented anti-fibrotic activity. BMP-7 is expressed only in select adult tissues, including the kidney, and its expression declines in rodent models of renal fibrosis. BMP-7 counteracts TGF-β1 induced induction of myofibroblasts and ECM in multiple models of organ fibrosis (Zeisberg et al., 2003; Weiskirchen and Meurer, 2013). Addition of exogenous BMP-7 can both prevent and even reverse fibrosis in models of kidney disease (reviewed in Weiskirchen and Meurer, 2013; Li et al., 2015).

BMP-7 mediates its anti-fibrotic activity through the type I receptor, ALK-3 [also termed BMPRIA, (Sugimoto et al., 2012)]. Expression of ALK-3 increases in the early stages of kidney disease, and loss of ALK-3 exacerbates TGF-β1 mediated fibrosis, suggesting that ALK-3 is protective for fibrosis. ALK-3 is also a docking receptor for related BMP ligands, BMP-2, BMP-4 and BMP-6. Both BMP-2 and BMP-6 can similarly attenuate kidney fibrosis in rodent models of kidney disease (Yang et al., 2009; Dendooven et al., 2011), and BMP-6 expression is increased in humans and mice suffering non-alcoholic fatty liver disease (NAFLD) and is protective for hepatic fibrosis (Arndt et al., 2015). Interestingly, loss of BMP-4 is speculated to drive opposing pro-fibrotic response in cardiac tissue (Sun et al., 2013), and increased BMP-9 in the liver induces a fibrotic-like response (Breitkopf-Heinlein et al., 2017).

Like activins, BMPs are secreted from cells in an activated state. Once secreted, many of the BMPs are sequestered to the ECM by propeptide-mediated binding to fibrillins. Fibrillin binding creates a local concentration of BMPs and is thought to facilitate release of the active BMP proteins (reviewed in Wohl et al., 2016). Notably, perturbed fibrillin/microfibril assembly is a feature of human fibrosis (Wohl et al., 2016), and likely alters the bioavailability of associated BMPs. BMP activity is also restricted by extracellular antagonists Gremlin, DAN, Chordin and Noggin (reviewed in Weiskirchen et al., 2009).

TARGETING TGF-β SIGNALING FOR THE TREATMENT OF FIBROSIS

TGF-β targeted therapies in fibrosis are designed to reduce activin/TGF-β signaling via SMAD-2/3, or alternatively, promote a BMP-mediated SMAD-1/5 signal. Many approaches are currently being explored, and some have reached the clinic (e.g., TGF-β3, follistatin, BMP-7). Leading approaches and emerging new strategies targeted to TGF-β mediated SMAD signals are described here.

TGF-β targeted therapies – A plethora of TGF-β targeted therapies have been explored (reviewed in Akhurst and Hata, 2012), some of which have been designated specifically for fibrosis. These include, TGF-β1 antibodies for kidney fibrosis (Akhurst and Hata, 2012; Voelker et al., 2017), peptides (Llopiz et al., 2009), and receptor decoys for lung fibrosis (Yamada et al., 2007). Interventions for TGF-β2 mediated fibrosis include peptides for cardiac and skin fibrosis (Santiago et al., 2005; Hermida et al., 2009), and antibodies for glaucoma-related scarring (Mead et al., 2003). TGF-β3 in the form of Juvista™ (Renova) was found to improve wound healing in phase I/II human clinical trials, but failed in phase III (Ferguson et al., 2009). However, few of these approaches have resulted in positive patient outcomes. Indeed, in a recent study testing a TGF-β1 specific antibody in a diabetic model of kidney fibrosis, disease progression was not improved (Voelker et al., 2017). Several factors likely impede the clinical effectiveness of these approaches, including the presence of multiple pro-fibrotic factors in advanced disease.

Despite their non-selectively, the small molecule inhibitors have advanced furthest owing to their economical production and ease of administration. Many of these approaches target the kinase activity of the TGF-β type I (TβRI, or ALK-5) and II (TGFβRII) receptors, including compounds GW788388 (Petersen et al., 2008) and SB-525334 (Grygielko et al., 2005), and have proven anti-fibrotic activities *in vivo*. Significantly, the small molecule inhibitor Pirfenidone™ (Azuma, 2012) has been approved for the treatment of lung fibrosis in humans. Though encouraging, the promiscuous nature of these small molecule inhibitors renders them more prone to side effects (Noble et al., 2011).

Interventions that target the intracellular phosphorylation of SMAD-2/3 proteins, can also reduce TGF-β triggered fibrosis (reviewed in Munoz-Felix et al., 2015). SMAD-7, which sequesters SMAD-2/3, protects against renal fibrosis upon viral gene-delivery in a mouse model of kidney fibrosis (Terada et al., 2002). SIS3, a selective compound that targets only SMAD-3 proved to reduce the expression of ECM proteins and delayed the progression of diabetic nephropathy in a mouse model (Li J. et al., 2010).

Activin and myostatin targeted therapies – Broad-spectrum TGF-β signaling inhibitors including SMAD-7, SIS3, and many of the small molecule kinase inhibitors can also block activin and myostatin induced signaling (Inman et al., 2002; Rodgers et al., 2014). The pleiotropic nature of these inhibitors is unfavorable for systemic use, and there is a pressing need for more tailored ligand therapies. 'Ligand traps' such as follistatin, soluble forms of the activin type II receptors (sActRII), and propeptides offer improved ligand selectivity, and their increased size favors serum retention (Wakefield et al., 1990).

Follistatin therapy is the leading approach for SMAD-2/3 blockade in activin/myostatin triggered fibrosis. Gene therapy approaches deploying follistatin have reached phase I/II clinical trials in humans (Mendell et al., 2015, 2017). Local expression of follistatin is sufficient to improve muscle function and reduce fibrosis in inflammatory myopathy (Mendell et al., 2017), and muscular dystrophy (Mendell et al., 2015). Similarly, the clinically relevant forms of sActRII (RAP-011 or ACE-011/sotatercept forms) can effectively reverse fibrosis in murine models of kidney disease (Agapova et al., 2016). However, clinical advancement of sActRII has been impeded by its apparent off-target vascular effects when used for human therapy (Attie et al., 2013; Campbell et al., 2016).

Propeptides, which are natural by-products of TGF-β assembly, and have an affinity for the ECM (Harrison et al., 2011), are attractive anti-fibrotic agents. We have shown that modified forms of the activin propeptides (propeptide-Fc fusion proteins) can both potently and specifically attenuate activin-mediated pathologies in mouse skeletal muscle (Chen et al., 2015, 2017). Similarly, we and others have found that the myostatin propeptide can revert muscle damage and attenuate fibrosis in mouse models (Qiao et al., 2008; Hamrick et al., 2010; Chen et al., 2017). This approach has also been demonstrated for the TGF-β propeptide, termed 'latency associated propeptide' or LAP, which has been shown to attenuate TGF-induced pathologies *in vivo* (Bottinger et al., 1996). However, as the TGF-β1 LAP has comparable affinity for all three TGF-β isoforms, and also GDF-8/-11, further modifications are required to improve its selectivity.

BMP-targeted therapies for fibrosis – The anti-fibrotic activity of BMP-7 encouraged its application as a human therapy. However, like many TGF-β proteins, BMP-7 is poorly made and processed in mammalian cells (Swencki-Underwood et al., 2008), and upon delivery, is likely rapidly cleared from the blood (Coffey et al., 1987). To address these limitations, Sugimoto et al. (2012), developed a small molecule BMP-7 mimetic (AA123), which has the same anti-fibrotic activity as recombinant BMP-7 in a mouse model of kidney disease. A similar BMP-7 mimetic is currently in phase II clinical trials for acute kidney injury (Thrasos Therapeutics, Canada).

CONCLUSION

Extracellular matrices provide a structural framework for cells and additionally serve as a scaffold for growth factors. Excessive ECM deposition, as observed in fibrosis, compromises tissue and organ structure and function and can lead to organ malfunction and failure. TGF-β proteins are major regulators of fibrosis, and the balanced activities of the pro-fibrotic and anti-fibrotic ligands ensure tissue homeostasis. TGF-β targeted therapies in fibrosis are designed to suppress the pro-fibrotic activity of TGF-β isoforms/activins/myostatin, or heighten the activity of anti-fibrotic BMPs. Promising anti-fibrotic TGF-β targeted therapies involve the use of ligand traps (follistatin, soluble receptors, propeptides) which sequester and deter activation of pro-fibrotic signals. Though encouraging, a major hurdle for clinical transition of these ligand traps is their *in vivo* stability, tissue-specificity, and minimisation of side effects. Indeed, one of the only drugs approved for TGF-β inhibition in lung fibrosis triggered gastrointestinal upset and the appearance of rashes (Noble et al., 2011) in treated patients, and activin blockade using the sActRII ligand trap caused vasculature complications (Attie et al., 2013; Campbell et al., 2016). Additionally, the importance of TGF-β ligands in non-fibrotic processes requires titrated treatments, so as not to ablate homeostatic functions. The ultimate goal for future TGF-β targeted fibrotic therapies is to identify ligand specific inhibitors with an extended signaling range that can act precisely within fibrotic tissue.

AUTHOR CONTRIBUTIONS

KW collected supporting evidence and wrote manuscript. KJ assisted with reference collection and collating. CH edited and provided guidance for the manuscript.

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Epigenetic Modifications to H3K9 in Renal Tubulointerstitial Cells after Unilateral Ureteric Obstruction and TGF-β1 Stimulation

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Introduction: Epigenetic regulation of fibrogenesis through post-translational histone modifications (marks) may be a key determinant of progression in renal disease. In this study, we examined the distribution and acquisition of histone 3 Lysine 9 (H3K9) marks after injury and stimulation with the pro-fibrotic cytokine TGF-β1. Our focus was on their presence in activated fibroblasts (myofibroblasts) and epithelial cells (epithelial-mesenchymal transition).

Methods and Results: Immunofluorescent microscopy was used to examine global H3K9 acetylation (H3K9Ac) and tri-methylation (H3K9Me3) after unilateral ureteric obstruction (UUO) in mice. Confocal, super resolution microscopy and flow cytometry were used to determine the *in vitro* effect of TGF-β1 on structural arrangement of these marks, and their relationship with α-smooth muscle actin (αSMA) expression, a marker of myofibroblasts and early EMT. The number of individual histone marks was increased 10 days after UUO ($p < 0.05$ vs. control), with both marks clearly seen in various cell types including proximal tubules and myofibroblasts. Sub-nuclear microscopy in primary rat renal fibroblasts and a proximal tubule cell line (NRK-52e) showed that H3K9Ac was co-localized with phosphorylated-Ser2 RNA polymerase II (pRNAPol II), while H3K9Me3 was not, consistent with permissive and repressive effects on gene expression respectively. In both cell types H3K9Ac was diffusely distributed throughout the nucleus, while H3K9Me3 was found in compartments resembling the nucleolus, and in the case of the fibroblast, also juxtapositioned with the nuclear membrane. TGF-β1 had no effect on H3K9Ac marks in either cell, but resulted in a redistribution of H3K9Me3 within the fibroblast nucleus. This was unrelated to any change in mitogenesis, but was associated with increased αSMA expression.

Conclusion: These findings highlight why it is important to consider the epigenetics of each cell individually, because whilst no overall enrichment occurred, renal myofibroblast differentiation was accompanied by distinct changes in histone mark arrangements.

Keywords: epigenetics, fibroblast, fibrosis, kidney, myofibroblast, histone, TGF-β1, proximal tubule

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INTRODUCTION

After acute renal injury occurs, normal tissue repair mechanisms are often able to restore kidney function. However, if such mechanisms are disrupted, the injury is severe, or the cause of the injury persists, acute kidney injury can progress into a chronic disorder characterized by non-recoverable organ remodeling and scarring (fibrosis) (Hewitson, 2009). Renal parenchymal fibrosis therefore represents the final common pathway in all progressive chronic kidney disease (CKD).

Histologically this process manifests itself as glomerular and vascular sclerosis and tubulointerstitial fibrosis, with the last of these the best predictor of disease progression (Hewitson, 2009). The interstitial fibroblast and tubular epithelial cell are therefore major effector cells in this process. Activation of these cells by paracrine and autocrine signals is responsible for a prodigious production of extracellular matrix, in particular the collagens that constitute scar tissue. In each case activation can be recognized by *de novo* expression of α-smooth muscle actin (αSMA), a marker of myofibroblast differentiation in fibroblasts, and epithelial-mesenchymal transition (EMT) in renal tubule epithelial cells (Hewitson, 2009).

Since its first mechanistic description in the kidney nearly 30 years ago (Border and Ruoslahti, 1990), transforming growth factor -β1 (TGF-β1) is widely considered to be the preeminent fibrogenic cytokine with a multiplicity of evidence showing that it enhances fibroblast recruitment, activation and fibrogenesis both *in vivo* and *in vitro* (Meng et al., 2016). Specific cellular targets of TGF-β1 in kidney disease include fibroblasts, mesangial cells, and tubular epithelial cells amongst others. We also increasingly recognize that such differentiation pathways are not only directly regulated through paracrine signals, but also indirectly regulated through epigenetic mechanisms which integrate pro-fibrotic signals and fibrogenesis (Reddy and Natarajan, 2015).

Epigenetics refers to changes in gene activity that are stable and can be inherited over cycles of cell division, but do not involve changes in the DNA sequence. Euphemistically termed a second genetic code, epigenetic regulation of gene expression in the kidney, as elsewhere, includes RNA interference, post-replicative DNA methylation, and histone modifications (Wing et al., 2013). Although DNA methylation (Bechtel et al., 2010), and miRNA regulation (McClelland et al., 2015; Gomez et al., 2016) of kidney fibrosis have received much attention, post-translational histone modifications (marks) in nucleosomes are poorly understood.

Nucleosomes, the building blocks of chromatin, are made up of chromosomal DNA wrapped around four core histone subunits (H2A, H2B, H3, and H4). Each histone has a flexible N-terminus (tail) that protrudes from the nucleosome. These amino acids are subject to post-translational modifications including phosphorylation, sumoylation, ubiquitination, acetylation (Ac) and methylation, the last being either monomethylation (Me), dimethylation (Me2) or trimethylation (Me3). Certain Lysine (K) amino acids, for example the 9th Lysine (K9) in the histone 3 tail (H3K9), are known to be particularly prone to modification. H3KAc is generally associated with relaxed chromatin and active gene expression. On the other hand, H3K methylation can serve as an active or repressive mark depending on the Lysine residue modified and the extent of methylation. These histone modifications serve as docking sites for coactivators, co-repressors, chromatin remodeling proteins, and various other proteins. The combined effects of the various histone marks form a 'histone code' that regulates transcription (Allis and Jenuwein, 2016).

Histone mark regulation of fibroblast-like differentiation is recognized in a multiplicity of pathologies including liver (Mann et al., 2010; Perugorria et al., 2012), nasal mucosa (Cho et al., 2012), cornea (Zhou et al., 2010), lung (Guo et al., 2009), and kidney (Irifuku et al., 2016) fibrosis. Consistent with this, TGF-β1 has been shown to enrich various active histone marks and decrease levels of repressive marks at pro-fibrotic gene promoters in renal mesangial cells (Sun et al., 2010; Yuan et al., 2013), epithelial cells (Sasaki et al., 2016), and fibroblasts (Irifuku et al., 2016; Sasaki et al., 2016). However, while these studies have clearly delineated functional significance, the emphasis has been on specific gene transcription.

To investigate the changes in phenotype during differentiation further we used a combination of *in vivo* and *in vitro* techniques to examine the distribution of histone marks in experimental progressive fibrosis, and how these are regulated by TGF-β1. In our study we have emphasized the effects on cell differentiation and the morphological changes in nuclear architecture that accompany this process. We were particularly interested in Lysine 9 on histone 3 (H3K9), as it can be both acetylated and methylated (Allis and Jenuwein, 2016), and H3K9Ac (Yuan et al., 2013) and H3K9Me3 (Sun et al., 2010; Perugorria et al., 2012) have been directly implicated in fibrogenesis.

MATERIALS AND METHODS

Animal Model

Male C57Bl6 mice were obtained from Monash Animal Research Platform (Melbourne, VIC, Australia) and maintained under normal housing conditions with a 12 h light/12 h dark lighting cycle and free access to food and water. After a 4–5 day acclimatization period unilateral ureteric obstruction (UUO) was performed in mice (aged 8–10 weeks) under inhalational general anaesthesia (Methoxyflurane, Abbott, Sydney, NSW, Australia). Eighteen animals were randomly allocated to 3 or 10 days of UUO or a non-UUO control group ($n = 6$ for each group). In the case of UUO, the left ureter of each animal was obstructed with a 0.4–1.0 mm microvascular clamp (S&T, Neuhausen, Switzerland), while the contralateral ureter was left intact. The incision was sutured and mice allowed to recover with Temgesic (Burprenorphine; Reckitt Benckiser, West Ryde, NSW, Australia) administration. These experiments were approved by the Monash University Institutional Animal Ethics Committee (MARP/2015/011), which adhere to the *Australian Code of Practice for the Care and Use of Laboratory Animals for Scientific Purposes*.

At 3 days (D3; when fibrogenesis occurs) and 10 days (D10; when fibrosis is established) post-UUO, animals were sacrificed by anaesthetic overdose and kidney tissue rapidly excised and cut

into transverse sections (each containing cortex and medulla) for immersion fixation in methyl carnoy's or 4% paraformaldehyde in phosphate buffered saline (PBS). A parallel control group consisted of tissue taken from control non-UUO animals (D0).

Immunohistochemistry

Immunoperoxidase staining was used to qualitatively examine myofibroblast recruitment (αSMA staining), and collagen I and IV deposition before and after UUO, as described previously (Hewitson et al., 2010). In brief, sections of 4% paraformaldehyde fixed, paraffin embedded tissue were labeled with mouse anti-αSMA (Dako, Glostrup, Denmark) conjugated to biotin (ARK kit; Dako). Labeling was visualized with 3′-diaminobenzidine (DAB; Dako) and hematoxylin counterstaining. Similarly, prepared methyl carnoy's fixed sections were stained with goat-anti-collagen IV (Southern Biotechnology, Birmingham, AL, United States) or rabbit anti-collagen I (Biodesign International, Saco, ME, United States). Unconjugated antisera were detected with biotinylated anti-IgG secondary antibody, and biotin amplified with avidin-biotin-complex (ABC Elite; Vector, Burlinghame, CA, United States). Again labeling was visualized with DAB (Dako) and hematoxylin.

Immunofluorescence Staining

For staining of histone marks, sections of tissue fixed in 4% paraformaldehyde and embedded in paraffin were dewaxed, boiled under pressure in citrate buffer (pH 6.0), and equilibrated in PBS for 30 min. Non-specific binding sites were blocked with 10% goat serum (Vector) in 3% bovine serum albumin (BSA)/PBS (pH 7.6) containing 0.1 M glycine for 1 h at room temp (RT). Slides were then incubated with rabbit monoclonal anti- H3K9Ac (Abcam, Cambridge, United Kingdom; cat# 61231) or anti-H3K9Me3 (Abcam; Cat# 8898) in 1% BSA in PBS overnight at 4°C. For co-staining of histones and αSMA, paraformaldehyde fixed sections were treated as above, before being incubated simultaneously with monoclonal anti-αSMA (Dako) and antibodies to individual histone marks in1% BSA/PBS for 2 h at RT. Binding was visualized by addition of Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 647 anti-rabbit IgG, and/or Alexa Fluor 594 anti-mouse IgG secondaries (Life Technologies, Carlsbad, CA, United States) in 1% BSA/PBS for 2 h at RT, as appropriate. In some cases sections were then incubated with fluorescein labeled Lotus tetragonolobus lectin (LTL) (Vector) in 1% BSA in PBS for 2 h at RT after secondary antibodies to identify proximal tubule brush borders. DAPI (2 μg/mL in PBS, 15 min, RT) was used as a nuclear stain. Finally, sections were washed in PBS and mounted in Vectashield Hard Set (Vector). Low power images were taken at 20x magnification using a Zeiss AXIOSKOP2 microscope (Carl Zeiss, Oberkochen, Germany), or visualized on a Leica SP5 confocal microscope (Leica, Buffalo Grove, IL, United States) with a 63x oil objective for high power imaging.

Cell Culture and Treatments

Primary banked cell cultures of fibroblasts propagated from fibrotic kidneys (3 days after UUO) of Sprague-Dawley rats were utilized for these studies (Grimwood and Masterson, 2009). Cultures were maintained in Dulbecco's modified Eagle Medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS; Biocore, Melbourne, VIC, Australia), 2.2% HEPES, 1% L-glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL) (all Sigma) in a humidified incubator at 37°C and 5% CO_2. For experimental work, cells were seeded into 6-well plates (Costar, Corning, NY, United States) at 1×10^6 cells/well for Western blotting or in 25 cm^2 flasks (Costar) at 5×10^6 cells/flask for flow cytometric analysis. After attachment overnight and removal of floating cells, fibroblasts were typically cultured for a further 24–48 h in maintenance growth medium before switching to FCS-reduced media (1% FCS) for 24 h before experiments.

NRK-52e cells were obtained from the American Type Culture Collection (Manassas, VA, United States) and maintained in DMEM, 10% FBS and supplements as above. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air and passaged twice a week. Cells were seeded in 6-well culture plates. Near confluent NRK-52e cells were subsequently transferred to serum-free Opti-MEM (Sigma) for overnight starvation prior to each experiment.

Fibroblasts and NRK-52E were treated with 1 and 10 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ, United States) in DMEM/5% FCS respectively for 48 h. In the control groups, cells were treated with DMEM/5% FCS only. The TGF-β1 dose administered was based on that previously shown to maximally stimulate αSMA expression in (myo)fibroblasts (unpublished observations) and NRK-52e (Doi et al., 2011).

Immunocytochemistry

Fibroblasts and NRK-52e were seeded onto glass coverslips (Carl Zeiss) in 6 well plates at low density (1×10^5/well), and treated with 5%FCS/DMEM or 5%FCS/DMEM with TGF-β1 (PeproTech) for 48 h. After treatment, coverslips were washed with warm DMEM, and cells simultaneously fixed and permeabilised in 4% paraformaldehyde 0.2% Triton X-100 in PBS for 10 min at RT. After washing in PBS, cells were post-fixed in 4% paraformaldehyde in PBS for 5 min. Cells were then blocked in 10% normal goat serum/3% BSA/0.1 M glycine in PBS for 45 min. For staining, coverslips were incubated with primary antibodies (in isolation or combination) diluted in blocking buffer for 2 h at RT in a humidified chamber, washed three times in PBS (5 min each), and then incubated in Alexa Fluor-conjugated goat anti-species secondary antibodies (Life Technologies) diluted in blocker for 60 min in the dark at RT. Primary antibodies used were mouse anti-αSMA (Dako), rabbit anti-vimentin (Abcam), mouse anti-E-cadherin (BD Biosciences, San Jose, CA, United States), rabbit anti-H3K9Ac (Abcam; Cat#61231), rabbit anti-H3K9Me3 (Abcam; Cat# 8898), anti-phosphorylated RNA polymerase II (pSer2 RNA pol II; Abcam) and mouse anti-nucleoporin 62 (NUP62; BD Biosciences). Nuclear staining of DNA was performed with DAPI (1 μg/mL diluted in PBS) for 15 min at RT. Finally, coverslips were washed three times in PBS (5 min each) and mounted in Vectashield HardSet (Vector). Low power images were captured with Zeiss AXIOSKOP2 microscope (Carl Zeiss) for cell characterization, while high power images for cytoskeletal

and nuclear morphology were prepared using a Leica SP5 confocal microscope using a 63x oil immersion objective. In a separate experiment, super-resolution OMX blaze microscopy (DeltaVision, GE Healthcare, Pittsburg, PA, United States) was used to examine simultaneous fluorescent labeling of H3K9Me3 and NUP62 in fibroblasts.

For initial characterization studies, coverslips of NRK-52e were also prepared as above and incubated with the proximal tubule specific biotinylated lectin Phaseolus hemaggutinin-l (Pha-L) (Vector), or mouse anti- vimentin (Abcam) followed by a biotinylated secondary anti-mouse antibody (Vector). Labeling was visualized using an ABC Elite kit (Vector), DAB (Sigma), with hematoxylin counterstaining.

Image Deconvolution and Volume Rendering

Confocal and OMX 3D image stacks were directly imported into Huygens Professional (Scientific Volume Imaging, Hilversum, Netherlands) for deconvolution and volume rendering. All images were deconvoluted using a theoretical point spread function for each channel, and the classical likelihood estimation algorithm. The signal to noise ratios and background intensities were automatically determined and taken into account during processing.

Morphometrics

To quantify the presence of histone marks in tissue sections, a minimum of three micrographs for each section were taken using a x20 objective (Zeiss AXIOSKOP2) in a blinded fashion, at equivalent settings. Fiji-Image (Schindelin et al., 2012) was then used to quantitate the number of histone marks in each field (0.25^2 mm) by thresholding. A similar analysis of DAPI staining at each time point was used as a measure of cellularity.

For co-localisation of signal intensity in individual nuclei, we manually plotted a line at the central nuclear plane. The "plot profiler" function of Fiji-Image was used to measure signal intensity of each fluorochrome channel across the length of that line. Individual signal intensities were normalized and plotted on the same axis using GraphPad Prism (GraphPad software, La Jolla, CA, United States).

Western Blotting Analysis

Western blot analysis with previously described techniques (Hewitson et al., 2016) was variously used to determine changes in αSMA, the epithelial cell transmembrane junction protein E-cadherin, and individual histone marks, in (myo)fibroblasts and NRK-52e.

Protein samples were isolated from cell cultures using RIPA (Sigma), with total protein concentration determined by the BCA

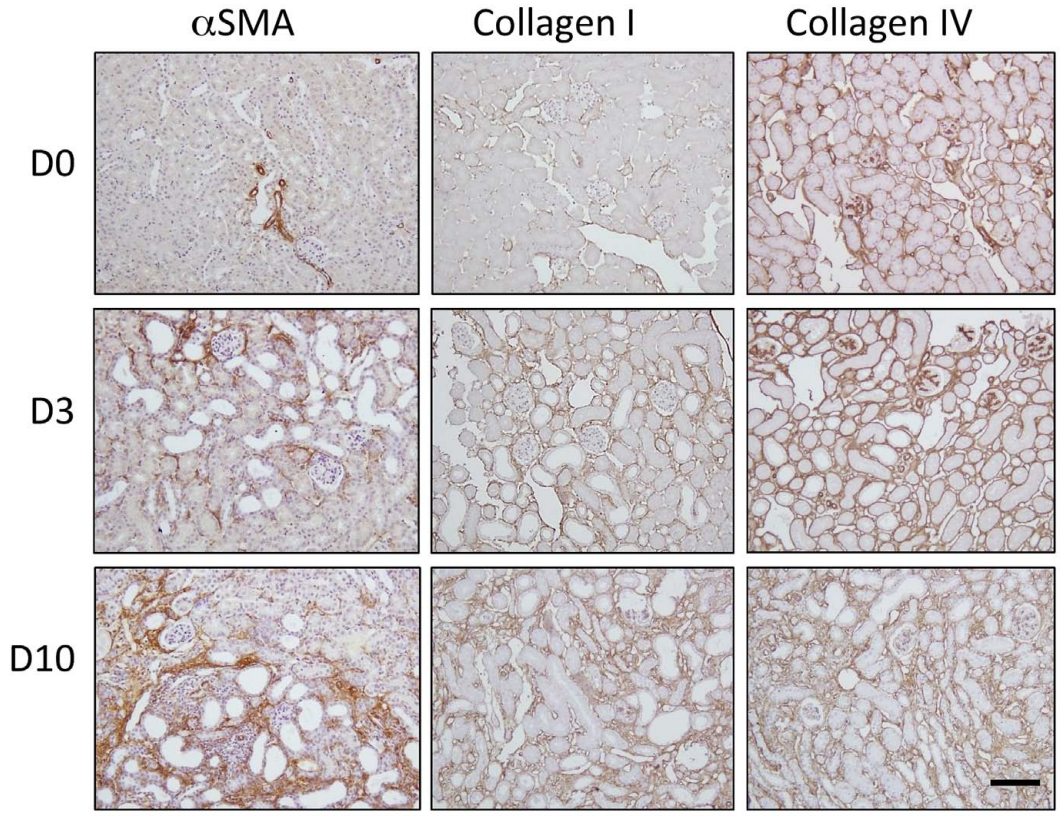

FIGURE 1 | Progression of fibrosis in the mouse after unilateral ureteric obstruction (UUO). Immunohistochemical staining for αSMA (a marker of myofibroblast recruitment) and the extracellular matrix proteins collagen I and collagen IV. Representative micrographs are shown from normal kidneys (D0) and kidneys 3 days (D3) and 10 days (D10) post-UUO. Nuclei were counterstained with hematoxylin. Scale bar = 100 μm.

assay (Thermo Scientific, Rockford, IL, United States). Histone extracts were isolated from cells using Histone Extraction Kit (Abcam). Briefly, harvested cells were resuspended in pre-lysis buffer and placed on ice for 10 min, spun 9,300 *g* for 1 min, before being resuspended in lysis buffer and incubated on ice for 30 min. After a further centrifugation (5 min, 4°C, 13,400 *g*) the supernantant fraction was collected, Balance-DTT buffer was added and the sample was stored at −80°C until analysis. Proteins (5–20 μg/lane) were separated on 10% Mini-PROTEAN TGX stain free pre-cast gels (Bio-Rad, Hercules, CA, United States) and transferred onto PVDF using the Trans-Blot Turbo transfer system (Bio-Rad). Non-specific protein binding sites were blocked with BLOTTO (Thermo Scientific) for 2 h at RT before being probed with mouse anti-αSMA (Dako), mouse anti-E-cadherin (BD Biosciences), rabbit anti H3K9Ac (Abcam; Cat# 61231), or rabbit anti-H3K9Me3 (Abcam; Cat# 8898) antibodies in incubation buffer (5% BSA supplemented with 0.1% Tween-20 in TBS) overnight at 4°C with agitation. Membranes were rinsed the following day in TBS containing Tween-20 (Bio-Rad). Primary antibody binding was identified with rabbit HRP-conjugated anti-IgG specific for the primary antibody (Abcam), and visualized using SuperSignal West Dura chemiluminescence reagent (Thermo Scientific). Following development, blots were re-probed for β-tubulin (Abcam) or anti-H3 (Cell Signaling Technology, Danvers, MA, United States), as appropriate, to confirm equivalent loading.

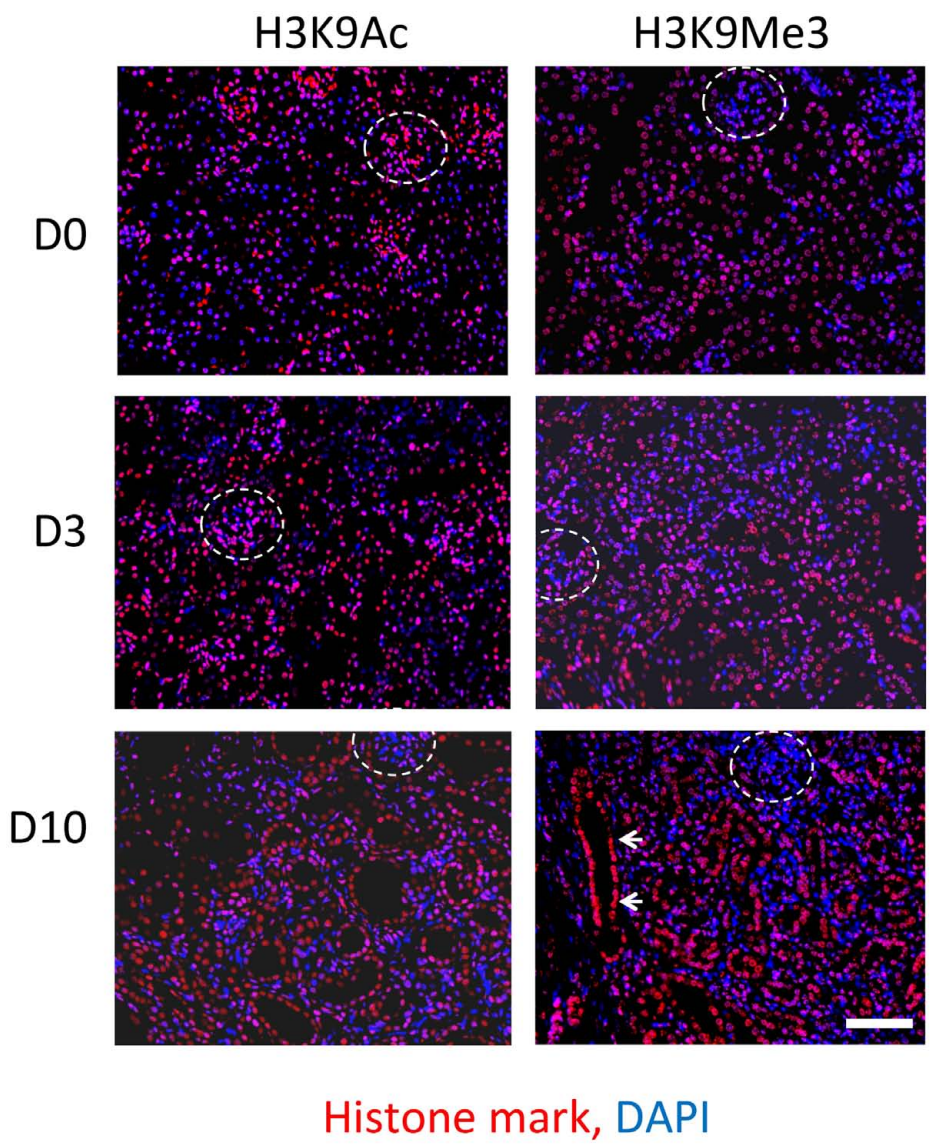

FIGURE 2 | Immunofluorescent staining of H3K9 acetylation (H3K9Ac) and tri-methylation (H3K9Me3) before and after UUO. Comparison of staining in normal mouse kidney (D0) and 3 days (D3) and 10 days (D10) after UUO. Merged images show respective histone marks (red) and nuclear DNA with DAPI (blue). Glomeruli are outlined by white dotted lines. White arrows highlight increased intensity in a dilated tubule. Scale bar = 100 μm.

Flow Cytometric Analysis

Flow cytometric analysis was used to identify changes in epigenetic cell states (Obier and Muller, 2010) and corresponding changes in αSMA expression simultaneously.

Intracellular staining of H3K9Ac, H3K9Me3, and αSMA were performed using standard protocols for indirect detection. Briefly, cells were detached from culture vessels using trypsin, and pelleted with centrifugation (x300 *g*). Cells were resuspended in 1 ml of flow cytometry staining buffer (eBioscience, Thermo Scientific), spun for 5 min at 300 *g*, with the pellet resuspended in 100 μl of BD Cytofix/Cytoperm Fixation/Permeabilisation solution (BD Biosciences). After incubation on ice for 30 min, 1 ml of BD Perm/Wash buffer (BD Biosciences) was added, and cells spun for 5 min at 300 *g*. The pellet was resuspended in 200 μl of 10% DMSO in FCS (v/v) before storage at −80°C for subsequent batched analysis. At analysis, aliquots (2×10^5 cells) were incubated with antibodies specific to each mark for 60 min at 4°C. After washing, cells were resuspended in staining buffer containing fluorochrome-labeled secondary antibodies and incubated for 30 min at 4°C in the dark. Cells were then washed and resuspended in ice-cold staining buffer for analysis. Isotype controls were from Abcam (rabbit monoclonal IgG; mouse monoclonal IgG; rabbit polyclonal IgG). For αSMA intracellular staining, fibroblasts were detached with Trypsin/EDTA and incubated with viability dye as described above. Cells were fixed with BD Cytofix fixation buffer (BD Biosciences), permeabilised with BD Phosflow Perm Buffer III (BD Biosciences), and stained with Alexa Fluor 647 rabbit monoclonal anti-αSMA (Abcam) for 30 min at RT in the dark. Cells were washed in Perm/Wash buffer (BD Biosciences) and analyzed on a BD FACSVerse (BD Biosciences) alongside cells stained with appropriate isotype control (Alexa Fluor 647 rabbit monoclonal IgG; Abcam).

Cell Cycle Analysis

Cell cycle analysis by flow cytometry with a DNA intercalating dye (propidium iodide) was used to measure the frequency of cells in the G0/G1 phase of the cell cycle. Cells were harvested with trypsin, washed twice with PBS, fixed in cold 70% (vol/vol) ethanol, and stored at 4°C until use. Before flow cytometric analysis, cells were washed with PBS and centrifuged, and the cell pellets were resuspended in a solution of RNAse (1 mg/ml) and propidium iodide (80 μg/ml) in PBS for 30 min. Stained cells were analyzed with a BD FACS Verse (BD Bioscience) flow cytometer. For each group, the software was used to calculate the proportion of cells in the G0/G1 phase of the cell cycle.

Statistical Analysis

Data are expressed as individual data points or mean ± SD, as indicated. Results were analyzed by one-way ANOVA, using the Newman–Keuls and Sidak *post hoc* tests to correct for multiple comparisons between groups. Two-tailed $p < 0.05$ was considered statistically significant.

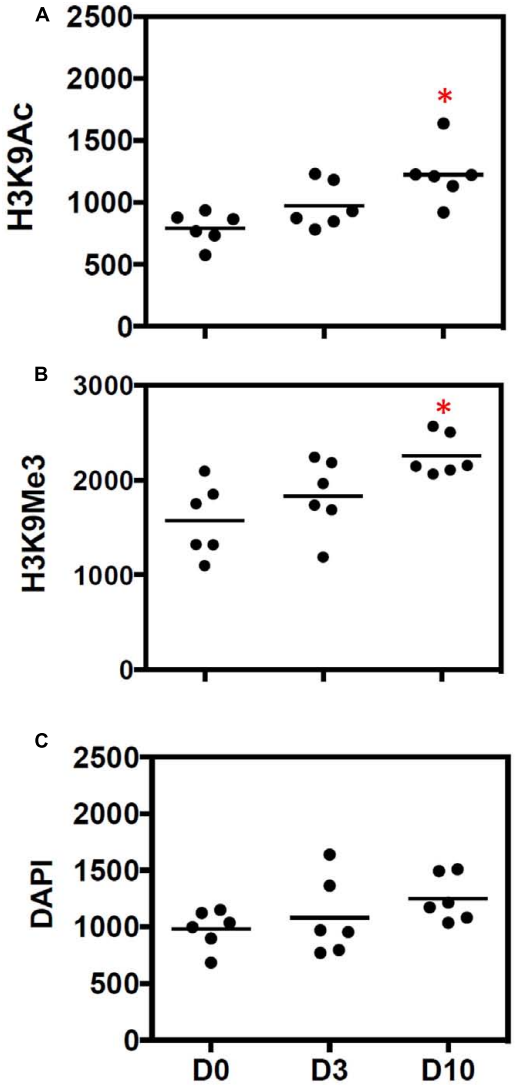

FIGURE 3 | Quantitative increase in histone marks during progressive fibrosis. Figures show the number of **(A)** H3K9Ac and **(B)** H3K9Me3 marks before (D0) and after 3 days (D3) and 10 days (D10) of UUO. Results are expressed as number of individual histone marks per 0.25 mm^2, with the average for each group shown with a bar. **(C)** DAPI staining was used to show the total DNA. $n = 6$ animals each group, $^*p < 0.05$ vs. respective D0.

RESULTS

De novo Accumulation of Myofibroblasts and Progression of Fibrosis in Obstructive Uropathy

Representative micrographs are shown from normal kidneys (D0) and kidneys 3 days (D3) and 10 days (D10) post-UUO (**Figure 1**). In the normal kidney, αSMA staining was confined to the vasculature. Collagen I and collagen IV were restricted to the interstitium and basement membranes respectively. Consistent with the well-described natural history in this model (Chevalier et al., 2009), UUO resulted in *de novo* interstitial staining

for αSMA, a marker of myofibroblast recruitment. This was paralleled by a rapid and progressive tubulointerstitial increase in collagen subtypes normally associated with the interstitium (collagen I) and the tubule basement membrane (collagen IV).

Histone H3 Lysine 9 Acetylation (H3K9Ac) and Trimethylation (H3K9Me3) Before and After UUO

Immunofluorescent staining was used to compare the spatial distribution of Lysine 9 acetylation (H3K9Ac) and trimethylation (H3K9Me3) in both the normal kidney, and after UUO (**Figure 2**). Both marks were widely distributed in the tubulointerstitium of D0 kidneys and after UUO. Likewise both marks were found in glomeruli at all time points although H3K9Me3 marks were less common and intense than H3K9Ac. Visually at least, the intensity of H3K9Me3 staining in areas of overt pathology appeared greater 10 days after UUO. Whilst both histone marks were diffusely distributed, merged immunofluorescent staining for histone marks and DAPI showed that marks were not present in all cells at any time point.

The average number of marks (per 0.25 mm^2) was increased 10 days after UUO (**Figures 3A,B**). Enumeration of nuclei through DAPI staining showed no significant change in total cellularity/0.25 mm^2 (**Figure 3C**).

Expression of H3K9 Marks in Various Anatomical Compartments of the Kidney

Laser confocal microscopy at higher magnification and resolution was used to qualitatively characterize renal expression of the histone marks H3K9Ac and H3K9Me3 in the tubulointerstitium (**Figures 4A,B**), glomerulus (**Figures 4C,E**), and vasculature (**Figures 4D,F**). Combined confocal and brightfield microscopy of D10 sections with multiple labels showed the presence of H3K9Me3 marks in both proximal tubules (LTL lectin positive) and other tubules, and myofibroblasts (αSMA positive cells) (arrows **Figures 4A,B**). Both marks were expressed in glomeruli (**Figures 4C,E**). A merging of staining for each mark and αSMA showed that H3K9Ac and H3K9Me3 were also found in the vasculature (**Figures 4D,F**).

TGF-β1 Activation of Rat Renal Fibroblast and NRK-52e *In Vitro*

TGF-β1 expression induced by UUO is known to play an important role in the development of renal fibrosis (Chevalier, 1996). Given the fundamental importance of TGF-β1 to fibrogenesis, we set out to use an *in vitro* model to further

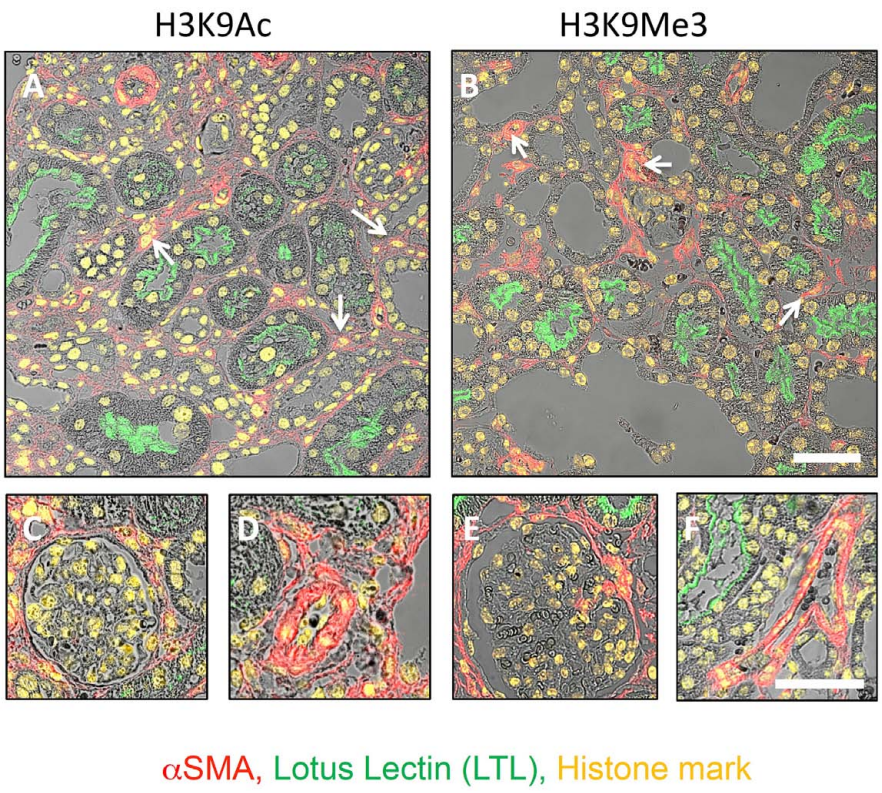

FIGURE 4 | Distribution of H3K9Ac and H3K9Me3 marks in the kidney after unilateral ureteric obstruction. Combined confocal and brightfield microscopy of sections from an animal 10 days after UUO triple labeled for each histone mark (yellow), LTL (proximal tubule brush borders; green) and αSMA (myofibroblasts; red). Micrographs illustrate the distribution of each mark in the tubulointerstitium **(A,B)**, glomerulus **(C,E)**, and vasculature **(D,F)**. Examples of histone mark positive myofibroblasts are shown with white arrows. Scale bar top panels = 50 μm, bottom panels = 25 μm.

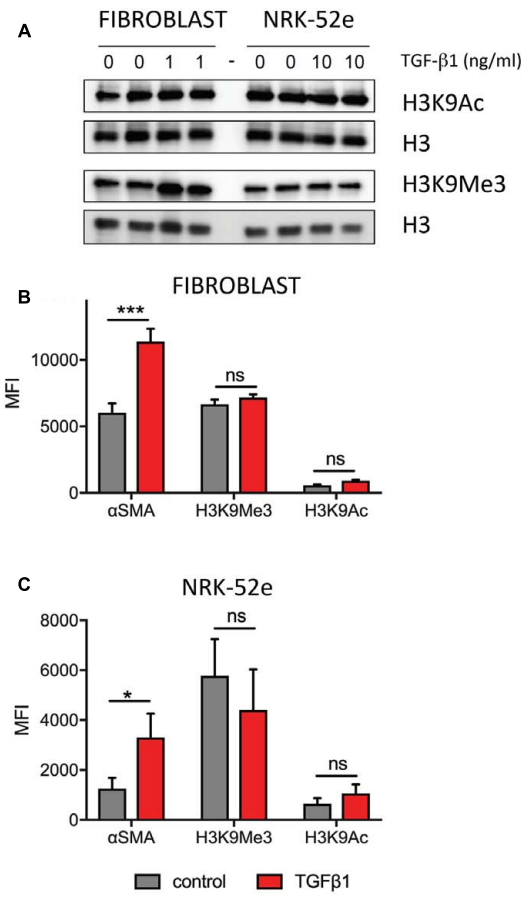

FIGURE 5 | Pooled analyses show no overall effect of TGF-β1 on H3K9 mark enrichment in (myo)fibroblasts and NRK-52e. (A) Western blotting of duplicate samples from fibroblasts and NRK-52e with and without 48 h of TGF-β1 stimulation. Parallel blots were probed for H3K9Ac and H3K9Me3. Each blot was stripped and re-probed for H3 to correct for loading. **(B)** Summary of flow cytometric analysis presented as mean fluorescent intensity (MFI) of triplicate experiments in **(B)** fibroblasts and **(C)** NRK-52e. Bars represent mean ± SD from three independent experiments. $*p < 0.05$, $***p < 0.001$ vs. control; ns, not significant.

investigate the H3K9 specific effects of TGF-β1 in individual cell populations.

To do this we firstly confirmed that our fibroblast and tubule cell cultures were responsive to TGF-β1 (Supplementary Figures S1, S2). In fibroblasts, flow cytometric analysis of forward scatter (FSC) was used to measure changes in cell size. The right shift in FSC indicated that cells grown for 48 h in media supplemented with 1 ng/ml TGF-β1 (red) are larger than their untreated counterparts (black) (Supplementary Figure S1A). Consistent with this, confocal microscopy localized αSMA to stress fibers (arrows) immediately beneath the cell membrane, a defining characteristic of activated fibroblasts, so-called myofibroblasts (Darby et al., 1990) (Supplementary Figure S1B). Treatment with 1 ng/ml TGF-β1 for 48 h increased both the number of cells staining for αSMA (myofibroblasts), and the presence of stress fibers in individual cells (Supplementary Figure S1B). Similar characterization studies of the widely used immortalized renal tubule cell, NRK-52e, confirmed their proximal tubule origin (PHA-L positive) (Supplementary Figure S2A). Cells were also positive for vimentin (Supplementary Figure S2A), a cytoskeletal protein marker of dedifferentiation in epithelia (Loghman-Adham et al., 2003). Immunofluorescent microscopy (Supplementary Figure S2B) and Western blotting (Supplementary Figure S2C) showed that αSMA expression in NRK-52e were likewise responsive to 10 ng/ml TGF-β1 over 48 h, although this effect was much more modest than in fibroblasts. This was paralleled by a reduction in the epithelial cell junction protein E-cadherin (Supplementary Figures S2B,C).

Histone Marks in Cell Cultures and Response to TGF-β1

Western blotting established expression of both marks, which was unchanged by TGF-β1 treatment (**Figure 5A**). A similar analysis was undertaken using flow cytometry to quantitate each mark, with results expressed as a mean fluorescent intensity (MFI) of three independent experiments. In agreement with the Western blot analysis, the MFI of each mark did not change in either fibroblast (**Figure 5B**) or NRK52e (**Figure 5C**) after TGF-β1 stimulation, despite an almost doubling of mean αSMA intensity in each case.

Quantitative Analysis of H3K9 Marks and Cell Phenotype

When pooling all results, there was no overall change in intensity, but analysis of individual replicate experiments showed that the MFI masked underlying subtle changes in histone marks with treatment. As in our characterization studies, flow cytometry indicated that the fibroblast cultures used consisted of two distinct populations of cells with respect to αSMA expression (**Figure 6A**). Again in accordance with our characterization studies, TGF-β1 treatment produced a single homogenous population with increased αSMA expression.

Similarly, two distinct populations of H3K9Me3 positive cells existed. Treatment with exogenous TGF-β1 also produced a homogenous population of cells with H3K9Me3 expression, albeit intermediate in intensity to that seen in untreated (myo)fibroblasts (**Figure 6A**). On the other hand, the presence of H3K9Ac was more homogenous and did not change quantitatively with TGF-β1 treatment.

The αSMA response to TGF-β1 in NRK-52e was more modest, but significant (**Figure 6B**). Again however flow cytometry showed a heterogeneous population of H3K9Me3 marks, with TGF-β1 producing a population of cells with intermediate characteristic. TGF-β1 did not result in any enrichment of H3K9Ac marks.

Sub-nuclear Distribution of Histone Marks after TGF-β1 Stimulation

As TGF-β1 seemed to induce more than simple enrichment of H3K9Me3, we used confocal microscopy to examine the sub-nuclear distribution of the two histone marks.

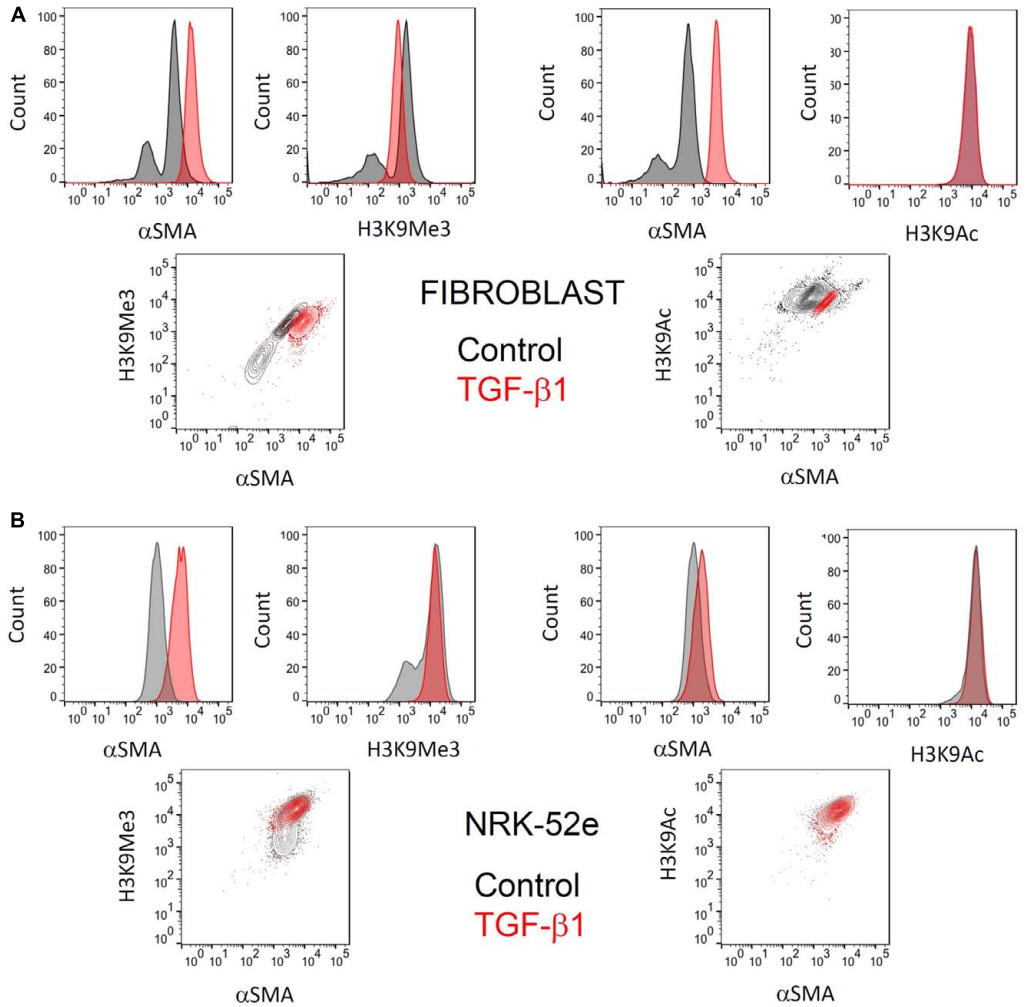

FIGURE 6 | Representative flow cytometric analysis comparing the effect of TGF-β1 stimulation on H3K9 marks and αSMA expression in (myo)fibroblasts and NRK-52e. Flow cytometric analysis of H3K9Ac and H3k9Me3 modifications, and their relationship with **(A)** myofibroblast differentiation after 48hrs of treatment with 1 ng/ml TGF-β1 and **(B)** αSMA expression in NRK52e after 48 h of stimulation with 10 ng/ml TGF-β1. Normalized frequency histograms with the control cell group shown in gray and TGF-β1 stimulated cells plotted in red. Contour plots depict changes in double-staining.

Confocal images of immunofluorescently stained (myo)fibroblast nuclei from cells incubated with and without exogenous TGF-β1 are shown in **Figure 7**. In control media, merged confocal micrographs show juxtapositioning of H3K9Me3 marks with the nuclear envelope protein nucleoporin 62 (NUP62) (**Figure 7A**). This relationship is graphically demonstrated in **Figure 7B** where the fluorescent intensity of each peaks at the nuclear periphery, with H3K9Me3 labeling adjacent but not overlapping with NUP62. Treatment with 1 ng/ml TGF-β1 resulted in a change in nuclear distribution of H3K9Me3, with loss of peripheral staining (**Figure 7B**). TGF-β1 stimulation had no effect on the distribution of H3K9Ac (**Figure 7A**).

In an additional experiment, we used OMX blaze super-resolution microscopy to resolve the relationship of H3K9Me3 with the fibroblast nuclear envelope. Individual nuclear pores can be seen with NUP62 staining. TGF-β1 resulted in a loss of peripheral H3K9Me3 enriched chromatin (**Figure 8** and Supplementary Figure S3).

The intense staining for H3K9Ac in NRK-52e was diffuse and distributed evenly throughout most of the nucleus (**Figure 7A**). The distribution was qualitatively unchanged by TGF-β1 treatment. Unlike in fibroblasts, the H3K9Me3 marks were not preferentially associated with the nuclear membrane when grown in control media, and did not change with TGF-β1 stimulation (**Figure 7A**).

Effect of TGF-β1 Stimulation on Cell Cycle Progression

Propidium iodide staining showed that TGF-β1 did not affect the proportion of cells in G0/G1 in either cell type. When grown in control media, flow cytometry showed that 72% of fibroblasts, and 86% of NRK-52e, were in G0/G1 phase. Parallel

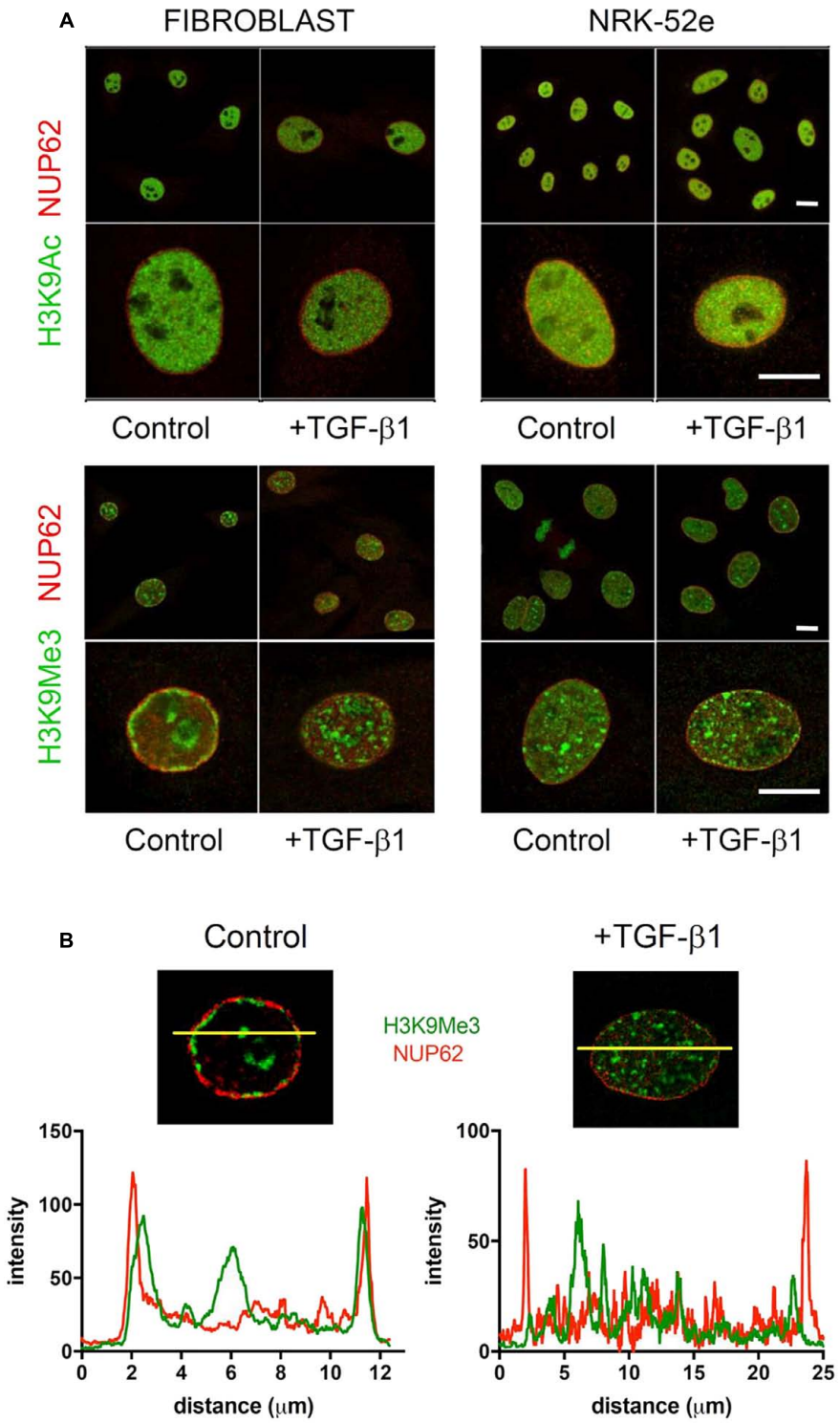

FIGURE 7 | Effect of TGF-β1 on the sub-nuclear distribution of histone marks. (A) Confocal images of immunofluorescently stained nuclei from fibroblasts and NRK-52e incubated with and without exogenous TGF-β1. Merged confocal micrographs show relative positioning of histone marks (H3K9Me3, H3K9Ac) and the nuclear envelope protein nucleoporin 62 (NUP62) in cells grown in control media, and media supplemented with TGF-β1. Scale bars = 10 μm.
(B) Co-localisation of staining intensity for H3K9Me3 and NUP62 at a central nuclear plane (yellow line) in a nucleus from each experimental group.

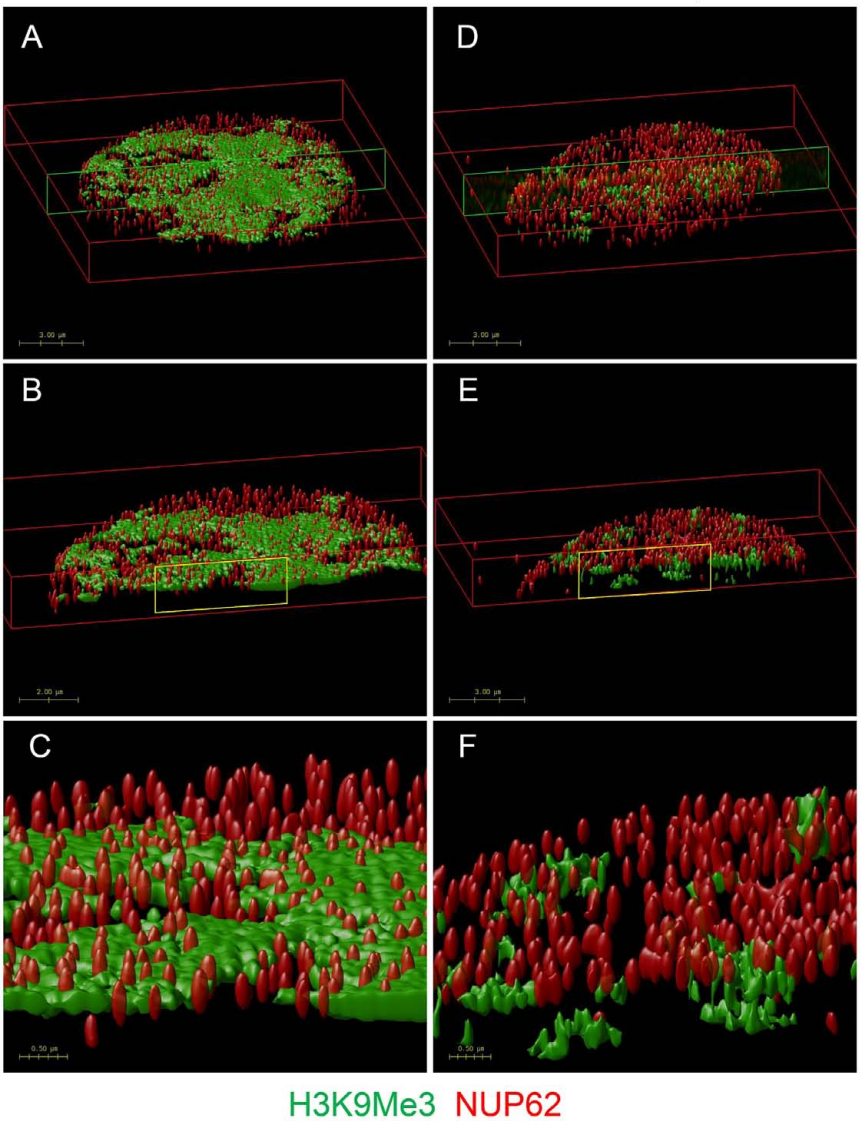

FIGURE 8 | OMX super-resolution microscopy demonstrating the effect of TGF-β1 on the nuclear distribution of H3K9Me3 marks. Volume rendered images showing effect of TGF-β1 on distribution of H3K9Me3, and its relationship with the nuclear envelope. H3K9Me3 marks are shown in green with individual nuclear pores seen with red NUP62 staining. Refer to Supplementary Figure S3 for representative optical cross sections of immunofluorescent staining before image stacking and volume rendering. Figure shows the top 2 μm of nuclei from cells grown in **(A)** control media and **(D)** media supplemented with 1 ng/ml TGF-β1. A transverse cross section of each is indicated by the green box, and illustrated in **(B,E)**. A higher magnification of the areas marked in yellow **(C,F)** clearly shows the relative position of NUP62 and H3K9Me3 with and without TGF-β1 stimulation.

groups treated with TGF-β1 had 72 and 88% of cells in G0/G1 respectively (**Figure 9**).

Sub-nuclear Localisation of Transcriptionally Permissive H3K9Ac and Transcriptionally Repressive H3K9Me3 during Fibroblast Activation

Rendered confocal images of fibroblast nuclei from cells incubated with and without exogenous TGF-β1 are shown in **Figure 10**. Merged micrographs show co-localisation (yellow) of H3K9Ac (green) and phosphorylated RNA polymerase II (pSer2 RNA pol II) (red), a marker of active transcription. Conversely, localisation of H3K9Me3 and pSer2 RNA pol II are quite distinct, which was confirmed in an analysis of staining intensity (**Figure 10B**). As above, treatment with TGF-β1 had no effect on the distribution of H3K9Ac but resulted in changes in the spatial distribution of H3K9Me3. There was a parallel loss of peri-nuclear membrane marks, and an increase in H3K9Me3 enriched chromatin

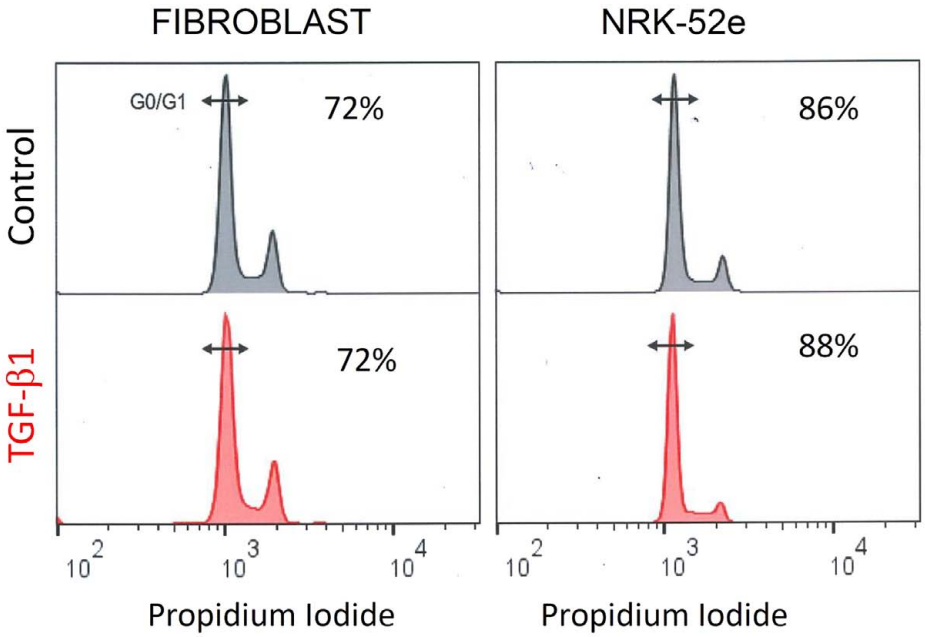

FIGURE 9 | TGF- β1 stimulation does not affect cell cycle progression in fibroblasts and NRK-52e. Representative cell cycle analysis of propidium iodide stained fibroblasts and NRK-52e epithelial cells. The cell populations were analyzed for DNA content after propidium iodide staining using flow cytometry. Percentage of cells in the G0/G1 phase for each group are indicated.

within the nucleus, in compartments that resembled the nucleolus.

Again the diffuse staining for H3K9Ac (green) and pSer2 RNA pol II (red) were co-localized (yellow) in NRK-52e nuclei, which was unchanged by TGF-β1 (**Figure 10A**). As seen previously, H3K9Ac was not preferentially localized to the nuclear periphery or compartmentalized in these cells, and was unchanged with TGF-β1 stimulation (**Figure 10A**).

DISCUSSION

In this study, we have examined the distribution and acquisition of H3K9 modifications in response to injury and TGF-β1 stimulation. Principal findings are that the histone marks H3K9Ac and H3K9Me3 are widely expressed in the normal kidney, with a small quantitative change during progressive fibrosis. *In vitro*, TGF-β1 caused a remodeling of the nuclear landscape, that was both cell and mark dependent but had no overall effect on mark enrichment.

A growing body of evidence suggests that epigenetic regulation is important in renal fibrogenesis. Most attention to date has focused on the role of non-coding micro RNA (miRNA) (Gomez et al., 2016) and DNA methylation (Bechtel et al., 2010). Less well-understood however is the role of histone modifications in this process.

How the cell deciphers the multitude of modifications that constitute the histone code has long been controversial, although most available evidence now suggests that both direct and indirect models are involved (Taverna et al., 2007; Greer and Shi, 2012). Acetylation can directly affect chromatin compaction by neutralizing the interaction between basic histones and negatively charged DNA to relax or de-condense chromatin (euchromatin), thereby increasing access for transcription machinery. In the indirect model, histone marks are read by protein effector modules termed readers, which alter transcription by recruiting various other molecules to either stabilize chromatin or facilitate transcription (Bannister et al., 2001).

To our knowledge, most contemporary studies have examined aggregate changes in histone marks using Western blotting techniques, with few studies examining the *in vivo* distribution of marks. Those that do, have not reported findings in the normal kidney (Marumo et al., 2010). The presence of both H3K9 marks in the normal kidney was more ubiquitous than we expected, although they were not found in every cell. The higher resolution offered by confocal microscopy was used to characterize tubulointerstitial distribution of marks after injury. Both H3K9Ac and H3K9Me3 were diffusely expressed in interstitial myofibroblasts, and tubule epithelial cells, including, but not limited to, proximal tubules. The quantitative changes after UUO were relatively modest, but significant and independent of changes in overall cellularity. Nonetheless, these quantitative *in vivo* changes do however suggest that progression of fibrosis involves divergent changes in gene expression regulation given their reciprocal action on transcription.

In vitro, untreated cultures consisted of a single population of H3K9Ac expressing cells by flow cytometry, the diffuse staining pattern of H3K9Ac in both fibroblasts and epithelial cells being consistent with the diffuse distribution of transcriptionally

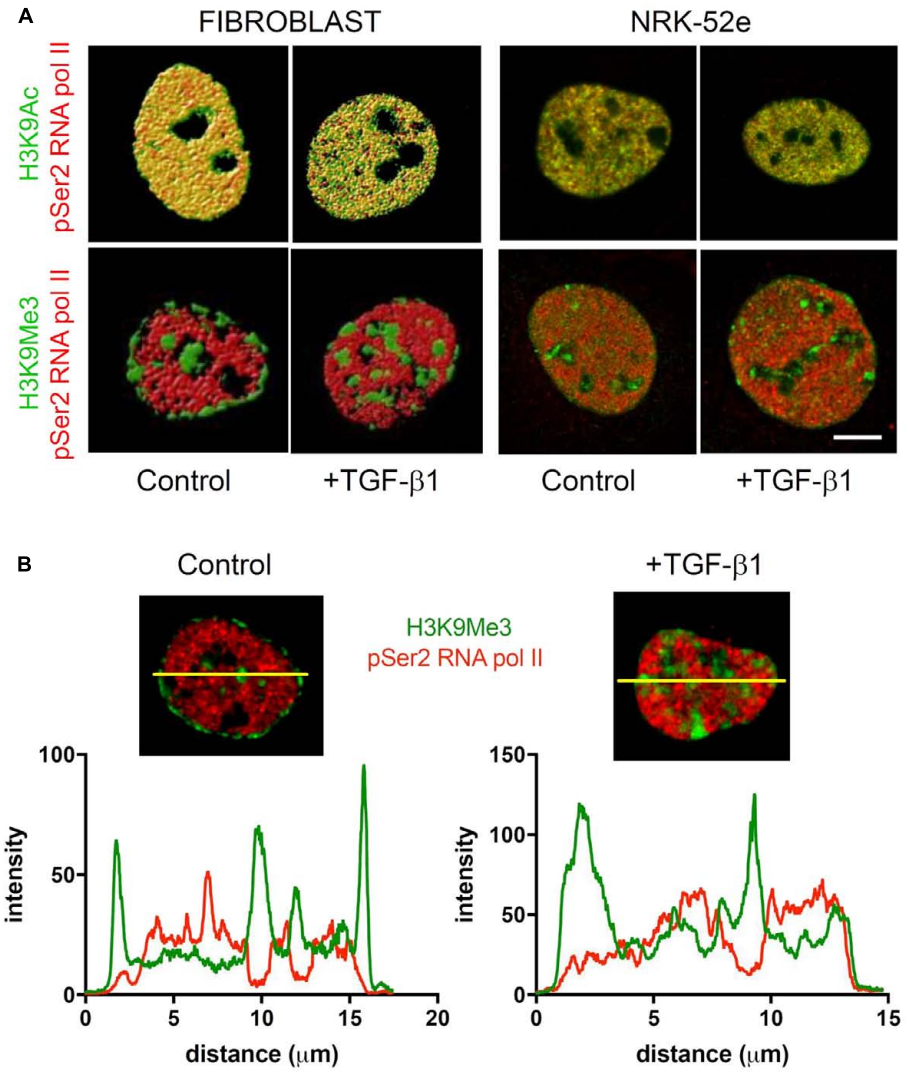

FIGURE 10 | Sub-nuclear localisation of transcriptionally permissive H3K9Ac and transcriptionally repressive H3K9Me3 in response to TGF-β1. **(A)** Volume rendered confocal images of nuclei from cells incubated with and without exogenous TGF-β1. Merged micrographs compare localisation of H3K9Ac (green)/H3K9Me3 (green) with phosphorylated RNA polymerase II (pSer2 RNA pol II) (red) in fibroblasts and proximal tubules (NRK-52e). Yellow indicates co-localisation of mark and pSer2 RNA pol II. Scale bar = 5 μm. **(B)** Co-localisation of staining intensity for H3K9Me3 and pSer2 RNA pol II at a central nuclear plane (yellow line) in a nucleus from each group.

active euchromatin (Reddy and Natarajan, 2011). Conversely, both fibroblast and epithelial cultures contained heterogeneous populations of H3K9Me3 expressing cells. However, the nuclear distribution in each was different with H3K9Me3 localized to the nuclear sub-compartments in both fibroblasts and tubule epithelial cells, and in the case of fibroblasts, also adjacent to the nuclear membrane. Transcriptionally silent heterochromatin tends to be positioned at the nuclear periphery (Andrulis et al., 1998), with stepwise methylation of histone H3K9 emerging as one pathway of assembly and heterochromatin-induced gene silencing (Towbin et al., 2013). Accordingly, we were able to co-localize H3K9Me3 with the nuclear pore protein NUP62.

In agreement with the transcriptionally permissive and repressive role of each mark (Barski et al., 2007; Koch et al., 2007), H3K9Ac was co-localized with pSer2RNA polymerase II (pRNApol II), a marker of transcriptional elongation and therefore active mRNA transcription machinery (Ghamari et al., 2013), whereas, H3K9Me3 loci were distinct from staining for pRNApol II and therefore not associated with active mRNA transcription. Fibrogenesis is associated with divergent and complex spatiotemporal changes in gene expression, and not simply a generalized upregulation of pro-fibrogenic transcripts. The rearrangement of marks seen here is consistent with our recent gene expression profiling study of TGF-β1 signaling in these cells, where TGF-β1 stimulated the concomitant activation of some genes and suppression of others (Smith et al., 2017). Further work is required to determine which genes are altered by the subtle quantitative and spatial epigenetic modifications

identified here as well as the cell-specific significance of such changes.

Flow cytometry suggested that there were underlying changes in epigenetic states not discernible at the pooled level. In fibroblasts, a more detailed analysis of this data showed that H3K9Ac marks were uniformly expressed, were unchanged by TGF-β1 stimulation, and were unrelated to myofibroblast differentiation. Conversely, TGF-β1 seemed to result in a synchronization of cells to a homogenous population with respect to H3K9Me3 staining, which clearly correlated with myofibroblast differentiation (αSMA acquisition). Interestingly this did not involve simple enrichment, or loss of marks, as staining intensity was intermediate. Super-resolution microscopy revealed further qualitative effects of TGF-β1 on H3K9Me3, showing dissociation of this mark from the nuclear membrane. Importantly, these changes in distribution were not secondary to changes in mitotic activity or cell cycle progression, as both were unaltered by TGF-β1 treatment. Taken together, these findings in the fibroblast capture hitherto unrecognized changes in H3K9Me3 nuclear dynamics induced by TGF-β1, that are not apparent using aggregative quantitative analyses.

For many years the prevailing dogma was that histone methylation marks were inherently more stable and permanent than their acetylation counterparts (Bannister et al., 2001). Our observations may provide an additional insight if the redistribution seen represents a simultaneous demethylation of existing marks and *de novo* acquisition in other nuclear compartments, rather than simply a relocation of marks to a different compartment.

The apparent increased enrichment in more interior sub-nuclear compartments is also of particular interest. The size, distribution, lack of membrane, and lack of mRNA transcription in these compartments suggest that they are likely to be nucleoli. It will be interesting to see if there is a specific role for H3K9Me3 in regulating ribosome function in these cells during fibrogenesis (Drygin et al., 2010), similar to how nucleolar enrichment of methylation marks is regulatory in liver cancer (Yu et al., 2015).

Our studies also examined the effect of TGF-β1 stimulation of NRK-52e. Notwithstanding that these cells may have already been in a transitional state, as indicated by the constitutive expression of αSMA and vimentin (Franke et al., 1979; Loghman-Adham et al., 2003; Doi et al., 2011), the distribution of H3K9Ac was homogeneous, and quantitatively unchanged after TGF-β1 stimulation. Two populations of H3K9Me3 cells existed in control cultures, with TGF-β1 exposure again resulting in a single homogenous culture with intermediate H3K9Me3 expression as in the fibroblast. The molecular nature of this synchronization in both cell types awaits elucidation.

Although beyond the scope of this work, future studies will need to examine the downstream molecular and functional effects of these structural modifications, the enzymes involved, and their relationship with TGF-β1 signal transduction. Nevertheless, our findings provide evidence of the dynamic state of H3K9 modification during renal fibrogenesis, and in particular, complex spatial changes in the nuclear distribution of H3K9Me3 induced by TGF-β1 that go beyond simple quantitative augmentation. Given the fundamental importance of the myofibroblast and TGF-β1 to fibrogenesis, these findings are likely to have implications for our understanding of the response to injury in the kidney.

AUTHOR CONTRIBUTIONS

Participated in research design, acquisition, analysis and interpretation: TH, SH, S-JT, BW, CS, ES. Wrote or contributed to writing of manuscript: TH, SH, S-JT, BW, CS, ES. Have given final approval: TH, SH, S-JT, BW, CS, ES. Agree to be accountable for the work: TH, SH, S-JT, BW, CS, ES.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2017.00307/full#supplementary-material

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Is There a Potential Therapeutic Role for Caveolin-1 in Fibrosis?

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Fibrosis is a process of dysfunctional wound repair, described by a failure of tissue regeneration and excessive deposition of extracellular matrix, resulting in tissue scarring and subsequent organ deterioration. There are a broad range of stimuli that may trigger, and exacerbate the process of fibrosis, which can contribute to the growing rates of morbidity and mortality. Whilst the process of fibrosis is widely described and understood, there are no current standard treatments that can reduce or reverse the process effectively, likely due to the continuing knowledge gaps surrounding the cellular mechanisms involved. Several cellular targets have been implicated in the regulation of the fibrotic process including membrane domains, ion channels and more recently mechanosensors, specifically caveolae, particularly since these latter contain various signaling components, such as members of the TGFβ and MAPK/ERK signaling pathways, all of which are key players in the process of fibrosis. This review explores the anti-fibrotic influences of the caveola, and in particular the key underpinning protein, caveolin-1, and its potential as a novel therapeutic target.

Keywords: caveolae, caveolin-1, cardiac fibrosis, lung fibrosis, kidney fibrosis

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INTRODUCTION

Fibrosis, a process of wound reparation, is a cellular response to tissue damage or insult, preceded by an inflammatory response. Various etiologies can act as triggers to induce inflammation and subsequent tissue damage, including infection, autoimmunity and tumors (Wynn, 2007; Wick et al., 2013). In physiological conditions, inflammation is followed by repair processes that eliminate the initial insult, through the replacement and replenishment of injured cells. The repair process progresses with the replacement of connective tissue or fibrous tissue-producing mesenchymal cells such as fibroblasts, the primary cells involved in fibrosis. Of note, this resolution step initiates

Abbreviations: $^{-/-}$, knockout; AKT, Protein Kinase B; AngII, angiotensin II; AT1R, angiotensin type 1 receptor; Cav-, caveolin-; CSD, caveolin scaffolding domain; ECM, extracellular matrix; EEA-1, endosomal antigen-1; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transformation; EndoMT, Endothelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; FSP-1, fibroblast specific protein-1; HPMCs, human primary mesothelial cells; IL-, Interleukin-; IPF, Idiopathic pulmonary fibrosis; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; MEK, Mitogen-activated protein kinase kinase; MI, myocardial infarction; MMP, matrix metalloproteinase; MMT, Mesothelial-mesenchymal transition; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PD, Peritoneal dialysis; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; R-Smad, Receptor-regulated Smad; S6K1, S6 kinase 1; siRNA, Small interfering RNA; TAK1, TGF-β-activated kinase 1; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor-α; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; α-SMA, α-smooth muscle actin.

the recruitment of leukocytes and subsequently promotes the process of apoptosis (Akbar and Salmon, 1997; Buckley et al., 2001). When the resolution step becomes dysregulated, observed by a transition from an acute to a chronic inflammatory process, fibrosis occurs which manifests as scar tissue, further promoting end-organ damage and ultimately death (Wynn, 2007; Wick et al., 2013). Chronic inflammation is essential for the progression of fibrosis as it promotes long-term tissue remodeling and subsequent organ dysfunction (Wynn, 2007; Wick et al., 2013; Zeisberg and Kalluri, 2013). The onset of fibrosis begins with epithelial or endothelial activation, which results in the release of inflammatory mediators, for both clot formation (hemostasis) and inflammatory cell recruitment. Innate immune cells, such as neutrophils, release pro-fibrotic factors or cytokines, including matrix metalloproteinases (MMPs), elastases, and cathepsins, to cleave connective tissue and in turn disrupt the basement membrane, promoting cell migratory capacities (Wynn, 2007; Wick et al., 2013). In addition, macrophages are vital for fibroblast activation, through their release of tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1). Further, macrophages also release transforming growth factor β (TGF-β), which is essential for the onset of processes involved in fibrosis including epithelial-mesenchymal transformation (EMT) (Wick et al., 2013). Morphologically, caveolae are described as flask-like or Ω shaped membrane invaginations with a diameter of 50–100 nm (Palade, 1953; Yamada, 1955). There are two main protein components essential for the formation and function of caveolae—caveolins and cavins (Drab et al., 2001; Galbiati et al., 2001). The primary function of caveolae is yet to be determined, although it has been shown to play a mediatory role in cholesterol homeostasis (Frank et al., 2008; Zhang et al., 2008), vascular reactivity (Drab et al., 2001; Razani et al., 2001a), mechanotransduction (Park et al., 1998; Sinha et al., 2011; Shihata et al., 2016), and cellular signaling (Yang and Rizzo, 2007). Importantly, caveolin-1 (Cav-1), originally called vesicular integral membrane protein of 21 kD (VIP-21), is generally accepted as the predominant protein involved in caveolae's key regulatory function in intracellular signaling pathways evident in fibrotic processes observed in different organs including cardiac, lung, and kidney fibrosis (Drab et al., 2001).

CARDIAC FIBROSIS

Cardiac fibrosis is the abnormal accumulation of extracellular matrix (ECM) in the myocardial tissue. Key to the pathogenesis of cardiac fibrosis is the limited regenerative ability of the adult mammalian heart (Soonpaa and Field, 1998). This is due to the transition phase during neonatal stages, where there is a switch from hyperplastic to hypertrophic growth, observed by an increase of myocardial mass, independent of proliferation. In the event of cardiac damage or injury, an inflammatory process is followed by the replacement of cardiomyocytes with fibrotic tissue in place of a replenished supply of cardiomyocytes (Soonpaa and Field, 1998). In the cardiac setting, Cav are present as various subtypes, however Cav-1 and Cav-3 are the predominant isoforms expressed by endothelial cells and cardiomyocytes, respectively (Tang et al., 1996).

The deletion of the gene encoding Cav-1 leads to adverse cardiac remodeling following myocardial infarction (MI). It has been demonstrated that this is due to Cav-1 knockout ($Cav1^{-/-}$) mice having an impaired inflammatory reaction, where alternative or M2 macrophages, known for promoting scar formation, accumulate more in $Cav1^{-/-}$ mice compared to WT mice. Further adoptive transfer studies, where macrophages from $Cav1^{-/-}$ mice were transferred into WT mice and vice versa, showed an increase in the survival rate of $Cav1^{-/-}$ mice that received macrophages from WT mice and decreased survival rates for WT mice who received $Cav1^{-/-}$ macrophages (Shivshankar et al., 2014). Similarly, a study evaluated the degree of fibrosis in the heart of mice with overexpression of Cav-3 compared to WT mice. Following transverse aortic constriction (TAC) procedures, mice that overexpressed Cav-3 displayed lower levels of fibrosis, and improved natriuretic levels, relative to the WT mice (Horikawa et al., 2011). Further, it has been shown that reconstitution of endothelial Cav-1 into Cav-$1^{-/-}$ knockout mice reduces gene expression of TGF-β1, collagen I and III, ultimately decreasing fibrotic lesions in the heart and improving cardiac function (Murata et al., 2007). Importantly, several studies have highlighted the major contributory role of TGF-β in the pathogenesis and regulation of cardiac fibrosis and hypertrophy (Brooks and Conrad, 2000; Deten et al., 2001; Dewald et al., 2004).

The main regulatory mechanism for TGF-β signal transduction is endocytosis regulated by Cav-1-associated lipid rafts and early endosomal antigen-1 (EEA-1) non-lipid rafts. While EEA-1 increases TGF-β1 signaling, Cav-1-associated internalization reduces, and in some cases even abolishes TGF-β1 signaling (Di Guglielmo et al., 2003; Ito et al., 2004). Without downregulation from Cav-1, ligand-receptor binding of TGF-β1 initiates the assembly of a heteromeric receptor complex by transphosphorylating the TGF-β type II receptor (TβRII), which then activates TGF-β type I receptor (TβRI) (Wrana et al., 1994). Further, TβRI induces Smad signaling by phosphorylating Smads, receptor-regulated Smad (R-Smad) 2 and 3. Smad2 and Smad3 relay the signaling process from the plasma membrane to the nucleus, via R-Smads and Smad4 that translocate to the nucleus where transcriptional changes occur (Miyazono et al., 2000). Cav-1 negatively regulates Smad signaling by binding the caveolin scaffolding domain (CSD) component, the key functional component of Cav-1, to TβRI, which in turn diminishes the downstream signaling of Smads via the TGF-β receptors (Razani et al., 2001b). The interaction between Cav-1 and TβRI impairs the phosphorylation process, and in turn inhibits the heteromerization with Smad4, which is essential for the initiation of transcriptional changes (Razani et al., 2001b). In addition to Smad signaling pathways, TGF-β also exerts its actions through non-canonical, non-Smad pathways including Mitogen-activated protein kinase (MAPK) pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase/Protein Kinase B (PI3K/AKT) pathways. Cav-1 has also been demonstrated to negatively regulate the activation state of p42/44 MAPK cascade in cardiac

fibroblasts (Galbiati et al., 1998). This finding is also supported by morphological evidence demonstrating ERK-1/2 co-localization to caveolae in an *in vivo* model of cardiac fibrosis (Liu et al., 1997).

LUNG FIBROSIS

Lung fibrosis, such as idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonitis, is the excess accumulation of ECM in lung tissue. IPF is defined as a chronic, progressive lung fibrosis condition without a specific cause and a mild level of inflammation, predominantly in older populations (Raghu et al., 2011; Travis et al., 2013). The role of Cav-1 in lung fibrosis was investigated by Kasper et al. where they induced fibrosis by irradiating alveolar cell types I and II of rats and mini pigs (Kasper et al., 1998). This study revealed downregulated Cav-1 gene expression in epithelial cells following radiation, resulting in fibrosis. Morphologically, the epithelial cells showed reduced expression of caveolae, coupled with attenuated signal transductory capacities (Kasper et al., 1998). Moreover, a five-fold increase in collagen expression via Mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) activation was observed in human lung fibroblasts when Cav-1 gene expression was reduced by 70% (Tourkina et al., 2005). Similarly, a study by Wang et al. observed a two-fold decrease in Cav-1 gene expression in lung tissue from IPF patients compared to control patients (Wang et al., 2006b). Furthermore, they investigated the therapeutic effects of Cav-1 in rat lungs using bleomycin, an antineoplastic antibiotic that causes pulmonary fibrosis as a major side effect. In another injury model, mice transfected with an adenovirus vector containing the Cav-1 gene, demonstrated resolved lung fibrosis, observed by reduced fibrotic area in the lung relative to untreated mice (Wang et al., 2006b). Like cardiac fibrosis, the formation of scar tissue in lung fibrosis is also driven by TGF-β (Khalil et al., 1991, 1994, 1996; Verma and Slutsky, 2007; Del Galdo et al., 2008). *In vitro* models of lung fibrosis have demonstrated that the anti-fibrotic effects observed are regulated by TGF-β1 via ERK and JNK activation, which are mediated by Cav-1. Indeed, TGF-β1 induces the gene expression of collagen type I via ERK1, while it regulates the production of fibronectin via c-Jun N-terminal kinase 1 (JNK1) (Wang et al., 2006b). Of note, TGF-β signaling can also mediate Cav-1 expression via non-SMAD signaling pathways. Indeed, downregulated Cav-1 has been observed in TGF-β-treated human lung fibroblasts through the activation of p38 MAPK (MEK/ERK signaling) (Sanders et al., 2015). The anti-fibrotic therapeutic potential of Cav-1 has further been highlighted in studies utilizing pharmacologically administration of CSD peptide. Similar to knockout studies, collagen deposition was reduced by more than 95% in less than 5 h in mice treated with the CSD peptide relative to the untreated mice (Tourkina et al., 2008). CSD peptide-dependent reductions in fibrosis were accompanied by inhibitions in MEK, ERK, JNK, and AKT activity as well as altering their cellular localization, confirming the anti-fibrotic effects of Cav-1 (Tourkina et al., 2008).

Like EMT, Endothelial-mesenchymal transition (EndoMT) has emerged as a key source of fibroblasts and myofibroblasts critical in the pathogenesis of fibrotic disease (Piera-Velazquez et al., 2011; Lin et al., 2012). EndoMT has been shown to be regulated via TGF-β (Li and Jimenez, 2011), with TGF-β being a potent inducer of EndoMT in pulmonary endothelial cells via the transcription factor, SNAIL1. In addition, the internalization of TGF-β receptors expressed on the cell membrane have been attributed to the cellular capabilities of Cav-1 (Razani et al., 2001b). Interestingly, pulmonary endothelial cells isolated from Cav-$1^{-/-}$ mice spontaneously underwent EndoMT. This cellular transition was further increased with the administration of TGF-β, highlighting the importance of both pathways (Li et al., 2013). Although the exact mechanisms involved in EndoMT are relatively unclear, SNAIL1 has been found to be a requirement for the induction of this process (Kokudo et al., 2008). This is supported by increased SNAIL1 gene expression observed in Cav$1^{-/-}$ mouse endothelial cells, which is reversed by the reconstitution of Cav-1, emphasizing the key mediatory role of Cav-1 in EndoMT (Strippoli et al., 2015).

Fibroblast proliferation is normally regulated by polymerized collagen via the inhibition of the PI3K-AKT-S6-kinase 1 (S6K1) signal pathway, by high phosphatase and tensin homolog (PTEN) (Xia et al., 2008, 2010). This negative feedback mechanism limits the proliferation of fibrotic tissue following injury in physiological conditions, however when the process is impaired, fibrotic conditions such as IPF may occur (White et al., 2006). Protein expression of both Cav-1 and PTEN was significantly reduced in the cellular membrane of myofibroblasts within fibroblast foci in lung cells of IPF patients' relative to control patients, compared to surrounding epithelial cells. In addition, a correlation in Cav-1 and PTEN levels was observed, which could be attributed to PTEN suppressing PI3K/AKT activation (Xia et al., 2008, 2010). This occurs through the translocation from the cytoplasm into the cellular membrane, where it is activated and in turn inhibits the signaling process. Cav-1 as an integral protein regulates PI3K/AKT transduction, where augmented expression of Cav-1 subsequently results in reduced levels of PTEN in the cellular membrane. This phenomenon is observed in Cav-1 null mice which demonstrate low expression of PTEN in the cellular membrane compared to wild type mice. Following reconstitution of Cav-1 using an adenovirus vector in the Cav-1 null fibroblast cells, a higher association of PTEN with cellular membrane and lower PI3K/AKT signaling was observed. This association was further supported by amino acid sequence analysis, showing a specialized binding sequence domain for PTEN which directly interacts with Cav-1 (Xia et al., 2008, 2010). Similarly in cardiac fibrosis and IPF, a downregulation in both Cav-1 and PTEN protein expression was observed in cardiac and pulmonary fibroblasts, respectively, relative to controls (Gao et al., 2014).

Further, MAPK pathways have been reported to play a mediatory role in TGF-β1 signaling, key for fibrotic processes, and have also been shown to be regulated by Cav-1 (Yue and Mulder, 2000; Fujita et al., 2004; Wang et al., 2006a). It has been demonstrated that Cav-1 regulates TGF-β1-induced ECM production via MAPK pathway in the lung. Over-expression of Cav-1 via transfection of adenovirus

vector inhibited TGF-β1-induced ERK and JNK activation, consequently resulting in decreased ECM production (Wang et al., 2006b). Despite the above findings highlighting the anti-fibrotic role of Cav-1, studies have also demonstrated contrasting roles for Cav-1 in tissue fibrosis. Cav-1 deficiency has been linked with significant inhibition of premature senescence of fibroblasts (Volonte and Galbiati, 2009), whereas elsewhere it has been shown that Cav-1 is needed to induce senescence in fibroblasts (Dasari et al., 2006). Indeed, Cav-$1^{-/-}$ mice were found to be protected against fibrosis induced by bleomycin relative to WT mice (Shivshankar et al., 2012). When exposed to bleomycin to induce fibrosis, WT mice displayed significant collagen deposition, whereas Cav-$1^{-/-}$ mice who did not present a significant increase (Shivshankar et al., 2012). This blunted fibrotic response could be due to reduced epithelial cell senescence and apoptosis in the Cav-$1^{-/-}$ mice. However, in contrast to these findings, a study demonstrated increased fibrosis, upregulated apoptosis and cellular senescence in Cav-$1^{-/-}$ mice exposed to bleomycin (Linge et al., 2007). The disparity in these findings could be due to the timing of fibrotic measurements and the expression of Cav-1, where it is very likely that in the early stages of exposure to bleomycin, Cav-1 is expressed which is linked with upregulated apoptosis and senescence in epithelial cells, whereas in the later stages, Cav-1 is decreased, which may be associated with the progression of fibroblast-mediated fibrosis (Shivshankar et al., 2012).

PERITONEAL AND KIDNEY FIBROSIS

Patients with terminal stage of kidney failure require either hemodialysis or peritoneal dialysis (PD). The common adverse effects following frequent long term PD is acute inflammation, which leads to chronic inflammation and eventually fibrosis (Grassmann et al., 2005; Strippoli et al., 2015). The predominant cause of acute inflammation is the continuous exposure of the peritoneal membrane of the kidney to hyperosmotic, hyperglycaemic, and acidic dialysis solutions. This leads to a specific EMT process of the mesothelial cells, known as mesothelial-to-mesenchymal transition (MMT). Like EMT, MMT is characterized by features such as loss of E-cadherin and cytokeratin, and upregulated α-smooth muscle actin (α-SMA) and fibroblast specific protein-1 (FSP-1) expression (Yanez-Mo et al., 2003; Strippoli et al., 2015). Of note, TGF-β is one of the early pro-fibrotic factors detected during PD treatment in human patients relative to control patients (Yanez-Mo et al., 2003). Further, p38 is also implicated as a key regulator of MMT, slowing down the transition process by promoting E-cadherin gene expression. E-cadherin is important for cell-to-cell adhesion and it is preserved by p38 via its inhibition of TGF-β via TGF-β-activated kinase 1-nuclear factor kappa-light-chain-enhancer of activated B cells (TAK1-NF-κB) signaling (Strippoli et al., 2010). The main suppressor of E-cadherin is the transcription factor SNAIL1, which modulates its effects via ERK or NF-κB. SNAIL1 is known as one of the major inducers of EMT and is also implicated in the downregulation of cytokeratin gene expression (Strippoli et al., 2008). Studies using Cav-1 gene silencing in human primary mesothelial cells (HPMCs) as well as mesothelial cells from Cav-$1^{-/-}$ mice have shown a loss of the typical cell structure and the acquisition of a spindle-like shape, characteristic of fibroblasts. This morphological change is also accompanied by suppression of E-cadherin gene expression. In addition, Cav-$1^{-/-}$ mice presented with increased α-SMA and collagen type I gene expression coupled with hyper-activation of ERK1 and ERK2, even in the basal state, relative to the WT mice (Strippoli et al., 2015). This was attributed to the inhibitory nature of Cav-1 on the ERK1/2 pathway. Moreover, HPMCs deficient of Cav-1 presented with enhanced ERK1/2 activity when stimulated by TGF-β. The hyper-activation of ERK1/2 leads to a stronger repression of E-cadherin gene expression via SNAIL1, accelerating the progression of MMT to fibrosis. Lentiviral overexpression of Cav-1 in HPMCs restored and reduced E-cadherin and α-SMA gene expression, respectively (Strippoli et al., 2015). The role of Cav-1 in MMT could be ascribed to the activation of ERK1/2, which directly upregulates SNAIL1 and suppresses E-cadherin and or to the diminished Smad2/3 signaling through the inhibitory effects of Cav-1 on TGF-β receptors (Xu Y. et al., 2008).

However, similar to fibrotic conditions in the lung, the role of Cav-1 is not definitive in kidney fibrosis, with several studies suggesting that Cav-1 plays a critical role in maintaining the fibrotic condition. A study conducted by Chen et al. (2012) demonstrated increased epidermal growth factor receptor (EGFR) association with phosphorylated Cav-1 in renal proximal tubular epithelial cells exposed to angiotensin II (AngII). An increase in reactive oxygen species (ROS) production was observed following angiotensin type 1 receptor (AT1R) activation, promoting Src kinase activation, Cav-1 and EGFR phosphorylation and consequently resulting in prolonged EGFR-ERK signaling which induces prolonged EMT (Chen et al., 2012). Further, this study also observed that silencing Cav-1 gene via small interfering RNA (siRNA) or via knockdown model leads to inhibited AngII activation, suggesting that EGFR association with Cav-1 leads to prolonged activation (Chen et al., 2012). These findings are in contrast to studies which have concluded that EGFR interactions with Cav-1 in caveolae or lipid rafts result in the inactivation of the EGFR (Orth and McNiven, 2006). Moreover, Chen & colleague's findings contradict a study conducted by Forrester et al. (2017) which found worsened perivascular fibrosis and hypertrophy in kidney tissue of Cav-$1^{+/+}$ compared to Cav-$1^{-/-}$ mice when administered with AngII, coupled with attenuated VCAM-1 expression in the endothelium and adventitia layer of Cav-$1^{-/-}$ mice relative to Cav-$1^{+/+}$ mice (Forrester et al., 2017).

LIVER FIBROSIS

Liver cirrhosis, the end-stage of various liver disease, is characterized by the replacement of normal physiologic hepatocyte cells with fibrotic tissue and ultimately organ failure (Elsharkawy et al., 2005; Asrani et al., 2013). Liver injury commonly results from "sinusoidal" portal hypertension,

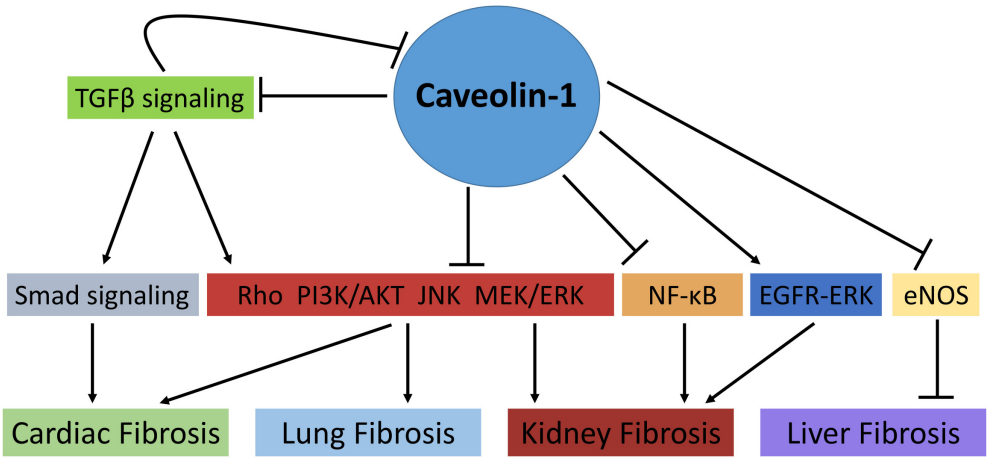

FIGURE 1 | The role of Caveolin-1 in cellular signaling mechanisms involved in fibrosis. Caveolin-1 (Cav-1) directly and indirectly regulates fibrotic processes in various tissues. In cardiac and lung fibrosis, Cav-1 prevents collagen deposition, fibroblast proliferation and TGFβ signaling through its negative regulation of Smad and non-Smad signaling pathways such as Rho-like GTPase, PI3K/AKT, MAPK (MEK/ERK), and JNK signaling pathways. Similarly, in kidney fibrosis, Cav-1 modulates fibrotic processes via the aforementioned pathways as well as NF-κB signaling. Of note, TGFβ signaling can also mediate Cav-1 expression via the activation of non-SMAD signaling pathways. Conversely, Cav-1 has been shown to promote kidney fibrosis by prolonging EGFR-ERK signaling. Moreover, in liver fibrosis, Cav-1 promotes liver cirrhosis through its negative regulation of eNOS (↓, activation; ⊥, inhibition).

primarily caused by intrahepatic shunts and hepatocyte swelling (Sherman et al., 1990; Yokomori et al., 2002). A key factor involved in liver cirrhosis is endothelial nitric oxide synthase (eNOS), which regulates the blood flow, a central parameter in portal hypertension, through its production of the potent vasodilator, nitric oxide (NO). Indeed, with reduced activity of eNOS, the liver is more susceptible to portal hypertension, leading to fibrosis and eventually cirrhosis (Matei et al., 2006). Cav-1 is a major negative regulator of eNOS, which impacts the enzyme through direct interaction of both C- and N- terminals of Cav with oxygenase domain of eNOS (Ju et al., 1997). Unlike in most organs discussed, Cav-1 is suggested to play a pro-fibrotic role in the liver. In fact, Cav-1 protein levels are increased in experimental liver disease as well as a murine model of Niemann-Pick disease type C (NPC), a disease characterized by impaired cholesterol homeostasis (Garver et al., 1997; Shah et al., 1999). Moreover, rats treated with dimethylnitrosamine (DMN) to induce liver cirrhosis demonstrate enhanced Cav-1-eNOS binding paired, with a positive correlation between Cav-1 protein expression and the degree of liver fibrosis (Xu B. et al., 2008). Similarly, Cav-1 was localized in liver sinusoidal endothelial cells (LSECs) of cirrhotic liver human samples, with an overexpression of Cav-1 in late-stage cirrhosis (Yamazaki et al., 2013). The role reversal of Cav-1 in liver fibrosis could be due to the fact that the cirrhotic liver contains higher levels of cholesterol, and Cav-1 is a major cholesterol binding protein, thus Cav-1 expression may be stimulated in chirrotic liver (Bist et al., 1997).

CONCLUSION

The pathophysiology of organ fibrosis has been studied extensively, and the anti-fibrotic role of caveolae and caveolins has been explored. Indeed, novel studies using both animal and pharmacological models have identified Cav-1 as a potential anti-fibrotic target in a number of fibrotic settings including cardiac, lung, and kidney fibrosis. Although the relationship between Cav-1 and key regulatory molecules involved in fibrotic processes such as TGF-β, ERK1/2, and Smad is understood extensively, it is still relatively unclear how they interact at the intracellular level and whether these Cav-1-associated signaling pathways can be targeted as potential therapies in fibrosis. Moreover, the tissue-specific effects of Cav-1 highlights the gap in knowledge regarding its role in fibrotic conditions. Indeed, contrasting studies highlight the dynamic role of Cav-1 in organ fibrosis, specifically in the kidney and lungs (**Figure 1**). Thus, further studies are essential to completely understand possible therapeutic effects of caveolae/caveolin-1 in fibrosis and whether its therapeutic potential is limited between varying human fibrotic conditions.

AUTHOR CONTRIBUTIONS

WS and MP drafted the manuscript and contributed to the interpretation of data in the review. WS prepared the manuscript. WS, MP, and JC reviewed, edited, and approved the final version of the manuscript.

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MINI REVIEW
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Anti-fibrotic Potential of AT_2 Receptor Agonists

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There are a number of therapeutic targets to treat organ fibrosis that are under investigation in preclinical models. There is increasing evidence that stimulation of the angiotensin II type 2 receptor (AT_2R) is a novel anti-fibrotic strategy and we have reviewed the published *in vivo* preclinical data relating to the effects of compound 21 (C21), which is the only nonpeptide AT_2R agonist that is currently available for use in chronic preclinical studies. In particular, the differential influence of AT_2R on extracellular matrix status in various preclinical fibrotic models is discussed. Collectively, these studies demonstrate that pharmacological AT_2R stimulation using C21 decreases organ fibrosis, which has been most studied in the setting of cardiovascular and renal disease. In addition, AT_2R-mediated anti-inflammatory effects may contribute to the beneficial AT_2R-mediated anti-fibrotic effects seen in preclinical models.

Keywords: AT_2 receptor, compound 21, cardiac fibrosis, renal fibrosis, inflammation

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INTRODUCTION

The renin angiotensin system (RAS) is one of the most important systems to regulate hemodynamics, blood pressure, and tissue remodeling processes. The RAS is a circulating as well as a local hormonal system (Campbell, 1987) such that local generation of angiotensin occurs in many tissues including the brain, heart, kidney, and vasculature (Te Riet et al., 2015). Various components of circulating and tissue RAS that are important for regulating vascular and cardiac contractility, fluid and electrolyte homeostasis, as well as extracellular matrix (ECM) production have been reviewed elsewhere (Campbell, 1987; Te Riet et al., 2015).

There are two major subtypes of angiotensin receptors, the angiotensin II subtype 1 receptor (AT_1R) and angiotensin II subtype 2 receptor (AT_2R) (Herichova and Szantoova, 2013; Karnik et al., 2015). It is well established that activation of AT_1R by angiotensin II (Ang II) mediates pathophysiological effects such as vasoconstriction, proliferation, fibrosis, oxidative stress, and inflammation (Sadoshima and Izumo, 1993; Ferrario and Strawn, 2006; Whaley-Connell et al., 2013), which occurs in multiple organs including heart, kidney, liver, lungs, vascular smooth muscle, and brain (Mehta and Griendling, 2007; Karnik et al., 2015). On the other hand, activation of AT_2R is thought to counter-regulate the pathophysiological effects induced by AT_1R and exert vasodilator, anti-fibrotic, anti-proliferative, and anti-inflammatory effects (Widdop et al., 2003; Jones et al., 2008) as well as natriuretic and antihypertensive effects in renal disease (Carey, 2017).

The AT_2R is also very topical in the context of neuropathic pain as it has recently been reported that an old AT_2R antagonist has been repurposed to treat neuropathic pain. For general interest, an historical account of this recent discovery is also noted (see Keppel Hesselink and Schatman, 2017). However, the current review will focus on recent evidence for an anti-fibrotic effect due to the pharmacological stimulation of AT_2R in the context of cardiovascular disease.

CARDIOVASCULAR DISEASE (CVD)

Cardiovascular disease is the leading cause of morbidity and mortality globally (Moran et al., 2014). While progress is being made in addressing CVD risk factors such as high blood pressure, diabetes, obesity, and high cholesterol (Moran et al., 2014), less therapeutic intervention has been directed at some of the underlying pathological changes occurring in relevant organs. In particular, the ECM is now considered an important site for therapeutic intervention (Rockey et al., 2015).

In the heart, for example, hypertensive heart disease is characterized by myocardial ECM expansion due to excess collagen accumulation (Weber, 1989). Injurious stimuli such as myocardial inflammation, cardiac overload, or cardiomyocyte death may activate pro-fibrotic pathways. Several cell types are involved in this process directly by producing matrix proteins (fibroblasts) or indirectly by secreting fibrogenic mediators (macrophages, mast cells, lymphocytes, cardiomyocytes) that in turn promote fibroblast-mediated ECM production. Transforming growth factor (TGF)-β1 is considered the main pro-fibrogenic mediator and promotes the transdifferentiation of fibroblasts into myofibroblasts that contribute to myofibroblast-mediated collagen synthesis leading to excess collagen complex deposition in the ECM (Tomasek et al., 2002; Rockey et al., 2015). This is one of the key cellular events that drive cardiac fibrosis. The accumulation of collagen (scar tissue) replaces cardiomyocytes that leads to the loss of structural integrity of the myocardium (Weber, 1989; Weber et al., 2013). The distinction between reactive interstititial fibrosis and reparative fibrosis, as occurs following myocardial infraction (MI), is not always well defined (Schelbert et al., 2014). In any case, the consequences of ECM expansion such as increased myocardial collagen deposition in patients results in heart dysfunction (Anderson et al., 1993; Brilla et al., 2000; Diez et al., 2002; Weber et al., 2013; Schelbert et al., 2014). Indeed, it has been estimated that fibrotic diseases contributed to about 45% of mortality in Western countries and may be higher in developing countries (Rosenbloom et al., 2013).

TARGETING RAS AS THERAPEUTIC TREATMENT FOR CVDs

Angiotensin II subtype 1 receptor blockers (ARBs) and angiotensin converting enzyme (ACE) inhibitors (ACEi) are effective treatments for hypertension based on the concept of blocking the AngII–AT_1R-axis mediated pathological effects (Karnik et al., 2015). Both treatments are effective in hypertensive patients and their antihypertensive effects appear equivalent (Vijan, 2009; Bavishi et al., 2016), although both ARBs and ACEi exhibit only limited capacity to improve cardiovascular outcome in hypertensive patients beyond blood pressure reductions (van Vark et al., 2012; Bavishi et al., 2016). By contrast, anti-fibrotic effects of ACEi and ARBs were clearly demonstrated in tissue biopsies in small well-controlled trials (Brilla et al., 2000; Diez et al., 2002; Querejeta et al., 2004), which were designed to measure cardiac ECM status (although this is clearly not possible in large outcome trials). Therefore, it was not surprising that many studies subsequently combined ACEi and ARBs (dual RAS inhibition) in the hope that this strategy would maximize any potential cardiovascular remodeling (such as fibrosis reduction) to improve clinical outcomes. However, the impact was in fact the opposite: there was an increased risk of adverse renal events such as hyperkalemia and acute renal failure together with symptomatic hypotension (Yusuf et al., 2008; Messerli et al., 2010; Makani et al., 2013). Indeed, dual RAS inhibition is now contraindicated in most cardiovascular guidelines. Clearly, novel treatments are needed that can exert anti-fibrotic effects alone or in combination with individual RAS inhibitors.

AT_2R KNOCK OUT AND OVER-EXPRESSION STUDIES

Initially, there were conflicting reports on the anti-fibrotic effects of AT_2R deletion on cardiac remodeling evoked by pressure overload, Ang II infusion, or myocardial infarction (MI) that were most likely due to the background mouse strains [see Widdop et al. (2003) for review]. Generally, there is strong evidence demonstrating the protective role of AT_2R activation since AT_2R knock out mice exhibited enhanced cardiac perivascular (Akishita et al., 2000), renal (Ma et al., 1998; Chow et al., 2014), and liver (Nabeshima et al., 2006) fibrosis following pro-fibrotic stimuli. Furthermore, cardiac overexpression of AT_2R was protective against Ang II-induced fibrosis (Kurisu et al., 2003), cardiac hypertrophy in spontaneously hypertensive rats (Metcalfe et al., 2004), and during post-infarct remodeling (Yang et al., 2002; Bove et al., 2004; Isbell et al., 2007; Qi et al., 2012). While detrimental effects of cardiac AT_2R overexpression have been reported (Nakayama et al., 2005), recent evidence suggests that there is an optimal AT_2R transgene copy number required to protect against MI-induced cardiac hypertrophy and fibrosis (Xu et al., 2014).

DIRECT PHARMACOLOGICAL AT_2R STIMULATION IS ANTI-FIBROTIC

The development of the selective nonpeptide AT_2R agonist compound 21 (C21) (Wan et al., 2004) provided another approach for the understanding of AT_2R function. Compound 21 is highly AT_2R-selective (Wan et al., 2004; Bosnyak et al.,

2011) although some off target effects such as interference with cellular calcium transport have been reported (Verdonk et al., 2012), albeit at concentrations orders of magnitude greater than its AT_2R binding affinity, as we have previously discussed (McCarthy et al., 2013). Therefore, C21 can generally be considered as a selective AT_2R agonist and has been used in this context by many research groups. In a seminal study, Kaschina et al. (2008) reported that following 7 days of treatment with C21, the scarring associated with post-MI remodeling was reduced, which correlated with significantly improved cardiac function (Kaschina et al., 2008). In addition, Gelosa et al. (2009) reported that chronic treatment with C21 reduced kidney inflammation and fibrosis in stroke-prone spontaneously hypertensive rats (SHRSP), although the main focus of this study was on stroke protection (Gelosa et al., 2009). Subsequently, there have been a handful of studies published that clearly show that AT_2R stimulation using C21 exerts anti-fibrotic effects in hearts of SHRSP (Rehman et al., 2012), following chronic MI in rats (Lauer et al., 2014), in vasculature of rats treated with the nitric oxide synthase inhibitor L-NAME (Paulis et al., 2012), and in lungs during pulmonary hypertension (Bruce et al., 2015). While C21 is generally considered to be AT_2R selective (Bosnyak et al., 2011), not all these studies used the AT_2R antagonist to confirm an AT_2R effect. In addition, renal anti-fibrotic effects of C21 have also been reported in kidneys insulted by doxorubicin (Hrenak et al., 2013) or different forms of diabetic nephropathy (Castoldi et al., 2014; Koulis et al., 2015). Details of all aforementioned studies are provided in **Table 1**. Collectively, these studies document a protective role of the C21–AT_2R axis against organ fibrosis. Intriguingly, it was recently reported that the AT_2R may form heterodimers with other class A G-protein-coupled receptors, such as relaxin family peptide receptor (RXFP1) to regulate fibrosis progression, as the anti-fibrotic effects of relaxin in the kidney were actually prevented by genetic or pharmacological inhibition of AT_2Rs (Chow et al., 2014).

TABLE 1 | Summary of anti-fibrotic and related protective effects evoked by chronic treatment with C21.

CVD model	Effect of AT_2R stimulation by C21	Reference
Cardiac/vasculature effects		
Myocardial infarction (MI) in Wistar rats: MI @ 7 days exhibited reduced cardiac function, scar formation, and peri-infarct apoptosis and inflammation	C21 (0.03, 0.3 mg/kg/d IP) for 7 days post-MI: Improved MI-impaired cardiac function (echocardiography and cardiac catheterization); decreased scar (by MRI); Decreased inflammation (mRNA cytokines); and apoptosis (caspase 3, Fas ligand) in peri-infarct zone; C21 effects blocked by PD123319	Kaschina et al., 2008
Stroke-prone SHR (SP-SHR); 13 weeks old @ study end: Exhibited modest fibrosis and inflammation in heart and coronary and aortic vessels	C21 (1 mg/kg/d in chow) for 6 weeks: Prevented vascular fibrosis (coronary and aorta) and stiffness (mesenteric); reduced vascular inflammation and oxidative stress (aorta); Decreased cardiac interstitial and perivascular myocardial collagen; unchanged cardiac MMP2/9; Reduced renal inflammatory/T cell infiltration	Rehman et al., 2012
L-NAME-treated Wistar rat; 16 weeks old @ study end: Exhibited increased aortic wall thickness, stiffness, and fibrosis	C21 (0.3 mg/kg/d PO) for 6 weeks with L-NAME: Partially prevented vascular wall stiffening and fibrosis and reduced pulse wave velocity	Paulis et al., 2012
MI in Wistar rats: MI @ 6 weeks exhibited LV remodeling with increased collagen, TGF-β1, MMM2/9, and decreased TIMP1; associated with impaired function (by echo)	C21 (0.03 mg/kg/d IP) for 6 weeks post-MI: Improved MI-impaired cardiac function (echocardiography); Reduced cardiac interstitial fibrosis and TGF-β1 in LV; Decreased MMP2/9; increased TIMP1 and MMP9/TIMP1 ratio	Lauer et al., 2014
Pulmonary hypertension in Sprague Dawley rats; studied 4 weeks after monocrotaline (MCT): Exhibited increased RV pressure; lung fibrosis; RV fibrosis; and increased lung mRNA for TGF-β1, TNF-α, and IL-1β	C21 (0.03 mg/kg/d IP) for 2 weeks; started 2 weeks after MCT: Improved MCT-impaired RV function; Reversed lung and RV fibrosis; Reversed pro-fibrotic and pro-inflammatory cytokines in lungs (mRNA); C21 effects blocked by PD123319 or MasR antagonist	Bruce et al., 2015
Disease model	**Effect of AT_2R activation**	**Reference**
Renal effects		
SP-SHR (4 weeks old) fed high salt diet for ∼8 weeks: Exhibited early development of proteinuria, glomerulosclerosis, and renal fibrosis; later accompanied by brain lesions (by MRI)	C21 (0.75, 5, and 10 mg/kg/d PO) for duration of high salt: Highest C21 dose was effective and delayed brain lesions and delayed proteinuria; Reduced glomerulosclerosis, renal fibrosis, and macrophage infiltration; decreased epithelium/mesenchymal differentiation	Gelosa et al., 2009
Doxorubicin-induced renal toxicity in Wistar rats; studied 4 weeks later: Exhibited decreased glomerular density, increased renal oxidative stress	C21 (0.3 mg/kg/d PO) for 4 weeks post-doxorubicin: Renal fibrosis unchanged; Reduced oxidative stress and restored glomerular density	Hrenak et al., 2013
Zucker diabetic fatty rats; 20 weeks old @ study end: Exhibited diabetic nephropathy including glomerulosclerosis, albuminuria, and renal fibrosis	C21 (0.3 mg/kg/d IP) for 15 weeks; Reduced renal glomerular, tubulointerstitial, and perivascular fibrosis; Reduced macrophage infiltration, but modest reduction in albuminuria (only for first 6 weeks of C21)	Castoldi et al., 2014
Streptozotocin in $ApoE^{-/-}$ mice (5 weeks old); studied 20 weeks later: Exhibited diabetic nephropathy including glomerulosclerosis, albuminuria, increased pro-fibrotic and pro-inflammatory cytokines	C21 (1 mg/kg/d PO) for 20 weeks post-STZ; Reduced glomerulosclerosis, mesangial expansion, albuminuria; inhibited many markers of oxidative stress, inflammation, and fibrosis; increased MMP2/9	Koulis et al., 2015

POTENTIAL ANTI-FIBROTIC MECHANISMS OF AT_2R

A number of anti-fibrotic mechanisms are likely to be associated with the changes evoked by C21 (**Table 1**). The activation of the pro-inflammatory nuclear factor-κBα (NFκB) pathway is a central transcriptional effector of inflammatory signaling. Nuclear factor-κB activation triggers gene transcription of many inflammatory cytokines, chemokines, and vascular adhesion molecules such as TNF-α, IL-1β, and IL-6 in fibrotic hearts (Torre-Amione et al., 1996; Francis et al., 1998; Plenz et al., 1998).

Rompe et al. (2010) were the first to show that C21 could exert a direct anti-inflammatory effect as C21 inhibited NFκB activation leading to reduced TNF-α-mediated IL-6 release from human dermal fibroblasts. The anti-fibrotic effect caused by C21 was consistently associated with reduced inflammatory responses and inflammatory cell infiltration in a variety of animal models/organs (**Table 1**) and in other studies not directly assessing fibrosis (Matavelli et al., 2011, 2015; Sampson et al., 2016). In particular, C21-mediated renal anti-inflammatory effects occurred within 4 days in hypertensive rats (Matavelli et al., 2011) and modestly protected against diabetic nephropathy in a short-term (4 week) model in rats (Matavelli et al., 2015) whereas C21 consistently evoked renal anti-inflammatory and anti-fibrotic effects in a longer term model of diabetic nephropathy in rats (Castoldi et al., 2014) and mice (Koulis et al., 2015). Taken together, these studies suggest that C21 inhibits inflammatory responses during the development of fibrosis via activation of AT_2R.

TGF-β1 is a major pro-fibrotic factor that plays a key role in the development of tissue fibrosis (Lijnen et al., 2000). TGF-β stimulates fibroblasts to differentiate into pro-secretory myofibroblasts that in turn enhance ECM protein synthesis (Desmouliere et al., 1993; Tomasek et al., 2002; Berk et al., 2007). At the same time, matrix metalloproteinases (MMPs) degrade ECM proteins and this process is tightly controlled by tissue inhibitors of metalloproteinases (TIMPs) (Weber et al., 2013). However, in an injured organ, TGF-β1 upregulates the expression of protease inhibitors such as plasminogen activator inhibitor (PAI)-1 and TIMPs which contribute to ECM preservation (Schiller et al., 2004).

Given that macrophages are a source of TGF-β1, the inhibition of macrophage infiltration via AT_2R activation could contribute to reduced TGF-β1 stimulation of fibrotic pathways. In addition, direct stimulation of AT_2R is well known to increase nitric oxide and cyclic guanosine monophosphate (cGMP) levels, particularly in the kidneys (Siragy and Carey, 1996, 1997) and vasculature (see Widdop et al., 2003), noting that decreased cGMP levels following AT_2R stimulation have also been reported (Karnik et al., 2015). Importantly, *in vivo* treatment with C21 increased NO/cGMP levels in kidneys (Matavelli et al., 2011, 2015) in keeping with a predominant AT_2R-cGMP stimulatory effect. Interestingly, cGMP was reported to inhibit TGF-β signaling (Gong et al., 2011), thereby providing another mechanism for AT_2R stimulation to modify fibrosis production. Indeed, a number of the anti-fibrotic effects of C21 already described were associated with marked reductions in TGF-β1 in heart (Lauer et al., 2014), lung (Bruce et al., 2015), and kidney (Matavelli et al., 2011; Koulis et al., 2015), suggesting that the inhibition of the TGF-β1 cascade is a common mechanism of the anti-fibrotic effect caused by AT_2R activation. As TGF-β1 acutely increased AT_2R expression in skeletal muscle (Painemal et al., 2013), it is possible that a similar compensatory response to cardiovascular injury contributes to increased AT_2R expression in CVD, although the role of such interactions on AT_2R expression during chronic AT_2R stimulation is not known.

In terms of collagen metabolism affecting ECM turnover, the effect of AT_2R activation on collagen degradation and the regulation of the MMP/TIMP balance is likely to depend on the experimental conditions studied, such as whether the main driver for fibrosis is reparative (in the case of MI) or persistent reactive fibrosis (in the case of hypertensive heart disease). Associated with the anti-fibrotic effect of C21, MMP2/9 levels were either unchanged in SHRSP hearts (Rehman et al., 2012), increased

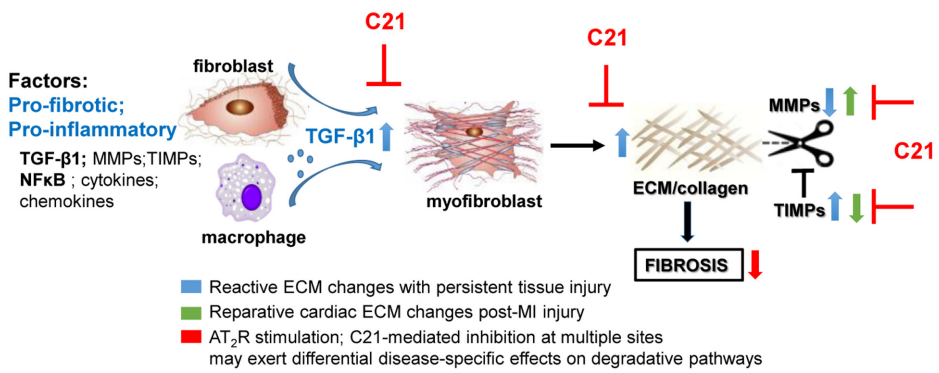

FIGURE 1 | Potential mechanisms involved in the anti-fibrotic actions of AT_2R stimulation based on the effects of C21 (inhibitory sites in red). AT_2R stimulation consistently reduces inflammatory and pro-fibrotic factors such as TGF-β1 thereby inhibiting myofibroblast differentiation and ECM production. However, the effects of AT_2R stimulation on ECM turnover may differ depending on the type of fibrosis/disease model studied. C21 inhibited the proteolytic left ventricular expansion associated with MI-induced injury (green arrows) whereas AT_2R stimulation is more likely to inhibit ECM preservation (blue arrows) associated with persistent injury (e.g., hypertension), thus facilitating ECM degradation.

in diabetic murine kidneys (Koulis et al., 2015), or decreased in MI-injured rat hearts (Lauer et al., 2014). These discrepant results are likely to reflect the different requirements of ECM in such models. For example, following MI, cardiac TGF-β1 and MMP levels were elevated whereas cardiac TIMP levels were reduced (Lauer et al., 2014). These somewhat opposing changes caused by MI itself, i.e., pro-fibrotic TGF-β1 activity together with increased proteolytic activity seen by raised MMP-9/TIMP-1 ratio, reflects the need to repair and remodel the heart following MI. In this instance, C21 appears to protect the heart by reducing widespread collagen production (decreased TGF-β1) and attenuating volume expansion (decreased MMP-9/TIMP-1 ratio). By contrast, the ability of C21 to reduce fibrosis in persistent reactive fibrotic models of CVD probably reflects both impaired collagen production (decreased TGF-β1 and collagen), as well as increased degradation due to raised MMP levels (Koulis et al., 2015), which is clearly different to abruptly developing MI-induced cardiac remodeling (**Figure 1**).

CONCLUSION AND FUTURE DIRECTIONS

Collectively, these studies demonstrate that pharmacological AT_2R stimulation evokes decreases in organ fibrosis, most studied in the heart and kidneys to date. The effects of C21 on cardiac ECM remodeling may differ depending on the preclinical fibrotic model studied (**Figure 1**), which is likely to reflect the prevailing circumstances in response to injury, i.e., replacement fibrosis following MI versus persistent reactive interstitial fibrosis seen in hypertensive heart disease. However, AT_2R stimulation also usually involves an anti-inflammatory effect that may contribute to the beneficial AT_2R-mediated anti-fibrotic effects. Most data related to chronic AT_2R stimulation have been obtained using C21, although there are a number of other AT_2R agonists beginning to emerge in the literature (Jones et al., 2011; Guimond et al., 2014; Del Borgo et al., 2015; Mahmood and Pulakat, 2015) that require rigourous *in vivo* testing in a similar manner to C21. Such studies will shed further light on the clinical potential of AT_2R agonists in CVD.

AUTHOR CONTRIBUTIONS

RW and CS conceived the review; YW wrote the first draft; MDB and DB provided literature searches and contributed to draft. YW, HL, BH, TG, and RW contributed and performed experiments in **Figure 1**. RW, CS, M-IA, and KD did major revisions to the draft manuscript and approved final submission.

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MINI REVIEW
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Mineralocorticoid Receptor Signaling as a Therapeutic Target for Renal and Cardiac Fibrosis

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Activation of the mineralocorticoid receptor (MR) plays important roles in both physiological and pathological events. Blockade of MR signaling with MR antagonists (MRAs) has been used clinically to treat kidney and cardiac disease associated with hypertension and other chronic diseases, resulting in suppression of fibrosis in these organs. However, the current use of steroidal MRAs has been limited by off target effects on other hormone receptors or adverse effects on kidney tubular function. In this review, we summarize recent insights into the profibrotic roles of MR signaling in kidney and cardiovascular disease. We review experimental *in vitro* data identifying the pathological mechanisms associated with MR signaling in cell types found in the kidney (mesangial cells, podocytes, tubular cells, macrophages, interstitial fibroblasts) and heart (cardiomyocytes, endothelial cells, vascular smooth muscle cells, macrophages). In addition, we demonstrate the *in vivo* importance of MR signaling in specific kidney and cardiac cell types by reporting the outcomes of cell type selective MR gene deletion in animal models of kidney and cardiac disease and comparing these findings to those obtained with MRAs treatment. This review also includes a discussion of the potential benefits of novel non-steroidal MRAs for targeting kidney and cardiac fibrosis compared to existing steroidal MRAs, as well as the possibility of novel combination therapies and cell selective delivery of MRAs.

Keywords: mineralocorticoid receptor, aldosterone, kidney, cardiac, fibrosis

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INTRODUCTION

The mineralocorticoid receptor (MR) is a ligand activated cytosolic receptor that has received increasing attention as a driver of cardiovascular and renal fibrosis. Although best known as an "aldosterone receptor" that regulates electrolyte and fluid homeostasis in the distal nephron and other epithelial tissues, the MR is expressed widely at low levels in the cardiovascular system, in podocytes and other kidney cells, central nervous system and adipocytes among others. While the primary mineralocorticoid ligand for the MR is aldosterone, the MR can also bind and respond to glucocorticoids; ligand selectivity for the MR in mineralocorticoid target tissues, including renal epithelial cells, colon, discrete nuclei in the brain, and the vessel wall, is thus maintained by pre-receptor metabolism of glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD2) (Chapman et al., 2013). In many tissues including cardiomyocytes, immune cells, and adipocytes, HSD2 is absent and cortisol/corticosterone, which circulate at higher levels than aldosterone, can bind and regulate the receptor.

A role for the MR in fibrosis was proposed by Brilla and Weber (1992) in studies demonstrating profibrotic effects of aldosterone infusion in high salt fed rats. The studies echoes the much earlier work of Selye (1958), who described granulomatous tissue and fibrosis in peripheral organs in dogs given high doses of the mineralocorticoid deoxycorticosterone (DOC), although it was thought to be a glucocorticoid effect at the time. The work of Brilla and Weber and other labs lead to the Randomized ALdactone Evaluation Study (RALES; Pitt et al., 1999), which formally demonstrated the therapeutic protective effects of spironolactone in all cause heart failure. However, the risk of hyperkalemia with the clinical use of MR blockers has limited their use. This review will discuss tissue and cell specific aspects of MR signaling in fibrosis of the kidney and heart and the potential strategies for better targeting MR in fibrotic disease.

MR SIGNALING IN KIDNEY FIBROSIS

Aldosterone and Chronic Kidney Disease

Glomerular and interstitial fibrosis are features of chronic kidney disease (CKD) which, if allowed to progress, can result in the development of end-stage renal failure and patients requiring renal replacement therapy (kidney transplantation or dialysis) to survive. CKD is associated with an adverse rise in circulating aldosterone levels with respect to extracellular volume, which increases as glomerular filtration rate falls. This state of relative hyperaldosteronism leads to activation of the MR in kidney cells which can facilitate proinflammatory and profibrotic responses, particularly in non-epithelial cells (Schwenk et al., 2015). Therefore, aldosterone-induced MR signaling may be a key factor in promoting fibrosis in CKD. Furthermore, current standard of care therapy for CKD, which involves blockade of the renin–angiotensin system (RAS) by angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs), will cause a paradoxical rise in aldosterone in 30–50% of patients, often referred to as "aldosterone breakthrough" (Schwenk et al., 2015). Hence, there is an important need to inhibit MR signaling in CKD.

Use of Mineralocorticoid Receptor Antagonists in Chronic Kidney Disease

Clinical trials have shown that MR antagonists (MRAs), including spironolactone (a first generation non-selective steroidal MRA), eplerenone (a second generation selective steroidal MRA) and finerenone (a third generation selective non-steroidal MRA) are all capable of providing protection against CKD. So far, most of these studies have involved the addition of spironolactone to RAS blockade with an ACEi or ARB. In diabetic nephropathy patients, spironolactone provides additional suppression of albuminuria compared to RAS blockade alone, and this protection appears to be partly independent of any effect on blood pressure (Guney et al., 2009; Mehdi et al., 2009; Esteghamati et al., 2013). Similar findings have been found in patients with albuminuria resulting from non-diabetic CKD (Furumatsu et al., 2008; Tylicki et al., 2008; Bianchi et al., 2010). Further analysis has shown that the protective effects of spironolactone are associated with reductions in the urine levels of transforming growth factor β TGF-β1 (Guney et al., 2009), collagen IV (Furumatsu et al., 2008), and amino-terminal propeptide of type III procollagen (Tylicki et al., 2008), suggesting that spironolactone is inhibiting renal fibrosis in these patients.

Clinical studies have also demonstrated that the addition of eplerenone or finerenone to RAS blockade provides greater suppression of albuminuria in patients with diabetic nephropathy (Epstein et al., 2006; Bakris et al., 2015) and non-diabetic CKD (Boesby et al., 2011). However, the specific effects of MRAs on renal fibrosis were not assessed in these studies.

Despite these benefits, the use of MRAs in CKD has drawbacks which can limit its clinical use. For example, spironolactone also binds to progesterone and androgen receptors which can lead to adverse progestational and anti-androgenic effects (Danjuma et al., 2014). In comparison, eplerenone is more selective, but has weaker affinity for binding MR and is less potent than spironolactone. A further downside of MRA therapy is that it can cause hyperkalemia in patients, which is a major clinical concern, particularly in the context of renal impairment, and necessitates withdrawal of this treatment (Mehdi et al., 2009; Bianchi et al., 2010; Esteghamati et al., 2013). This problem arises because MRAs inhibit the activation of ion channels in tubule cells by aldosterone, which is required for maintaining sodium and potassium homeostasis. Blocking this pathway elevates potassium levels, which is exacerbated during RAS blockade. However, recent preclinical and early phase clinical trial evidence suggests that this problem may be reduced using finerenone (a non-steroidal MRA) which can inhibit pathological MR signaling as effectively as spironolactone while having a minimal effect on potassium homeostasis (Bakris et al., 2015). A potential explanation for the lack of effect of finerenone on potassium levels has been identified by radioactive labeling studies showing that finerenone is almost equally distributed in heart and kidney which contrasts with steroidal MRAs (spironolactone and eplerenone) which show greater accumulation in the kidney (Kolkhof et al., 2014). This suggests that the protective effects of MR blockade in heart and kidney can be achieved with finerenone at a dose that has reduced risk of hyperkalemia, which is proposed to be due to structural differences that influence its selective uptake in target tissues.

Effects of Mineralocorticoid Receptor Antagonists in Animal Models of Renal Fibrosis

The anti-proteinuric effects of MRAs have also been seen in a variety of animal models of CKD, including diabetic nephropathy, hypertensive nephropathy, lupus nephritis, polycystic kidney disease, and cyclosporine A nephrotoxicity, which in some cases are also associated with protection of renal function (Guo et al., 2006; Monrad et al., 2008; Nemeth et al., 2009; Miana et al., 2011; Jeewandara et al., 2015; Sun et al., 2015). In models of diabetic nephropathy and hypertensive nephropathy, MRAs have been shown to provide additional protection when combined with an ACEi, compared to either therapy alone, and their benefit is often independent of an effect

on blood pressure (Kang et al., 2009; Nemeth et al., 2009; Zhou et al., 2016).

Analysis of tissues from acute and chronic animal models of kidney disease have shown that MRAs suppress the development of glomerulosclerosis and interstitial fibrosis, which is usually linked to reduced production or deposition of matrix proteins (e.g., collagen, fibronectin) and profibrotic molecules (e.g., TGF-β1, plasminogen activator inhibitor-1 [PAI-1], connective tissue growth factor [CTGF]) (Trachtman et al., 2004; Kang et al., 2009; Sun et al., 2015). In addition, MR blockade in these models can reduce podocyte injury (Nemeth et al., 2009), autoantibodies (Monrad et al., 2008), kidney leukocyte accumulation (Guo et al., 2006; Huang et al., 2014), and expression of molecules which drive inflammation (e.g., monocyte chemoattractant protein-1 [MCP-1], tumor necrosis factor [TNF]-α) (Huang et al., 2014; Zhou et al., 2016), suggesting that the anti-fibrotic effects of MRAs may, in part, arise indirectly from the inhibition of inflammation and apoptosis in the kidney.

Infusion of MR Ligands Promotes Renal Injury and Fibrosis in Rodents

Prolonged aldosterone infusion into rats and mice results in a model of hyperaldosteronism which causes hypertension, podocyte injury, kidney inflammation, proteinuria, and renal fibrosis (Blasi et al., 2003). This renal injury and fibrosis can be blocked with MRA treatment and is reduced in mice lacking galectin-3 (Calvier et al., 2015), PAI-1 (Ma et al., 2006), interleukin-18 (IL-18) (Tanino et al., 2016), or inflammasome activation in macrophages (Kadoya et al., 2015), suggesting their involvement in the MR-mediated renal fibrosis.

Studies have also shown that infusion of hydrocortisone or angiotensin II (AngII) can induce renal injury in aldosterone deficient rodents, which is inhibited by MRAs (Rafiq et al., 2011; Luther et al., 2012). This suggests that cortisol (an MR ligand) and AngII (via binding to its receptor) may also activate MR to cause renal injury. The potential for cortisol to activate MR is particularly high in kidney macrophages which, unlike most other kidney cells, lack HSD2 that normally converts cortisol to a metabolite that is incapable of activating MR (Brown, 2013).

Aldosterone Induces Fibrotic Responses in Kidney Cells

Aldosterone can directly promote fibrotic responses in cultured kidney cells. For example, aldosterone stimulates proliferation of mesangial cells and kidney fibroblasts via transactivation of epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) (Huang et al., 2009, 2012). Aldosterone can also induce myofibroblastic transdifferentiation in mesangial cells and tubular epithelial cells (Zhang et al., 2007; Diah et al., 2008). In addition, aldosterone can directly stimulate the gene expression and synthesis of profibrotic cytokines (TGF-β1, PAI-1, CTGF; Huang et al., 2008; Terada et al., 2012) and matrix proteins (fibronectin and collagens; Nagai et al., 2005; Lai et al., 2006; Diah et al., 2008; Chen et al., 2013) in mesangial cells and kidney fibroblasts. These responses were shown to be dependent on the generation of reactive oxygen species and signaling via Rho-kinase, phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), or small body size mothers against decapentaplegic-2 (SMAD2).

Lessons from MR Deletion in Specific Cell Types in Models of Kidney Disease

The specific roles of MR in some cell types (macrophages, podocytes, vascular smooth muscle cells, and endothelial cells) have been examined in models of kidney disease in transgenic mice where MR is selectively deleted in these cells. Studies have shown that deficiency of endothelial MR has no effect on renal injury induced by DOC acetate/salt (Lother et al., 2016) and deficiency of podocyte MR has no effect on the development of anti-glomerular basement membrane (anti-GBM) glomerulonephritis (Huang et al., 2014). In contrast, deficiency of MR in smooth muscle cells has recently been shown to limit ischemia-reperfusion injury in the kidney through effects on Rac1-mediated MR signaling (Barrera-Chimal et al., 2017). Remarkably, deficiency of MR in macrophages was found to have similar protection to eplerenone treatment in a model of anti-GBM glomerulonephritis (Huang et al., 2014). This suggests that macrophage MR signaling may be the major cause of MR-mediated injury in CKD driven by macrophage-dependent inflammation, which include progressive forms of glomerulonephritis and diabetic nephropathy.

MR SIGNALING IN CARDIAC FIBROSIS AND HEART FAILURE

A Role for MR Signaling in Heart Failure

Heart failure is defined as failure of the pump function of the myocardium and is caused by many factors including structural remodeling of the ventricle wall due to myocardial hypertrophy and elevated interstitial fibrosis. Cardiac fibrosis, due to increased deposition and crosslinking of extracellular matrix (ECM) proteins, results in increased stiffness of the tissue, which leads to poor ventricular relaxation and reduced contractile force (Travers et al., 2016). Together with "remodeling" of ion channel function and other cellular pathways (Cohn et al., 2000) these functional changes limit cardiac output, underpinning the transition from compensated to decompensated cardiac hypertrophy. Many signaling systems contribute to the development of cardiac pathological changes including increased activation of the MR.

As noted above, the RALES and other clinical trials investigated the benefits for MRA as add on therapy to current best practice therapy and demonstrated a role for MR signaling in all-cause heart failure, heart failure post-MI and in mild heart failure (Pitt et al., 1999, 2003; Zannad et al., 2010). These trials validated many preclinical studies of cardiovascular disease (CVD), as well as the renovascular disease models discussed above, that showed equivalent protection for MRA—spironolactone and eplerenone—in a range of rodent models of cardiac fibrosis including aldosterone or DOC infusion plus salt, elevated AngII, low nitric oxide (N-nitro-L-arginine methyl ester

[L-NAME]), pressure overload (transverse aortic constriction [TAC]), myocardial infarction (MI), spontaneously hypertensive rats and Dahl salt-sensitive rats (Young, 1995; Rocha and Funder, 2002; Oestreicher et al., 2003; Mihailidou et al., 2009; Lother et al., 2011). Moreover, it has also been demonstrated using subpressor doses of MRA or central administration of MRA to block hypertension that MR-mediated cardiac fibrosis is independent of blood pressure changes (Bauersachs and Fraccarollo, 2003). Together these studies underscore the importance of MR signaling, directly in the heart, for disease settings in which tissue injury, oxidative stress, and inflammation are common factors. Thus, MRA likely exert cardio-protective effects via direct blockade of cardiac and vascular MR.

Mechanisms of MR-Mediated Cardiac Fibrosis

The diffuse interstitial and perivascular collagen depots that characterize established cardiac fibrosis are preceded by early tissue injury and inflammation responses that can be detected within days of MR activation (Young et al., 2003; Wilson et al., 2009). Similar to the kidney, MR activation promotes oxidative stress in the vessel wall and the expression of inflammatory factors including chemoattractant proteins (MCP-1/chemokine (C-C motif) ligand 2 [CCL2], chemokine (C-X3-C motif) ligand 1 [CX3CL1], chemokine (C-C motif) ligand 5 [CCL5], etc.) and adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1]) that facilitate the recruitment of inflammatory cells to the myocardium (Fuller and Young, 2005). Activated tissue macrophages and T-cells release proinflammatory cytokines (TNF-α, inducible nitric oxide synthase [iNOS], osteopontin, etc.) to amplify type-1 proinflammatory responses in the tissue (Travers et al., 2016). Resolution of the inflammatory response and the transition to anti-inflammatory and repair phenotypes is essential to maintain normal myocardial function and involves expression of common trophic and profibrotic factors such as TGF-β, platelet-derived growth factor (PDGF), CTGF, and PAI-1 among others (Rickard and Young, 2009; Shen et al., 2016). Studies have also shown that oxidative stress in cardiomyocytes can induce MR activation in a Rac1-dependent, ligand-independent manner, and thereby promote cardiac injury (Nagase et al., 2012; Ayuzawa et al., 2016). In addition, cardiac overexpression of constitutively activated Rac1 promotes MR activation and cardiac fibrosis (Lavall et al., 2017). Elevated fibrosis is essential for the resolution of major insults such as MI in which reparative scar tissue is absolutely required to replace necrotic myocytes and maintain ventricle wall integrity. In contrast, reactive fibrosis in viable tissue in response to MR signaling only serves to limits cardiac function and is the focus of considerable efforts to identify therapeutic targets (Fraccarollo et al., 2011).

Experimental Models for MR-Dependent Cardiac Fibrosis

Mice with MR deficiency in specific cell types have been used to determine cell-selective MR signaling mechanisms in cardiac fibrosis. As noted above, the macrophage was identified as a novel site of MR signaling in cardiac remodeling; loss of macrophage MR signaling had a profound protective effect in terms of tissue inflammation and fibrosis, despite little or no changes in the recruitment of macrophages in some models (Rickard et al., 2009; Usher et al., 2010; Bienvenu et al., 2012). Specifically, mice lacking MR in macrophages are protected from DOC/salt and L-NAME/AngII mediated cardiac inflammation and fibrosis and most recently, atherosclerosis (Rickard et al., 2009; Bienvenu et al., 2012). The type 1 proinflammatory response is markedly reduced in these models as are pro-repair/pro-fibrotic signals and the expression of α-smooth muscle actin (α-SMA) expressing fibroblasts in the myocardium (Shen et al., 2016). In trying to explain this outcome, other investigators have suggested that the MR null macrophage has an M2 like phenotype, which may be immunomodulatory and limit tissue remodeling (Usher et al., 2010). In addition, evidence from different groups support a role for MR signaling in promoting an M1 proinflammatory phenotype in macrophages (Rickard et al., 2009; Martin-Fernandez et al., 2016). To this end, recent efforts to determine the cell signaling pathways regulated by MR signaling suggest that proinflammatory signaling via JNK/activating protein-1 (AP-1), and potentially nuclear factor-kappa B (NF-κB)/p65, transactivation pathways are MR-dependent (Shen et al., 2016; Sun et al., 2016). The MR is also involved in direct gene transcriptional responses in the macrophage, including for MR target genes such as p22phox, PAI-1 (Calo et al., 2004). Consistent with studies of transcriptional regulation of macrophages the overall effect of MR activation is most likely dependent upon macrophage phenotype (Barish et al., 2005). Taken together, these knockout studies validate the importance of macrophages and early inflammation in promoting the overall fibrotic response. However, MR signaling elsewhere in the cardiovascular system is also important.

Deletion of the MR from cardiomyocytes is sufficient to block reactive, but not reparative fibrosis in the heart. In a model of MI, cardiomyocyte MR null mice demonstrated improved reparative scar formation in the infarcted zone, increased revascularization of the tissue and improved cardiac function, but reduced reactive fibrosis in the viable ventricle wall (Fraccarollo et al., 2011). The improved remodeling profile was associated with lower inflammatory and profibrotic marker expression but more importantly, these data demonstrated a dual role for cardiomyocyte MR in the cardiac tissue response to an ischemic event that was highly dependent upon the tissue context. In support of these data, studies investigating the DOC/salt reactive fibrosis model and the TAC model showed that, whereas DOC/salt-mediated tissue inflammation and fibrosis were prevented in cardiomyocyte MR null mice (Rickard et al., 2012), pressure-overloaded hearts were not similarly protected (Lother et al., 2011). Despite these differences in tissue remodeling, cardiac function in each of the disease models was universally improved in hearts in which MR signaling was absent, indicating a separation of MR signaling effects in the heart. We also detected up-regulation of antifibrotic mechanisms including enhanced expression of ECM protein decorin, which can block TGF-β and CTGF (Rickard et al., 2012). These data

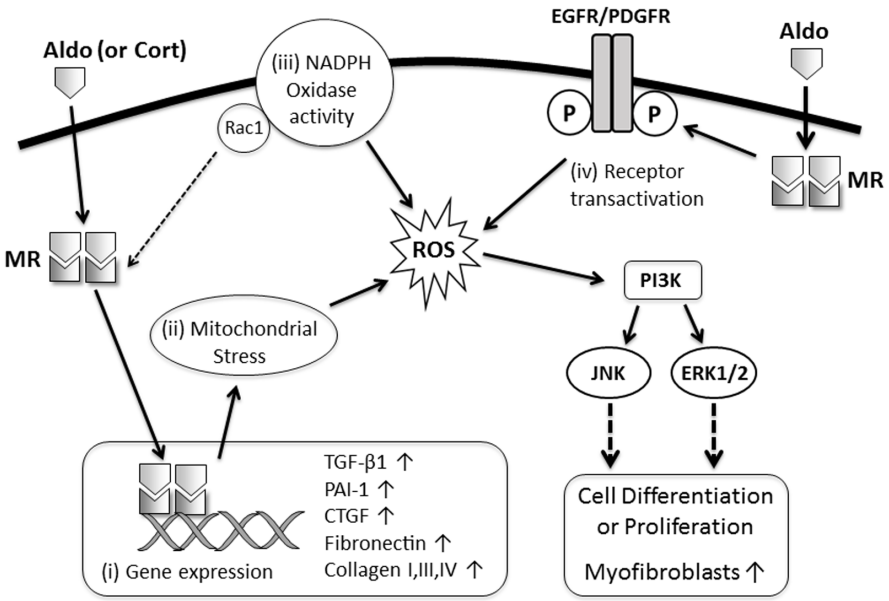

FIGURE 1 | Proposed mechanisms of MR-mediated fibrosis in the kidney and heart. MR ligands (Aldo, aldosterone; Cort, cortisol) bind with cytoplasmic MR resulting in: (i) translocation of the MR to the nucleus and activation of transcription of profibrotic genes (TGF-β1, PAI-1, CTGF, collagens, fibronectin); (ii) induction of oxidative stress in mitochondria resulting in transdifferentiation of epithelial and mesangial cells into a more profibrotic myofibroblast phenotype; (iii) increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and oxidative stress in macrophages, cardiomyocytes, and podocytes leading to enhanced MR activation (via Rac1) and worsening inflammation and injury which subsequently promote fibrosis; or (iv) transactivation of growth factor receptors, which facilitate oxidative stress, rapid activation of mitogen-activated protein kinase (MAPK) signaling and proliferation of fibroblast-like cells.

support earlier studies showing elevated aldosterone levels can block antifibrotic pathways.

MR in the endothelial cell and vascular smooth muscle cell (VSMC) layers of the vessel wall regulate important aspects of vascular physiology. Contractile and relaxation responses are MR-dependent and require MR signaling in both cells types, whereas cell-selective deletion studies of the MR shows that VSMC MR but not endothelial cell MR are required for age-related and other forms of hypertension (McCurley and Jaffe, 2012; Rickard et al., 2014). In fact, endothelial cell MR deficiency had negative effects on nitric oxide production and signaling that resulted in aberrant vascular functional responses in unchallenged tissues (Jaffe and Jaisser, 2014; Rickard et al., 2014). However, endothelial cell MR null mice are protected from cardiac inflammation and fibrosis induced by exogenous mineralocorticoids and cardiovascular remodeling and dysfunction due to diet-induced obesity (Schafer et al., 2013; Rickard et al., 2014; Jia et al., 2016). Although adhesion molecules such as ICAM-1 can be directly regulated by aldosterone/MR signaling in endothelial cells (Caprio et al., 2008), studies now suggest it may not be the primary regulator of MR-mediated fibrosis (Salvador et al., 2016). In contrast, recent work by Marzolla et al. (2017) has identified enhanced atherosclerotic plaques in aldosterone-treated ApoE$^{-/-}$ mice and has shown that macrophage recruitment in these lesions is dependent on MR-mediated ICAM-1 signaling and that ICAM-1 is directly regulated by MR in HUVEC cells. The complexity of the endothelial MR signaling was further demonstrated by research which validated its role in DOC/salt mediated cardiac fibrosis but not renal fibrosis, suggesting interaction with permissive signaling factors that are specific to organs or vascular bed (Lother et al., 2016).

MR signaling in VSMC can also regulate tissue fibrosis, either as fibrosis of the vessel wall and perivascular space or via facilitating inflammatory cell influx and activation. We and others have shown vascular inflammation and oxidative stress to precede the onset of aldosterone-mediated cardiac fibrosis (Rocha and Funder, 2002; Wilson et al., 2009; McCurley and Jaffe, 2012). This in turn promotes endothelial cell damage which further enhances oxidative stress and inflammation leading to uncoupling of endothelial nitric oxide synthase (eNOS), production of peroxynitrate species and further potentiation of vessel wall injury and the recruitment of monocytes and macrophages to the surrounding tissue. In addition to the important role of VSMC MR in blood pressure control, MR also plays a key role in VSMC in the pathological remodeling following MI (Gueret et al., 2016). The authors showed increased left ventricle compliance, preserved coronary vascular reserve and reduced tissue fibrosis in VSMC MR null versus wild type mice subjected to MI. The VSMC is also of interest in MR signaling given the evidence supporting crosstalk between the MR and AngII signaling pathways in pathological processes in cardiovascular tissues, which support the use of MRA as well as AngII signaling blockers for the management of CVD (Jaffe and Mendelsohn, 2005).

Insights into Pharmacological Targeting of MR in Kidney and Cardiovascular Disease

As noted previously, the first MRA, spironolactone, was succeeded by MR selective eplerenone following the success of the RALES trial to address side effects associated with binding at sex steroid receptors. However, hyperkalemia remained a significant side effect for these MRAs. Several non-steroidal MRAs, which potently and selectively inhibit MR, are now being examined for their impact on cardiac and renal diseases and hyperkalemia, of which finerenone (or BAY-94-8862 from Bayer) is in phase 3 trials for end stage renal failure. Finerenone, CS-3150 (from Daiichi Sankyo) and PF-03882845 (from Pfizer) have been investigated in DOC/salt mediated renal and cardiac fibrosis and other forms of kidney disease and show protective effects that are equivalent to eplerenone. The development of MRAs has recently been reviewed in detail (Kolkhof et al., 2016).

Recent insights into the mechanisms of MR-dependent fibrosis has raised the possibility of combining MRAs (at a dose that avoids hyperkalemia) with other therapies that target the downstream effects of MR signaling in order to minimize organ injury. Given that oxidative stress and MAPK signaling are important for mediating the profibrotic effects of MR signaling in the kidney and heart (**Figure 1**), it is likely that therapies which specifically target these mechanisms, if well tolerated, could be used in conjunction with MRAs to provide better suppression of MR-dependent fibrosis. In addition, there are other novel therapies which attenuate models of MR-mediated cardiac fibrosis, including the insulin sensitizing drug Metformin (Mummidi et al., 2016), antagonism of chemokine receptor (C-X-C motif) receptor 4 (CXCR4) (Chu et al., 2011) and treatment with a high fiber diet to enhance healthy gut microbiota (Marques et al., 2017). These therapies offer novel insights into the mechanisms of disease and could also be combined with MRAs or other injury-suppressing drugs to reduce fibrotic disease.

Studies involving cell selective deletion of MR in models of kidney and CVD have indicated the potential benefits of selectively delivering MRAs to macrophages, cardiomyocytes, and vascular smooth muscle cells. If MRAs could be selectively delivered to these cells with carriers that recognize cell surface molecules that are restricted to these cell types, then the problem of hyperkalemia could be avoided. Animal studies have shown that drugs can be selectively targeted to macrophages using a variety of nanoparticles (He et al., 2017), however, the development of these delivery systems is in the very early stages and needs further progress.

CONCLUSION

Cardiovascular and kidney diseases are a major health problem worldwide, with the incidences becoming more prevalent in an aging population and with the growing epidemic of obesity and type 2 diabetes. Given the critical role of MR signaling in kidney and cardiac fibrosis, effective and selective targeting of the pathological effects of MR signaling in these organs remains a high priority for treatment. Hopefully, this can be achieved with emerging novel MR inhibitors, combination therapies, or cell selective delivery of MRAs, leading to better patient outcomes.

AUTHOR CONTRIBUTIONS

GT wrote the section of this review on MR signaling in kidney disease and co-wrote the abstract, introduction, the insights into pharmacological targeting of MR and the conclusion. GT also developed **Figure 1**. MY wrote the section of this review on MR signaling in cardiac disease and co-wrote the abstract, introduction, the insights into pharmacological targeting of MR and the conclusion. Both authors approved of the final revised manuscript.

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Sphingosine 1-Phosphate Receptors: Do They Have a Therapeutic Potential in Cardiac Fibrosis?

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Sphingosine 1-phosphate (S1P) is a bioactive lipid that is characterized by a peculiar mechanism of action. In fact, S1P, which is produced inside the cell, can act as an intracellular mediator, whereas after its export outside the cell, it can act as ligand of specific G-protein coupled receptors, which were initially named endothelial differentiation gene (Edg) and eventually renamed sphingosine 1-phosphate receptors (S1PRs). Among the five S1PR subtypes, S1PR1, S1PR2 and S1PR3 isoforms show broad tissue gene expression, while S1PR4 is primarily expressed in immune system cells, and S1PR5 is expressed in the central nervous system. There is accumulating evidence for the important role of S1P as a mediator of many processes, such as angiogenesis, carcinogenesis and immunity, and, ultimately, fibrosis. After a tissue injury, the imbalance between the production of extracellular matrix (ECM) and its degradation, which occurs due to chronic inflammatory conditions, leads to an accumulation of ECM and, consequential, organ dysfunction. In these pathological conditions, many factors have been described to act as pro- and anti-fibrotic agents, including S1P. This bioactive lipid exhibits both pro- and anti-fibrotic effects, depending on its site of action. In this review, after a brief description of sphingolipid metabolism and signaling, we emphasize the involvement of the S1P/S1PR axis and the downstream signaling pathways in the development of fibrosis. The current knowledge of the therapeutic potential of S1PR subtype modulators in the treatment of the cardiac functions and fibrinogenesis are also examined.

Keywords: sphingosine 1-phosphate, sphingolipids, matrix metalloproteinases, cardiomyocytes, collagen accumulation, G-coupled receptor, cardiac fibrosis

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INTRACELLULAR AND EXTRACELLULAR ACTIONS OF S1P

Sphingosine 1-phosphate is the intermediate breakdown product of the catabolism of complex SLs (**Figure 1**), a class of lipids characterized by a C8 carboamide alcohol backbone that was discovered in the brain in 1870 (Thudichum, 1884). S1P is present in the plasma, where it binds to ApoM on HDL particles, and serum albumin (Christoffersen et al., 2011). This bioactive lipid is in all types of mammalian cells (Hannun and Obeid, 2008) and, systemic and local gradients of S1P are essential for immune cell homing (Olivera et al., 2013; Nishi et al., 2014). S1P is formed from Sph by two differently localized and regulated enzyme isoforms, SphK 1 and SphK2 (Maceyka et al., 2012).

Although SphK1 and SphK2 catalyze the same reaction, SphK1 inhibition/gene ablation decreases blood S1P, while SphK2 inhibition/gene ablation increases blood S1P. At the cellular level, studies have shown the involvement of SphK1 in cell survival and cell growth, whereas SphK2 is rather associated with growth arrest and apoptosis (Liu et al., 2003).

Sphingosine 1-phosphate acts inside cells as a signaling molecule that regulates specific targets (Maceyka et al., 2012), such as PHB2, a highly conserved protein that regulates mitochondrial assembly and function, TRAF-2, which is upregulated in fibroblasts, and NF-κB, which is crucially involved in inflammatory gene regulation (Xia et al., 2002; Alvarez et al., 2010).

However, in response to only partially known stimuli, S1P can be transported outside the cells by a specific S1P transporter, named Spns2, and upon binding to one or more of the five subtypes of G-protein-coupled receptors (GPCRs), named S1PR1-5, it triggers many downstream signaling pathways (Zu Heringdorf et al., 2013; Kihara et al., 2014; Nishi et al., 2014) (**Table 1**). Hla and Maciag (1990), by a differential display method, discovered the orphan GPCR Edg-1, and successively identified as a S1PR1 receptor based on the sequence homology with LPA1/Vzg-1/Edg-2. Later, other receptors, including Edg-5 and Edg-3, followed by Edg-6 and Edg-8 (now termed S1PR2, S1PR3, S1PR4 and S1PR5), were described (An et al., 1997, 2000; Goetzl et al., 1999).

Sphingosine 1-phosphate receptors are widely expressed and specifically coupled to distinct G-proteins as reported in **Table 1** (Blaho and Hla, 2014; Kihara et al., 2014; Pyne et al., 2015). Recently, studies on the crystal structure of S1PR1 (Hanson et al., 2012) have indicated that the ligand likely binds the receptor by lateral access (Rosen et al., 2013). Substantial evidence has demonstrated that S1PR-mediated signaling regulates many biological processes, such as cell growth and survival, migration, and adhesion (Maceyka et al., 2012; Kunkel et al., 2013; Proia and Hla, 2015); thus, the impairment of the SphK/S1P/S1PR axis leads to many disorders, including inflammation, fibrosis, and cancer (Schwalm et al., 2013, 2015; Newton et al., 2015; Pyne et al., 2015).

Abbreviations: 3KR, 3-keto reductase; AC, adenylate cyclase; Akt, serine/threonine-specific protein kinase 1; ApoM, apolipoprotein M; C1P, ceramide-1-phosphate; cAMP, cyclic adenosine monophosphate; CDase, ceramidase; Cer, ceramide; CerK, ceramide kinase; CerS, ceramide synthase; CTGF, connective tissue growth factor; DeS, desaturase; ECM, extracellular matrix; Edg-1, endothelial differentiation gene-1; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; GIRK, G protein-coupled inwardly rectifying potassium channels; GPCR, G-protein coupled receptors; GTP, guanosine triphosphate; HDAC, histone deacetylase; HDL, high density lipoproteins; IL, interleukin; LPA1, lysophosphatidic acid receptor 1; miR, microRNA; MMP, matrix metalloproteinase; MS, multiple sclerosis; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cell; PDGF, platelet-derived growth factor; PHB2, prohibitin 2; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PLC, phospholipase C; Rho GTPase, Ras homolog GTP hydrolase; RLX, relaxin; ROCK, Rho associated-protein kinase; RXFP1, relaxin/insulin like family peptide receptor 1; S1P, sphingosine 1-phosphate; S1PR, S1P receptor; siRNA, short interfering RNA; SL, sphingolipid; SM, sphingomyelin; SMA, smooth muscle actin; Smad, small mother aganist decapentaplegic; SMase, sphingomyelinase; SMS, sphingomyelin synthase; Sph, sphingosine; SphK, sphingosine kinase; SPL, S1P lyase; Spns2, Spinster 2 (S1P transporter); SPPase, S1P phosphatases; SPT, serine palmitoyl transferase; TGFBR, TGF receptor; TGFβ, transforming growth factor β; TIMP, tissue inhibitors of metalloproteinase; TNF, tumor necrosis factor; TRAF-2, TNF receptor-associated factor 2; UTR, untranslated region; Vzg-1, ventricular zone gene-1.

CARDIAC FIBROSIS

Cardiac fibrosis is a multistep disorder, which arises due to several circumstances, such as inflammation, ischaemia and senescence. Myocardial integrity is assured throughout life by fibrotic remodeling of cardiac tissue that becomes decisive in the progression of cardiac disease, thus contributing to the high risk of mortality for this disease (Cohn et al., 2000; Kong et al., 2014; Gyöngyösi et al., 2017).

Fibroblasts, the major producers of cardiac ECM (Krenning et al., 2010), provide the initial structural support in the neonatal heart, respond electrically to mechanical stretch and participate in the synchronization of cardiac tissue (Tomasek et al., 2002). In chronic conditions of ischaemia or altered oxygen tension, angiotensin-aldosterone mediated oxidative/redox stress, pro-inflammatory and pro-fibrotic factors activate circulating bone marrow-derived fibrocytes, epithelial cells and resident fibroblasts that adopt an hypersecretory myofibroblast phenotype (Porter and Turner, 2009; van den Borne et al., 2010; Lajiness and Conway, 2014). Myofibroblasts, by acting in an autocrine/paracrine manner, start to overproduce ECM. Accumulation of abundant type I/III fibrillar collagen and a variety of bioactive substances causes stiffening of the heart and decreased cardiac function (Gyöngyösi et al., 2017). Among the factors involved in the initiation and progression of cardiac fibrosis, transforming growth factor β (TGF-β) and the local renin-angiotensin-aldosterone system together with other cytokines, including tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and endothelin-1 (Davis and Molkentin, 2014; Bomb et al., 2016), trigger many signaling pathways, such as Smad protein and GTP hydrolase (GTPase) activation (Rosenkranz, 2004; Wynn, 2008; Porter and Turner, 2009; Leask, 2010; Creemers and Pinto, 2011; Wynn and Ramalingam, 2012). In cardiac matrix remodeling, other key players include the zinc-dependent matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Tyagi et al., 1993; Nagase et al., 2006; Spinale, 2007; Mishra et al., 2013). Aberrant levels of different MMPs and TIMPs are highly correlated with cardiac fibrosis (Moshal et al., 2005; Ahmed et al., 2006; Spinale et al., 2013). MMP-2 and MMP-9 have distinct spatial and temporal actions in cardiovascular remodeling; MMP-2 is constitutively expressed, whereas MMP-9 is inducible (Mishra et al., 2013). In addition to their role in ECM degradation, MMPs also act on non-matrix molecules, such as growth factors, allowing local induction-activation of signaling pathways which has been demonstrated after MMP-9 ablation (Mishra et al., 2010). Among the TIMP isoforms (TIMP1-4) implicated in cardiac fibrosis (Vanhoutte and Heymans, 2010), the level of TIMP1 increases in diseased hearts (Heymans et al., 2005) and high levels of TIMP4 are found in parallel to inhibition of MMP-9. Modulation of ECM turnover and activation of MMPs and TIMPs are controlled by many factors, including TNF-α, TGF-β and ILs (Parker and Schneider, 1991; Tsuruda et al., 2004; Wynn

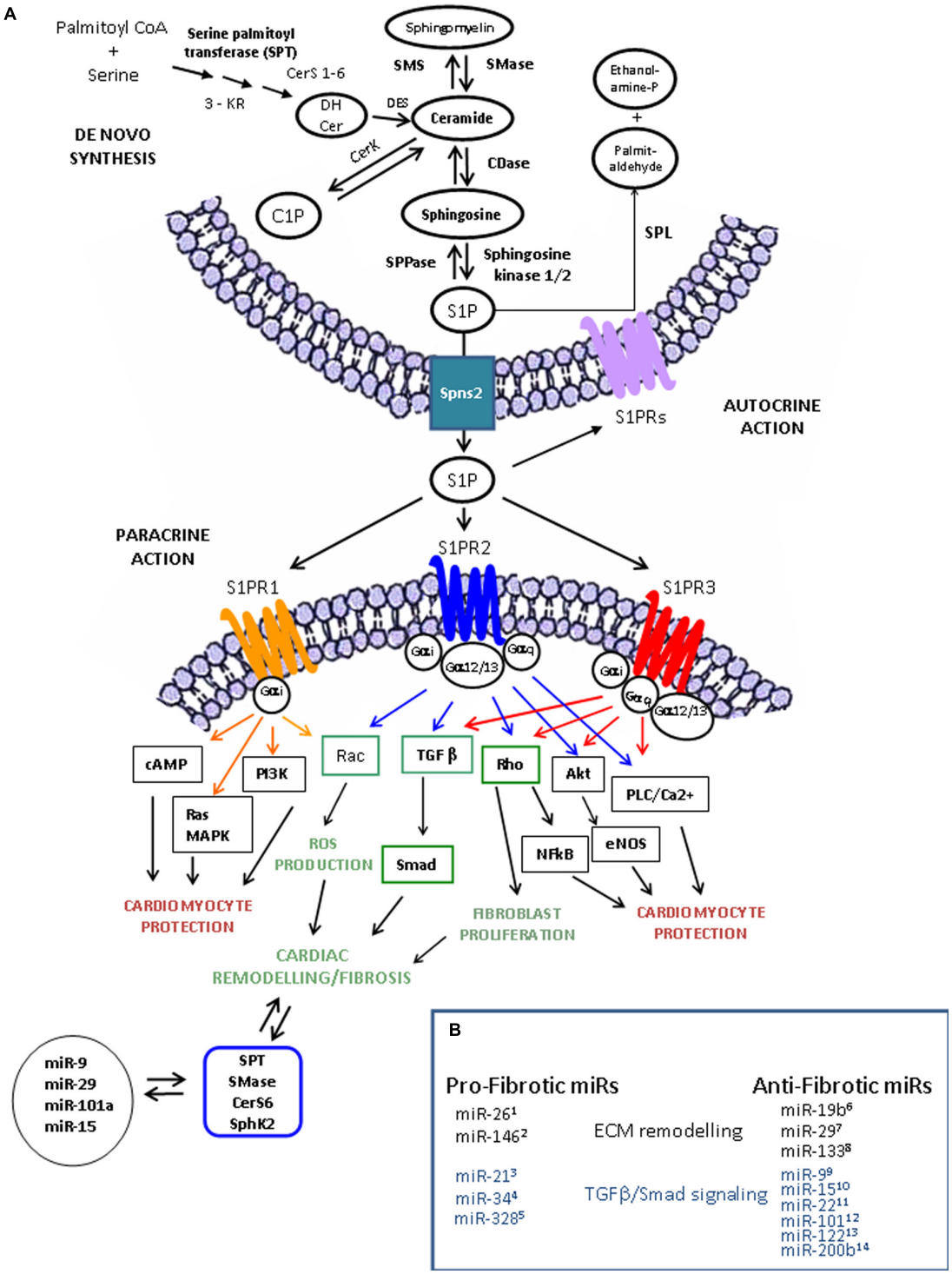

FIGURE 1 | Sphingolipid metabolism, S1P receptor-activated pathways and miR involved in cardiac remodeling and functions. **(A)** S1P is synthesized from sphingosine (Sph) by the sphingosine kinases, SphK1 and SphK2 and irreversible cleaved by S1P lyase (SPL), which generates hexadecenal and phosphoethanolamine. S1P is also a substrate of specific S1P phosphatases (SPPase). Ceramide derives via the sphingomyelin cycle or *de novo* sphingolipid synthesis involving serine palmitoyl transferase (SPT), 3-keto reductase (3KR), ceramide synthase (CerS), and desaturase (DeS), and converted reversibly to sphingosine (Sph) by ceramidase (CDase), or phosphorylated to ceramide-1-phosphate (C1P) by ceramide kinase (CerK) activity. S1P produced inside the cell can be transported in the intercellular space by an ATP-binding cassette transporter named spinster homolog 2 (Spns2). As ligand, S1P acts as autocrine and paracrine factor triggering specific signaling pathways by interacting with S1P specific heterotrimeric GTP binding protein-coupled receptors (GPCR), named S1PR. Three

(continued)

FIGURE 1 | continued
among five subtypes of S1PRs, S1PR-1 (orange), -2 (blue), and -3 (red), are expressed in cardiomocytes, cardiac fibroblasts and procursor cardiac cells. In heart, S1PR activation leads to different cardiac effects (profibrotic, green; antifibrotic and cardioprotective, black/red). The scheme exemplifies, in accordance with the current literature, the main pathways triggered by S1PR activation leading to cardiac cell protection and extracellular matrix (ECM) remodeling. Interestingly, the expression of the key enzymes involved in sphingolipid metabolism can be regulated by microRNAs (miRs) and some of them (i.e., miR-9[9], miR-19b[6], miR-15[10], and miR-29[7]) also regulate the fibrotic process by affecting extracellular matrix (ECM) remodeling through the modulation of metalloprotease (MMPs), TGF-β/TGFR and Smad protein expression. **(B)** miRNA in fibrosis. Several miRs have been described as regulators of cardiac fibrosis acting as pro-fibrotic or anti-fibrotic factors on ECM remodeling (black) and on TGFβ /Smad signaling (blue). [1]Wei et al. (2013). [2]Wang et al. (2015). [3]Thum et al. (2008); Roy et al. (2009), Liang et al. (2012); Dong et al. (2014), He et al. (2016). [4]Bernardo et al. (2012); Huang et al. (2014). [5]Du et al. (2016). [6]Zou et al. (2016). [7]van Rooij et al. (2008), Abonnenc et al. (2013), Zhang et al. (2014). [8]Duisters et al. (2009), Castoldi et al. (2012), Chen et al. (2014), Muraoka et al. (2014), Wang et al. (2016). [9]Li et al. (2016). [10]Tijsen et al. (2014). [11]Hong et al. (2016). [12]Pan et al. (2012). [13]Beaumont et al. (2014). [14]Feng et al. (2016).

and Ramalingam, 2012; Gyöngyösi et al., 2017). Among these factors, the hormone peptide relaxin (RLX) is a key regulator of ECM remodeling in reproductive and non-reproductive tissues (Samuel et al., 2007; Du et al., 2010; Nistri et al., 2012). Particularly, RLX inhibits pro-fibrotic cytokines (i.e., TGF-β1) and modulates the accumulation and degradation of ECM that acts on MMPs and TIMPs (Samuel et al., 2007; Bani et al., 2009; Du et al., 2010; Frati et al., 2015). Moreover, recent research has found that epigenetic factors, such as miRs play an important role in tissue remodeling by controlling MMPs and TGF-β/Smad signaling (**Figure 1**) (Care et al., 2007; Roy et al., 2009; Creemers and van Rooij, 2016; Biglino et al., 2017). Notably, miR expression can also be regulated by MMP-9 (Mishra et al., 2010). Therefore, this class of small non-coding RNAs, which inhibits gene expression by binding the 3′ UTRs of target mRNAs, can be crucial for the fibrotic process, acting as either pro-fibrotic or anti-fibrotic factors. Since dysregulation in miR expression has been reported in myocardial fibrosis (Gurha, 2016), miRs may represent a novel therapeutic strategy to counteract the fibrotic changes that occur in cardiac diseases (Wijnen et al., 2013).

ROLE FOR THE SPHK/S1P AXIS AND S1PR IN CARDIAC FIBROSIS

Many studies performed in cultured cells as well as in animal models have proposed that S1P possesses cardioprotective effects (Kupperman et al., 2000; Jin et al., 2002; Zhang et al., 2007; Karliner, 2013; Maceyka and Spiegel, 2014). In fact, S1P protects cultured rat neonatal cardiomyocytes from ischaemia-induced cell death (Karliner et al., 2001; Jin et al., 2002). Moreover, mice lacking the enzyme SPL that degrades S1P show reduced sensitivity to ischaemia/reperfusion injury and increased S1P level in both plasma and cardiac tissue (Jin et al., 2011) (**Figure 1**).

Regarding cardiac fibrosis, SphK1 appears to play a relevant role. SphK1 is induced by TGF-β and mediates TIMP-1 upregulation, and siRNA against SphK1 inhibited TGF-β-stimulated collagen production (Yamanaka et al., 2004; Gellings Lowe et al., 2009). Importantly, the neutralization of extracellular S1P with a specific anti-S1P antibody significantly reduced TGF-β-stimulated collagen production, indicating the involvement of an "inside-out" signaling of S1P after SphK1 activation in the pro-fibrotic action (Gellings Lowe et al., 2009). Moreover, apelin, an adipocyte-derived factor, inhibits TGF-β-stimulated activation of cardiac fibroblasts by reducing SphK1 activity (Pchejetski et al., 2011).

Notably, high S1P production/accumulation in cells is deleterious. In fact, transgenic mice that overexpressed SphK1 at high level develop spontaneous cardiomyocyte degeneration and fibrosis (Tao et al., 2007; Takuwa et al., 2010) and are characterized by increased levels of Rho GTPases and phospho-Smad3, suggesting that these pathways are downstream of SphK1/S1P.

Recently, we have reported that SL metabolism can be activated by RLX at concentrations similar to those previously reported to elicit specific responses in cardiac muscle cells (van der Westhuizen et al., 2008). In both neonatal cardiac cells and H9C2 cells, RLX induces the activation of SphK1 and S1P production. The silencing and pharmacological inhibition of SphK1 alters the ratio MMPs/TIMPs, and CTGF expression elicited by RLX indicates that hormone peptides promote an ECM-remodeling phenotype through the activation of endogenous S1P production and SM metabolism (Frati et al., 2015).

The role of SphK2 in heart tissue is less clear. Previous research has shown that maternal-zygotic SphK2 is fundamental for cardiac development in zebrafish (Hisano et al., 2015). Moreover, SphK2 knockout sensitizes mouse myocardium to ischaemia/reoxygenation injury (Vessey et al., 2011), and mitochondria obtained from Sphk2 knockout mice exhibit decreased oxidative phosphorylation and increased susceptibility to permeability transition, suggesting a role as protective agent (Gomez et al., 2011). SphK2 appears to be less involved in tissue fibrosis than SphK1 (Schwalm et al., 2015). For example, protein expression of SphK1, but not SphK2, was significantly elevated in lung tissues from patients with idiopathic pulmonary fibrosis (Huang et al., 2013). Presently, the role of SphK2 in cardiac fibrosis is still unclear. Interestingly, Hait et al. (2009) have found that SphK2 is associated with histone H3, and endogenous S1P that is formed in the nucleus via SphK2 inhibits the action of HDACs (Hait et al., 2009; Ihlefeld et al., 2012). Since HDAC activity is increased in patients with cardiac fibrosis (Liu et al., 2008; Pang and Zhuang, 2010), there is a potential link between SphK2, nuclear S1P and the epigenetic regulation of gene expression that is involved in cardiac fibrosis.

Very recently, a strict correlation between the levels of miRs involved in cardiac fibrosis and SL metabolism has been demonstrated. In fact, SphK, SPT, acid SMase and ceramide synthase 6 (CerS6) can be regulated by several miRs (**Figure 1**).

TABLE 1 | Sphingosine 1-phosphate receptors and their intracellular signaling pathways and functions.

S1PR	Knock out phenotype	Intracellular mediators	Biological effects	Cardiac function	Agonists	Antagonists	Indications	Clinical trial
S1PR1 (Edg1) Most tissues **Kd (nM)** (8–20) **Coupled to:** Gαi	Normal until E11.5 then lethal, Liu et al., 2000 abnormal yolk sacs, defective blood and smooth muscle, vessels maturation	(–) AC (+) ERK,Rac, PI3K, Akt	Angiogenesis Lymphocytes migration Cardiomyocytes survival	S1PR1 agonist: Improves cardiac function following myocardial infarction S1PR1 antagonists Stroke and ischaemic pre- and post-conditioning cardioprotection	SEW2871 FTY720-P KRP203 BAF312 ACT-128800 ONO-4641	VPC23019 VPC44116 W416	Multiple Sclerosis Crohn's disease Polymyosites and Dermatomyosites Lupus erythematosus	FTY720-P FDA approved ACT-12880 Phase II ONO-4641 phase II CS-0777 Phase I CYM-5442 Phase III BAF-312 Phase II KRP-203 Phase III
S1PR2 (Edg5) Most tissues **Kd (nM)** 16–27 **Coupled to:** $G\alpha_i$, $G\alpha_q$ et $G\alpha_{12/13}$	Normal until 3–5 weeks, Kono et al., 2004 Excitability at cortical neurons, severe inner ear defects heart development	(–)AC (+) AC, PLC p38MAPK Rho	Vestibular funtion Vascular tone (contraction) Neuronal excitability Cardiomyocyte survival		Not identified	JTE013		
S1PR3 (Edg3) Heart Lung Spleen Kidney Intestine **Kd (nM)** 23–26 **Coupled to:** Gαi/o, Gαq, and Gα12/13	Normal Smaller litter size, Ishii et al., 2001	(–)AC (+) ERK, Rac, eNOS, PLC, Akt	Cardiac rytm regulation Vascular tone (relaxation) Cardiomyocytes survival	S1PR3 antagonists Inhibits the S1P-mediated reduction in coronary flow in perfused rat hearts Partially inhibits FTY720-induced bradycardia in rats *in vivo*	FTY720-P KRP203	VPC23019 CAY1044		
S1PR4 (Edg6) Lymphoid tissues Blood cells Lung smooth muscle **Kd (nM)** 12–63 **Coupled to:** $G\alpha_i$ and $G_1\alpha_{2/13}$	Normal, Gräler et al., 1998; Schulze et al., 2011	(+) AC, ERK, PLC, Rho	Megakaryocyte Differentiation Vascular tone (contraction)		FTY720-P KRP203	Not identified		

(Continued)

TABLE 1 | Continued

S1PR	Knock out phenotype	Intracellular mediators	Biological effects	Cardiac function	Agonists	Antagonists	Indications	Clinical trial
S1PR5 (Edg8) Brain Skin natural killer cells	Normal Aberrant natural killer cells, Im et al., 2001	(−) AC, ERK, (+)JNK	Oligodendrocytes survival Mielinization process		FTY720-P KRP203 BAF312	Not identified	Polymyosites and Dermatomyosites	BAF-312 Phase II
Kd (nM) 2–6 **Coupled to:** $G\alpha_i$ and $G\alpha_{12/13}$								

S1P binds to their specific GPCRs, which activate heterotrimeric G-proteins (defined here by their α subunits) to initiate signaling cascades leading to specific biological effects and functions in cardiac tissue. Agonists and antagonists for each specific S1PR subtypes are reported together with the clinical trials in which the drug have been successfully used. AC, adenylate cyclase; Akt, serine/threonine-specific protein kinase 1; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PLC, Phospholipase C; p38MAPK, p38 Mitogen activated protein kinase; Rac, Ras related monomeric GTP hydrolase; Rho GTPase, Ras homolog GTP hydrolase; Kd, dissociation constant; (+) activation; (−) inhibition; Clinical trial: FTY720 (Gilenya, Fingolimod) NCT00662649 and NCT01436643; ACT-128800 (Actelion, Ponesimod) NCT01006265; ONO-4641 (Merck/Ono pharmaceuticals, Ceralifimod) NCT01081782; CS-0777 (Daiichi Sankyo Inc.) NCT00616733; CYM-5442 (Celgene, Ozanimod) NCT02531113; BAF-312 (Novartis, Siponimod) NCT01665144 and NCT00879658; KRP-203 (Novartis) NCT01294774 US National Library of Medicine. https://clinicaltrials.gov.

miR-613 and miR-124 inhibit SphK1 (Yu et al., 2017; Zhao et al., 2017). miR-137, miR-181c, miR-9, and miR-29 regulate SPT (Geekiyanage and Chan, 2011). miR-15a modulates acidic SMase (Wang et al., 2015), and miR-101a targets Cer6 (Suzuki et al., 2016) (**Figure 1**). Interestingly, miR release into exosome particles depends on the ceramide-dependent pathway (Kosaka et al., 2010).

Although the importance of SphK/S1P system has been thoroughly reported, very little is known about the S1P/S1PR axis in the context of cardiac fibrosis. Given that specific anti-S1P antibodies significantly reduce TGF-β-stimulated collagen production by interfering with the binding of exogenous S1P to its specific receptors, a few years ago, the role of "inside-out" S1P signaling in the fibrotic process was proposed (Gellings Lowe et al., 2009). Three of the five S1PRs (S1PR-1, -2, -3) are the major subtypes expressed in the heart (Peters and Alewijnse, 2007; Means and Brown, 2009). Major candidates for the exogenous S1P-mediated control of the fibrotic process are S1PR2 and S1PR3 (Takuwa et al., 2008, 2010, 2013) that preferentially mediate the two following parallel signaling pathways crucially involved in the fibrotic process: Ras homolog GTPase/Rho-associated-protein kinase (Rho/ROCK) and Smad proteins. Particularly, S1PR3 promotes the activation of Rho signaling and the transactivation of TGF-β (Theilmeier et al., 2006; Takuwa et al., 2010). Under chronic activation of SphK1/S1P signaling, S1PR3 mediates pathological cardiac remodeling through ROS production (Takuwa et al., 2010). Moreover, S1PR3-mediated Akt activation protects against *in vivo* myocardial ischaemia-reperfusion (Means et al., 2007). Characterization of S1PR3-deficient mice also indicates that HDL and S1P promote cardiac protection through nitric oxide/S1PR3 signaling, and exogenous S1P induces intracellular calcium increase through the S1PR3/PLC axis (Theilmeier et al., 2006; Fujii et al., 2014).

Furthermore, S1PR3 can mediate cardioprotection in Langendorff-perfused mouse hearts against ischaemia/reperfusion injury via Rho/NFkB signaling (Yung et al., 2017). Although, S1PR3 is the most prevalent subtype in cardiac fibroblasts (Takuwa et al., 2013), myofibroblast differentiation and collagen production are mainly mediated by S1PR2 signaling (Schwalm et al., 2013). In fact, the silencing of S1PR2, but not of S1PR1 or S1PR3, can block S1P-mediated α-SMA induction (Gellings Lowe et al., 2009), and S1PR2 knock out mice show reduced fibrosis markers expression (Ikeda et al., 2009).

In the heart, the signaling pathways downstream of S1PR1 inhibit cAMP formation and antagonize adrenergic-mediated contractility activation (Means and Brown, 2009). Through S1PR1, S1P induces hypertrophy of cardiomyocytes *in vitro* (Robert et al., 2001) and decreases vascular permeability (Camerer et al., 2009). In bleomycin-induced injury, S1PR1 functional antagonists increase the pro-fibrotic response (Shea et al., 2010), suggesting an antifibrotic action of S1PR1 in the lung tissue.

There is evidence showing that S1P exhibits cross-talk with pro-fibrotic signaling pathways, such as TGF-β (Xin et al., 2004) and PDGF (Alderton et al., 2001). The involvement of S1PRs in the pro-fibrotic effects that are mediated by the cross-talk

between S1P and TGF-β has been demonstrated by inhibition of this effect in primary cardiac fibroblasts by the murine anti-S1P antibody, Sphingomab (Gellings Lowe et al., 2009). Specifically, the role of S1PR3 has been reported in the transactivation of the TGF-β/small GTPases system (Takuwa, 2002; Brown et al., 2006). Evidence has also been provided by a study in which the S1PR1 agonists FTY720 mimicked TGF-β action by promoting the differentiation of fibroblasts to myofibroblasts, but failed to act on S1PR3$^{-/-}$ fibroblasts (Keller et al., 2007). Differently from TGF-β, S1P and FTY720-P do not promote Smad signaling to induce ECM synthesis but rather activate PI3K/Akt and ERK1/2 (Sobel et al., 2013). Moreover, TGF-β2 stimulates the transactivation of S1PR2 in cardiac fibroblasts, and the silencing of TGF-β receptor II or co-Smad4 reduces the upregulation of CTGF expression induced by FTY720-P in mesangial cells (Xin et al., 2006).

Recently, our group has demonstrated that extracellular S1P inhibits the effects of RLX on MMP-9 release and potentiates hormone action on CTGF expression and TIMP-1 expression through a S1PR subtype-mediated signaling (Frati et al., 2015). Although the action of RLX on S1PRs expression is unknown, a transactivation between S1PRs and the RLX-specific receptor RXFP1 is worthy of investigation (Bathgate et al., 2013).

MicroRNAs can regulate S1PRs in several pathological conditions; for example, S1PR1 expression is upregulated by the deregulation of miR-148a, leading to TGF-β-dependent epithelial-mesenchymal transition (Heo et al., 2014). TNF-α significantly increases S1PR2 expression in human endothelial cells by reducing miR-130a level (Fan et al., 2016). No data are currently available on the role of miRs that are involved in cardiac fibrosis and S1PR expression.

S1PR MODULATORS IN CARDIAC FUNCTIONS

S1PR1 Agonists

Fingolimod (FTY729), synthesized from myriocin, is an immunosuppressive product isolated from *Isaria sinclairii* and has received approval from the Food and Drug Administration and from the European Medicines Agency as a drug for the treatment of MS (Gilenya, Novartis) (Chun and Hartung, 2010; Chun and Brinkmann, 2011; Cohen and Chun, 2011). The phosphorylated form, phospho-FTY720 **(FTY720-P)** that is formed by SphK2 *in vivo* (Brinkmann et al., 2010), is a structural analog of S1P that binds and activates S1PR1-3-4-5, but not S1PR2. Notably, the long-term activation of S1PR1 by FTY720-P determines receptor internalization and degradation, thus acting as a S1PR1 antagonist (Graeler and Goetzl, 2002; Matloubian et al., 2004; Brinkmann et al., 2010; Gonzalez-Cabrera et al., 2012). Due to the binding to various S1PRs, FTY720-P is responsible for several collateral effects on MS patients, such as cardiac effects (bradycardia and atrioventricular block) (Sanna et al., 2004; Camm et al., 2014; Gold et al., 2014). Such effects have been attributed to the activation of S1PR3, and in some cases, to S1PR1. Long-term S1PR1 down-regulation contributes to the disruption of Ca^{2+} homeostasis and attenuation of ischaemic preconditioning (Keul et al., 2016).

The phosphorylated form of Fingolimod takes part in cardioprotection in heart transplantation related ischaemia-reperfusion (*I/R*) injury (Santos-Gallego et al., 2016) and acting as potent anti-inflammatory (Aytan et al., 2016) and anti-oxidant agents may lead to reduce myocardial damage as a consequence of reduced cardiomyocytes death.

A new selective S1PR modulator, **ceralifimod (ONO-4641)**, has been recently designed and tested for its ability to limit the cardiovascular complications of Fingolimod (Krösser et al., 2015). Similarly, **Amiselimod (MT-1303)**, a second-generation S1PR modulator, has potent selectivity for S1PR1 and S1PR5 and has almost fivefold weaker GIRK activation (G-protein-coupled inwardly rectifying potassium channel) than FTY720-P (Sugahara et al., 2017). Other interesting compounds that can act on cardiac functions have been reviewed elsewhere (Vachal et al., 2006; Guerrero et al., 2016; Xiao et al., 2016) (**Table 1**). **SEW2871** is structurally unrelated to S1P, and its phosphorylation is not required for binding to S1PR1. SEW2871 promotes lymphopenia by reducing inflammatory cells, especially $CD4^+$ T cells (Lien et al., 2006), attenuates kidney ischaemia/reperfusion injury (Lai et al., 2007), and improves cardiac functions following myocardial infarction (Yeh et al., 2009). However, some evidence has indicated that SEW2871 can exacerbate reperfusion arrhythmias (Tsukada et al., 2007). SEW2871 and **AUY954,** an aminocarboxylate analog of FTY720 (Zhang et al., 2009), directly prevent allograft rejection in rat cardiac transplantation through the regulation of lymphocyte trafficking (Pan et al., 2006). Moreover, AUY954 significantly inhibited expressions of IL-17 and MMP-9 in rat sciatic nerves (Zhang et al., 2009). Repeated AUY954 administration enhanced pulmonary fibrosis by inducing vascular leak (Shea et al., 2010), suggesting caution in the use of this drug. **CYM-5442,** which binds to S1PR1 in a structural hydrophobic pocket different from Fingolimod (Gonzalez-Cabrera et al., 2008), induces lymphopenia and promotes eNOS activation in endothelial cells, thus playing a role in vascular homeostasis (Tölle et al., 2016). **Compound 6d** lacks S1PR3 agonism and induces lymphopenia with reduced collateral effects on the heart (Hamada et al., 2010). **BAF-312** is a next-generation S1PR modulator, which is selective for S1PR1 and S1PR5 (Fryer et al., 2012), and has species-specific effects on the heart. BAF-312 induces rapid and transient bradycardia in humans through GIRK activation (Gergely et al., 2012). Notably, different doses may be used to limit cardiac effects (Legangneux et al., 2013). **KRP-203** reduced chronic rejection and graft vasculopathy in rat skin and heart allografts (Takahashi et al., 2005), and in an experimental autoimmune myocarditis model, it significantly inhibited the infiltration of immune cells into the myocardium, reducing the area of inflammation (Ogawa et al., 2007). Currently, KRP-203 is undergoing a clinical trial for subacute lupus erythaematosus and in patients undergoing stem cell transplantation for hematological malignancies.

S1PR1 antagonists were reported to have therapeutic potential, but their use requires attention. **VPC23019,** acting as S1PR1 and S1PR3 antagonist, has been used in ischaemic pre- and post-conditioning cardioprotection that is promoted

by endogenous S1P in *ex vivo* rat hearts (Vessey et al., 2009). **W-146,** which was initially reported to increase the basal leakage of the pulmonary endothelium (Sanna et al., 2006), has been used to demonstrate the role of the S1P pathway in TGF-β1-induced expression of α-SMA in human fetal lung fibroblasts (Kawashima et al., 2012).

S1PR2 Agonists

S1PR2 agonists are mainly used in the treatment of hearing loss (**Table 1**). A study has shown that **CYM-5478** has vascular effects, which is indicated by enhanced ischaemia-reperfusion injury *in vivo* (Satsu et al., 2013).

S1PR2 Antagonists

JTE-013 was initially reported to affect coronary artery contraction (Ohmori et al., 2003). At long-term S1PR2 antagonism induces several collateral effects, such as a high incidence of B cell lymphoma (Cattoretti et al., 2009).

S1PR3 Antagonists

Selectively blocking S1PR3 is very difficult. For example, **VPC25239** antagonizes both S1PR3 and S1PR1, affecting smooth muscle cell functions, whereas VPC01091 leads to neointimal hyperplasia by preferentially blocking S1PR3 (Wamhoff et al., 2008). Other antagonists for S1PR3 are as follows: **CAY10444 (or BML-241)** (Koide et al., 2002) that can inhibit the prosurvival effect of HDLs after hypoxia-reoxygenation and **TY-52156** that suppresses the bradycardia induced by FTY-720 *in vivo* and promotes vascular contraction (Murakami et al., 2010).

The therapeutic use of monoclonal antibodies can be a valid alternative to synthetic compounds. A **monoclonal antibody, 7H9,** functionally blocks S1PR3 activation both *in vitro* and *in vivo* (Herr, 2012), reduces the growth of breast cancer tumors and prevents systemic inflammation, representing an effective approach against the morbidity of sepsis (Harris et al., 2012). To date, no specific antagonists for S1PR4 and S1PR5 are available, although (*S*)-FTY720-vinylphosphonate acts as a pan-antagonist that fully antagonizes S1PR1, S1PR3 and S1PR4 and partially antagonizes S1PR2 and S1PR5 (Valentine et al., 2010).

CONCLUSION

Targeting S1P signaling might be an intriguing new strategy for the treatment of cardiac fibrosis. However, the double face of the "sphinx" should be carefully considered, and the potential of the multiple collateral effects of S1PR modulators should be evaluated with caution.

AUTHOR CONTRIBUTIONS

EM and LM have partecipated in design the main structure of the minireview and in the revision of literature and in the preparation of the text. AV, FP, and AF have participated in reviewing the literature and writing the manuscript and prepare the figure.

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Sphingosine 1-Phosphate Receptor Modulator Fingolimod (FTY720) Attenuates Myocardial Fibrosis in Post-heterotopic Heart Transplantation

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Background and Objective: Sphingosine 1-phosphate (S1P), and S1P receptor modulator fingolimod have been suggested to play important cardioprotective role in animal models of myocardial ischemia/reperfusion injuries. To understand the cardioprotective function of S1P and its mechanism *in vivo*, we analyzed apoptotic, inflammatory biomarkers, and myocardial fibrosis in an *in vivo* heterotopic rat heart transplantation model.

Methods: Heterotopic heart transplantation is performed in 60 Sprague–Dawley (SD) rats (350–400 g). The heart transplant recipients ($n = 60$) are categorized into Group A (control) and Group B (fingolimod treated 1 mg/kg intravenous). At baseline with 24 h after heart transplantation, blood and myocardial tissue are collected for analysis of myocardial biomarkers, apoptosis, inflammatory markers, oxidative stress, and phosphorylation of Akt/Erk/STAT-3 signaling pathways. Myocardial fibrosis was investigated using Masson's trichrome staining and L-hydroxyline.

Results: Fingolimod treatment activates both Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways as evident from activation of anti-apoptotic and anti-inflammatory pathways. Fingolimod treatment caused a reduction in myocardial oxidative stress and hence cardiomyocyte apoptosis resulting in a decrease in myocardial reperfusion injury. Moreover, a significant ($p < 0.001$) reduction in collagen staining and hydroxyproline content was observed in fingolimod treated animals 30 days after transplantation demonstrating a reduction in cardiac fibrosis.

Conclusion: S1P receptor activation with fingolimod activates anti-apoptotic and anti-inflammatory pathways, leading to improved myocardial salvage causing a reduction in cardiac fibrosis.

Keywords: sphingosine 1-phosphate, cardiac fibrosis, ischemia/reperfusion injury, cardioprotective, heart transplantation

INTRODUCTION

Heart transplantation is the ultimate treatment option for heart failure (HF) (Koerner et al., 2000), which is a major health problem worldwide, with a prevalence of 23 million worldwide and 5.8 million in the United States alone (Benjamin et al., 2017). HF has been singled out as an emerging epidemic in the world with higher morbidity and mortality in 65 years old people and elders (Roger, 2013). Cardiac fibrosis is one of the main factors in the development and progression of HF. Therefore, targeting the development and progression of cardiac fibrosis is a critical goal in the treatment of HF (Miner and Miller, 2006; Opie et al., 2006).

Myocardial fibrosis can be categorized in two distinct forms: (1) ischemia/reperfusion (I/R) injury as a result of myocardial infarction, cardiac arrest, cardioplegic arrest, and cardiac transplantation due to reparative process by replacement of cardiomyocytes with collagen matrix and (2) interstitial or perivascular fibrosis as a consequences of trauma caused by volume or pressure overload, hypertrophic, or dilated cardiomyopathies (Dobaczewski and Frangogiannis, 2009; Krenning et al., 2010). Most common cause of cardiac dysfunction and mortality in cardiac transplant patients is due to I/R injury (Murata et al., 2004; Tanaka et al., 2005). Currently, low potassium solutions give some positive evidence for better cardiac graft preservation and demonstrate attenuation of grafted heart damage related to cold storage and I/R injury (Rudd and Dobson, 2009). However, it has been observed that this solution is not sufficient to protect from myocardial damage, due to decreased cellular energy reserves, release of oxidants, myocardial stunning, and myocardial dysfunction. Till now, there is no efficient strategy that can reduce cardiac fibrosis due to heart procurement and transplantation.

Sphingosine 1-phosphate (S1P) is a bioactive lipid that is characterized by a peculiar mechanism of action. In fact S1P, which is produced intracellularly, can act as an intracellular mediator, whereas after its export outside the cell, it can act as ligand of specific G-protein coupled receptors, which were initially named endothelial differentiation gene (EDG) and eventually renamed sphingosine 1-phosphate receptors (S1PRs). Currently five S1P (S1P1-5) receptors are known. The S1P1, S1P2, and S1P3 receptors are ubiquitously expressed whereas the expression of S1P4 and S1P5 receptors is highly restricted to the immune and nervous system (Ishii et al., 2004; Chun et al., 2010). In cardiac tissue, S1P1 receptor is the predominant receptor, whereas the expression of S1P2 and S1P3 receptors is relatively very low (Zhang et al., 2007). Keul et al. (2016) demonstrated in experimental study that S1P1 receptor is vital for regulating cardiac function by modulating ion channels and mediates myocardial protection by ischemic preconditioning.

Sphingosine 1-phosphate receptor modulator fingolimod is a lipophilic agent derived from the fungus *Isaria sinclairii* (Chiba and Adachi, 2012). S1P may also take part in cardioprotection against I/R injury (Somers et al., 2012). S1PR modulator fingolimod has potent anti-inflammatory and anti-oxidant properties, by inhibiting oxygen free radical and leading to reduced myocardial fibrosis, as a consequence of less cardiomyocytes death (Aytan et al., 2016).

Fingolimod activates Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways, leading to inhibition of pro-apoptotic proteins and activation of anti-apoptotic proteins (Liu et al., 2013). We hypothesized that pre-conditioning with fingolimod might achieve optimal cardioprotection and reduce fibrosis by activating anti-apoptotic, anti-inflammatory, and pro-survival pathways. Our aim was to investigate the cardioprotective role of fingolimod on apoptosis, inflammation, oxidative stress, nitrative stress, activation of Akt and Erk1/2 signaling pathways, and cardiac fibrosis in rat heterotopic heart transplantation model.

MATERIALS AND METHODS

Animals

Sprague–Dawley (SD) Rats (350–400 g) were obtained from Harlan Laboratories (Udine, Italy). The rats were fed standard rat chow with an access to *ad libitum*. The rats were housed at a density of 4–6 per cage and maintained on a 12 h light/dark cycle at 21°C. All the animal experiments were carried out according to the regulations (Declaration of Helsinki and "Guide for the Care and Use of Laboratory Animals" – Institute of Laboratory Animal Resources – National Institutes of Health) after experimental protocols were reviewed and approved by the Ethics Committee of University of Verona and the Italian Ministry of Health (341/2016-PR).

Experimental Design

In this study, 120 animals were used, 60 donors and 60 recipients. The 60 recipient rats were divided into two main groups (control group and fingolimod treated group). The experimental protocol used was as follows: the animals were pre-treated 15 min before heart explantation, by intravenous (i.v.) injection of either saline (control group) or fingolimod [treated group, 1 mg/kg; (Santos-Gallego et al., 2016)]. Hearts were harvested and transplantation was performed within 1 h. Abdominal cavity was closed and animals were allowed to recover. Transplanted animals were then divided into two subgroups: first subgroup was sacrificed after 24 h of R and second group was sacrificed 30 days after transplant ($n = 15$ for each group). The 24 h group was used to collect blood aliquots and hearts for cardiac, inflammatory, apoptotic, and oxidative markers while 30 day group was used to study cardiac fibrosis using Masson's trichrome staining and L-hydroxyproline. Schematic presentation of the experimental plan is illustrated in **Figure 1**.

Surgical Technique

Recipient rats were anesthetized with 5% isoflurane in 50% O_2 administered through a facial mask. Median abdominal incision was practiced. Sub-renal aortic and inferior vena cava (IVC) are exposed and isolated. Two vascular clamps were positioned superiorly and inferiorly to the site of transplanted heart implantation. Two adequate incisions were performed on aorta and IVC and the lumen of the two vessels was perfused with saline solution and heparin to avoid thrombus formation.

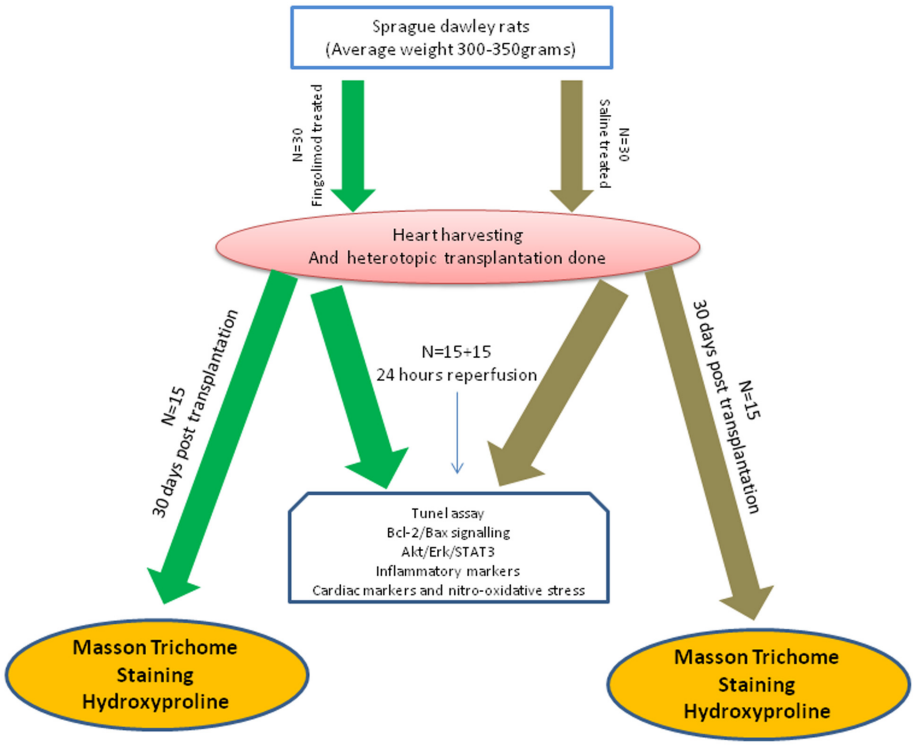

FIGURE 1 | Schematic presentation of experimental design.

While an operator sets up the recipient rat another operator prepares the donor rat. Anesthesia was performed by intraperitoneal administration of sodium thiopental (Pentotal®) 60 mg/kg. The animal was intubated orotracheally with an atraumatic tube consisting of a 16 G venous cannula and was mechanically ventilated (Harvard Model 687; Harvard Apparatus, Holliston, MA, United States). Tidal volume was set to 10 ml/kg and respiratory rate 60 breaths/min with an air–oxygen mixture/FiO_2 = 0.5). Anesthesia was maintained with isoflurane (2%) during the whole procedure.

The chest was opened through median sternotomy and heart was exposed. The right superior vena cava (SVC) was tied with Ticron 3.0 suture, ascending aorta and pulmonary artery are resected at 3–4 mm from their origin with an angled scissor.

Ten milliliters of cold S. Thomas cardioplegic solution was injected to stop the heartbeat, and after that IVC was tied and closed with Ticron 3.0 suture. The heart was then lifted up and with a single ligature pulmonary veins and left SVC were tied and closed. The heart was immediately dipped in a cold cardioplegia bath and another 10 ml of S. Thomas solution was injected into the heart through ascending aorta.

The explanted heart was then placed in the abdomen of the recipient rat and positioned in order to avoid twisting. The first anastomosis was performed between the abdominal aorta of the recipient rat and the ascending aorta of the donor heart using polypropylene 8.0 continuous suture. After completing this anastomosis, the anastomosis between IVC of the recipient rat and the pulmonary artery of the donor heart was performed. The heart was topically cooled by irrigating intermittently with saline solution at 4°C during the transplantation period. Time for the execution of the two anastomoses was between 35 and 40 min. Afterward, the vascular clamps were removed and if no stenosis or bleeding is evident, the implanted heart spontaneously starts beating. Warm saline solution was poured into the abdominal cavity and the abdomen was closed through continuous suture of the muscle wall and of the skin with a Polypropylene 4.0 suture. Sedation was suspended and after few minutes rat wakes up.

TUNEL Assay

Paraffin-embedded hearts from control and treated animals were deparaffinised with xylene and gradually hydrated in ethanol (EtOH) solutions (100, 95, 70, and 50%). Apoptotic cells were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the *In Situ* Cell Death Detection Kit (Roche, Cat. 11684809910). Nuclei were stained with Hoechst 33342 solution and quantified using ImageJ software (United States National Institutes of Health).

Histology and Myocardial Fibrosis Quantification

Myocardial tissues from the left ventricle were fixed in 10% formaldehyde in phosphate-buffered saline (PBS) (pH 7.2), after fixation these blocks were embedded in paraffin and 3 μm thick sections were prepared for histological staining (Song et al., 2014). Myocyte diameter was determined from haematoxylin–eosin stained ventricle sections and expressed in micrometer.

Cardiac fibrosis was analyzed from tissue sections stained with Masson's trichrome staining.

L-Hydroxyproline Assay

The L-hydroxyproline content is a type of protein that indicates collagen deposition. Using the L-hydroxyproline assay, collagen deposition was evaluated to assess tissue damage. Cardiac tissue was minced and hydrolyzed with HCl at 125°C and 200 psi for 2 h. After serial evaporation and addition of water final product was reconstituted in 5 ml distilled water. Each sample (2-ml aliquot) was oxidized with 1 ml of chloramine-T for 20 min. The remaining chloramine-T was neutralized with 1 ml of perchloric acid (3.0 M). The sample was mixed with 1 ml of *p*-dimethylaminobenzaldehyde and incubated in a hot water bath at 60°C and cooled to room temperature (RT) afterward. The absorbance was measured at 557 nm and the L-hydroxyproline concentration was measured using a standard curve.

Western Blotting

The heart tissue was homogenized in buffer containing 1% Triton X-100 with phosphatase and protease inhibitors cocktail (Sigma–Aldrich, Milan, Italy) as described previously (Giani et al., 2007). The tissue extracts were centrifuged at 16,000 × *g* for 15 min at 4°C to remove the insoluble material, and supernatants were collected in separate aliquots. Protein concentration was measured by using the BCA Assay Kit (Beyotime Institute of Biotechnology, China) according to manufacturer's instruction. The phosphorylation levels of Akt1/2 and ERK1/2 equal amounts of solubilized proteins (35 μg) were denatured by boiling for 5 min at 100°C in reducing buffer, resolved by SDS-PAGE, and protein transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, membranes were blocked in blocking solution (5% BSA in TBS-T) for 1 h. The membranes were then incubated with primary antibodies (1:1000) overnight at 4°C. The membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibodies (1:10,000). The bands were visualized by using Syngene Western Blotting detection system (Syngene, Cambridge, United Kingdom). The quantification of protein band densities was performed by ImageJ software (United States National Institutes of Health). GAPDH was used as loading control. The antibodies used were: total Akt1/2 (Abcam, Cambridge, United Kingdom), anti-phosphorylated Akt1/2 (rabbit, Cell Signaling), total ERK1/2 (Abcam, Cambridge, United Kingdom), anti-phosphorylated ERK1/2 (Cell Signaling, Denver, CO, United States), anti-STAT3 (Cell signaling, Denver, CO, United States), and HRP-conjugated goat anti-rabbit IgG (Abcam, Cambridge, United Kingdom). The remaining reagents were purchased from Sigma–Aldrich (St. Louis, MO, United States).

Immunohistochemical Staining

Paraffin-embedded left ventricle sections were heated at 60°C for 1 h and rehydrated with xylene for 20 min and with graded EtOH solutions (100, 95, 70, and 50%). Antigen retrieval solution was applied (0.01 M citrate buffer, pH 6.0) for 30 min in a boiling water bath, followed by slow cooling and rinsing with PBS. Endogenous peroxidase activity was suppressed by incubation with 3% hydrogen peroxide in TBS-T. Myocardial tissue sections were incubated with primary antibodies Bax (Abcam, Cambridge, United Kingdom) and Bcl-2 (Dako, Glostrup, Denmark) were incubated overnight at 4°C. Afterward, the sections were incubated with HRP-conjugated secondary antibody for 30 min, and diaminobenzidine and hydrogen peroxide chromogen substrate (DAB, Dako Corp.). All sections were counterstained with haematoxylin and mounted. The negative controls were incubated with non-immune rabbit IgG instead of primary antibody. All histological sections were studied using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY, United States).

Detection of Cardiac Enzymes and Oxidative Stress

After transplantation and R, blood was taken from the carotid artery and was placed at RT for 30 min to allow coagulation. The serum was then collected by centrifugation and placed at −70°C for preservation. Serum CK-MB and cardiac troponin-I were measured using CK-MB and Cardiac Troponin-I Assay Kits (Sigma–Aldrich, United Kingdom) and Oxidative Stress Kit (Thermo Fisher Scientific, United States) according to manufacturer's instruction.

Statistical Analysis

To compare treatment and control groups, all measurements and results are presented as mean ± SEM. Both groups were compared using Student's *t*-test or Mann–Whitney *U* nonparametric test. A *p*-value of less than 0.05 was considered to be statistically significant. Analyses were performed using SPSS software version 21 (SPSS Inc., Chicago, IL, United States).

RESULTS

Expression of Bcl-2 and Bax

Myocyte apoptosis is hallmark of reperfusion injury after HT. Therefore, the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 was analyzed in our HT model. As presented in **Figure 2**, after 24 h of reperfusion there was a massive expression of Bax in control group (**Figure 2B**) which was abolished by S1PR agonist fingolimod (**Figures 2C,G**). Likewise, the expression of anti-apoptotic Bcl-2 was significantly enhanced in fingolimod treated group (**Figures 2E,F,H**).

Effect of Fingolimod on Apoptosis

Myocyte apoptosis was analyzed by TUNEL assay. As shown in **Figures 3D,E,F** massive apoptosis was observed in the control group after 24 h of transplantation compared to healthy non-operated hearts (**Figures 3A–C**). This was significantly attenuated in fingolimod group (**Figures 3G,H,I**).

Collagen Deposition and Cardiac Fibrosis

Development of cardiac fibrosis was analyzed by measuring the collagen deposition in the cardiac tissue after 30 days

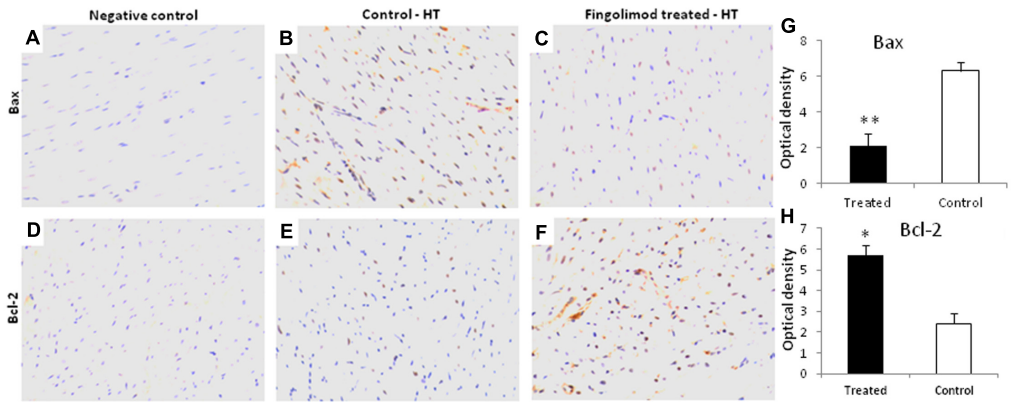

FIGURE 2 | Representative images (n = 15 for each group control and treated group) of myocardial tissue expression levels of Bcl-2 and Bax after 60 min of ischemia and 24 h reperfusion in fingolimod treated and control group. The protein expression levels of Bcl-2 and Bax were determined by an immunohistochemistry. **(A,D)** Negative control, **(B,E)** Bax and Bcl-2 expression in HT-control group, respectively, **(C,F)** Bax and Bcl-2 expression in HT-fingolimod treated group, **(G,H)** graphical presentation of Bax and Bcl-2 protein in fingolimod treated and control group. Data presented as a mean ± SEM. P-value < 0.05 considered as significant. (*p < 0.05 and **p < 0.001, treated vs. control).

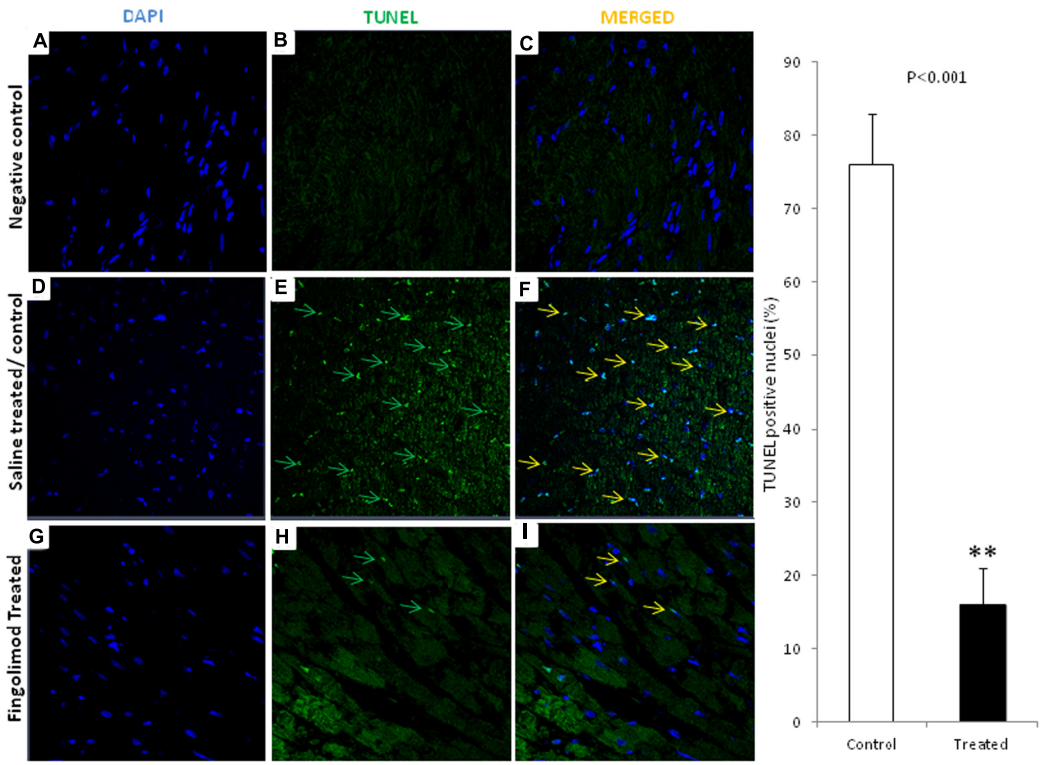

FIGURE 3 | Representative images (n = 15 for each group control and treated group) of immunofluorescent staining for TUNEL-positive nuclei in negative control, HT-control, HT-fingolimod treated groups. **(A,D,G)** Shows only DAPI in myocardial tissue, **(B,E,H)** TUNEL signals, and **(C,F,I)** merged images. TUNEL-positive myocytes were much lower in numbers frequently in control HT group than in HT-fingolimod treated group. Green and yellow arrows indicate apoptotic nuclei and apoptotic nuclei merged with DAPI, respectively. Original magnification 40×. Data presented as a mean ± SEM. P-value < 0.05 considered as significant. (**p < 0.001, treated vs. control).

of the reperfusion. Collagen deposition was analyzed by Masson's trichrome staining. Extensive collagen deposition in the ventricular tissue as indicated by blue staining was observed after 30 days of HT (**Figures 4A,B**). Interestingly, hearts previously perfused with fingolimod before transplantation were protected from fibrosis as indicated by much reduced collagen deposition (**Figures 4B,C**). L-Hydroxyproline assay has been performed to investigate collagen content in myocardial tissue

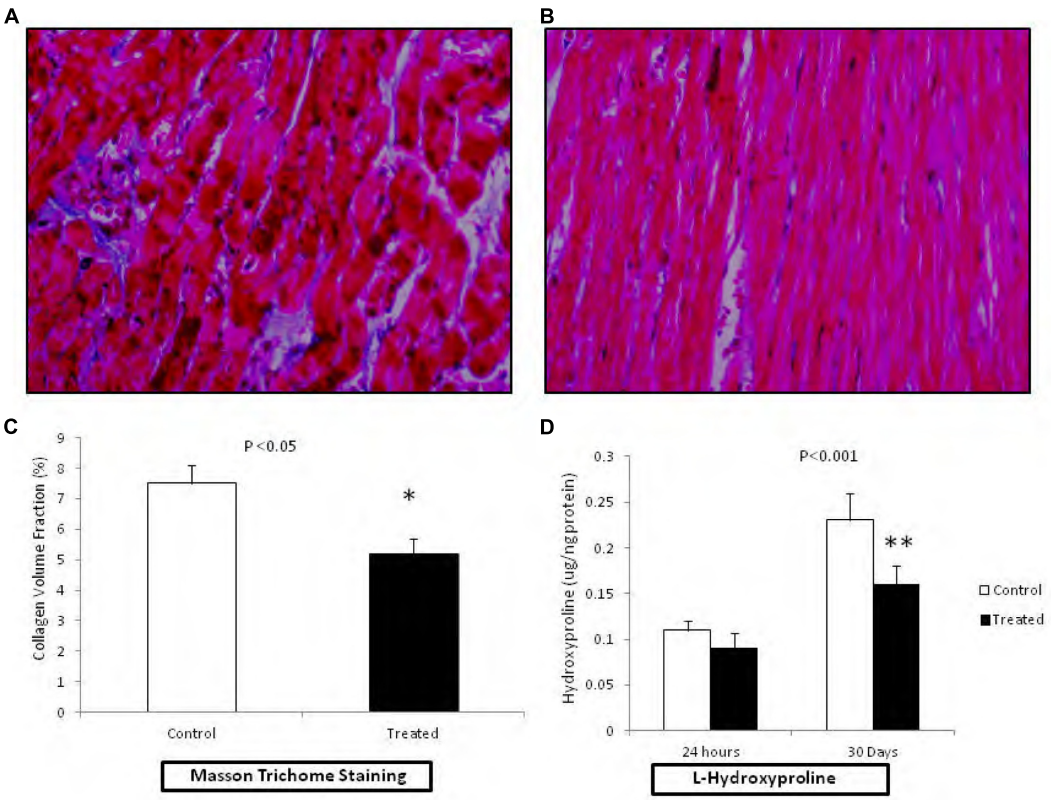

FIGURE 4 | Representative photomicrograph (n = 15 for each group control and treated group) showing the collagen deposition in rats related to HT I/R injury (20× magnification). Fingolimod decreased post-HT myocardial in myocardial fibrosis. The viable myocardium is stained bright red. Fibrosis is stained bright blue. **(A)**; HT control after 30 days of reperfusion and **(B)**; HT fingolimod treated after 30 days of reperfusion. **(C)** Graphical presentation of cardiac fibrosis, and **(D)** graphical presentation of L-hydroxyproline at early and late phase of R injury related to transplantation. Data presented as a mean ± SEM. P-value < 0.05 considered as significant (*p < 0.05 and **p < 0.001, treated vs. control).

post-cardiac transplantation at early and late phase of reperfusion (**Figure 4D**). This quantitative assay confirmed our histological findings that showed reduction of cardiac fibrosis with treatment of fingolimod prior to heart transplantation.

Serum Levels of Inflammatory Mediators

The inflammatory mediator's contribution in tissue injury has previously been identified in the heterotopic HT models (Mori et al., 2014). During heterotopic HT-related I/R injury a significant elevation in the plasma levels of cytokines mainly TNF-α, IL-1β, IL-6, and ICAM-1 may result (Vinten-Johansen et al., 2007). Like previous reports, increased serum levels of these mediators were observed in HT animals after 24 h of R (**Figure 5**) which were significantly attenuated in animals transplanted with hearts pre-treated with fingolimod.

Effect of Fingolimod on Erk42/44, Akt1/2, and STAT3 Phosphorylation

Since fingolimod pre-treatment caused a profound reduction in apoptosis of myocardial tissue, activation of pro-survival pathways was analyzed by measuring the phosphorylation state of the pro-survival Erk, Akt, and STAT3. As shown in **Figure 6**, pre-treatment with fingolimod resulted in a significant increase in phosphorylation of Erk42/44, Akt1/2, and STAT3.

Oxidative Stress

Uncontrolled production of reactive oxygen species (ROS) is one of the major causes inducing apoptosis in cardiac tissue after R. In order to evaluate whether fingolimod-mediated cardiac protection is due to its effect on cardiac ROS generation, levels of ROS and aldehydes (lipid peroxidation derivatives) were analyzed in the frozen perfused myocardial sample. As shown in **Figure 7**, levels of ROS and malondialdehyde were significantly reduced in cardiac tissues pre-treated with fingolimod.

DISCUSSION

In this study, we investigated cardioprotective and anti-fibrotic effect of pharmacological preconditioning during prolonged organ preservation using an *in vivo* heart transplantation model. We found that fingolimod, a S1PR modulator, efficiently works as ischemic preconditioning agent. Our findings suggested that fingolimod plays an important role in the reduction of apoptosis, inflammation, oxidative stress, and cardiac fibrosis. To the

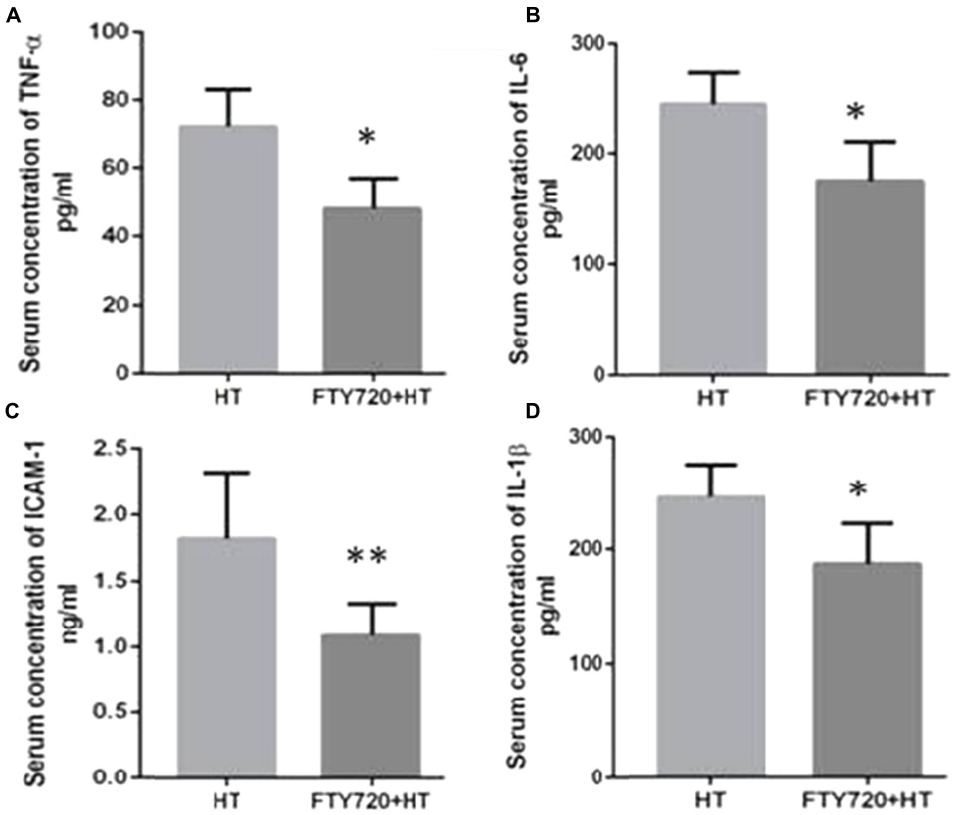

FIGURE 5 | Myocardial production of TNF-α **(A)**, IL-6 **(B),** ICAM-1 **(C)**, and IL-1β **(D)** after 24 h of R. **(A)** HT model without fingolimod treatment shows high expression of TNF-α as compared to fingolimod treatment. **(B)** HT-R induced significant high IL-6 after 24 h of R compared with the fingolimod treated. **(C)** Fingolimod treated group remarkably reduced the production of the ICAM-1 release as compared to Control. **(D)** This section of the panel presents production of 1L-1β higher in control vs. fingolimod treated group in HT-R group. Each bar height represents the mean ± SEM (each group $n = 15$) ($^{\#\#}p \leq 0.01$ vs. baseline, $^{*}p \leq 0.05$ and $^{**}p \leq 0.001$ vs. HT-control group).

best of our knowledge, the present study is first to investigate cardioprotective and anti-fibrotic role of fingolimod in preclinical heart transplantation model.

Fingolimod is one of the FDA approved treatment option in prevention of multiple sclerosis relapse (Bergvall et al., 2014; Totaro et al., 2015). In addition to its immune-modulating effect, fingolimod has a number of additional useful actions, including anti-inflammatory, anti-apoptotic, anti-oxidative, and anti-nitrative stress (Behjati et al., 2014). These properties are predicted to improve the myocardial insult related to I/R during heart transplantation. Previous studies have reported that pre-treatment with S1PR modulator protected myocardium from I/R injury (Li et al., 2016). In the present study, we show that fingolimod treatment remarkably reduced myocardial fibrosis.

The role of fingolimod in cytoprotection in different organs has been reported including pancreatic transplantation in diabetics (Tchorsh-Yutsis et al., 2009), fingolimod pre-treatment reduces I/R injury in liver transplantation (Anselmo et al., 2002), in renal transplantation (Tedesco-Silva et al., 2006), and against acute ischemic stroke (Zhu et al., 2015). These all models are suggestive for cytoprotective role of fingolimod in I/R injury.

Reperfusion after transient I in myocardium leads to cardiomyocytes apoptosis and cardiac dysfunction (Khan et al., 2006; Oh et al., 2013). TUNEL assay is the gold standard method (Darzynkiewicz et al., 2008) to measure the extent of apoptosis. Consistent with previous results, the transplanted hearts treated with fingolimod 15 min before explant expressed a lower frequency of TUNEL positive nuclei compared to control. This indicates the cardioprotective role of fingolimod by activating anti-apoptotic cascade.

In order to investigate the possible mechanisms of cardio-protective effects of fingolimod, its effects on activation of survival pathways were analyzed. Activation of the RISK and SAFE pathways by fingolimod has previously been reported (Lecour et al., 2002; Zhang et al., 2007). The RISK (Akt1/2, Erk1/2, and GSK 3β) and SAFE pathways (JAK and STAT3) are the main sources for regulation of apoptotic pathways because of control on mitochondrial permeability transition pore (Heusch et al., 2011; Heusch, 2013, 2015). Consistent with previous findings, activation of RISK and SAFE signaling pathways was observed, followed by decreased level of apoptosis in the treated group as compared to

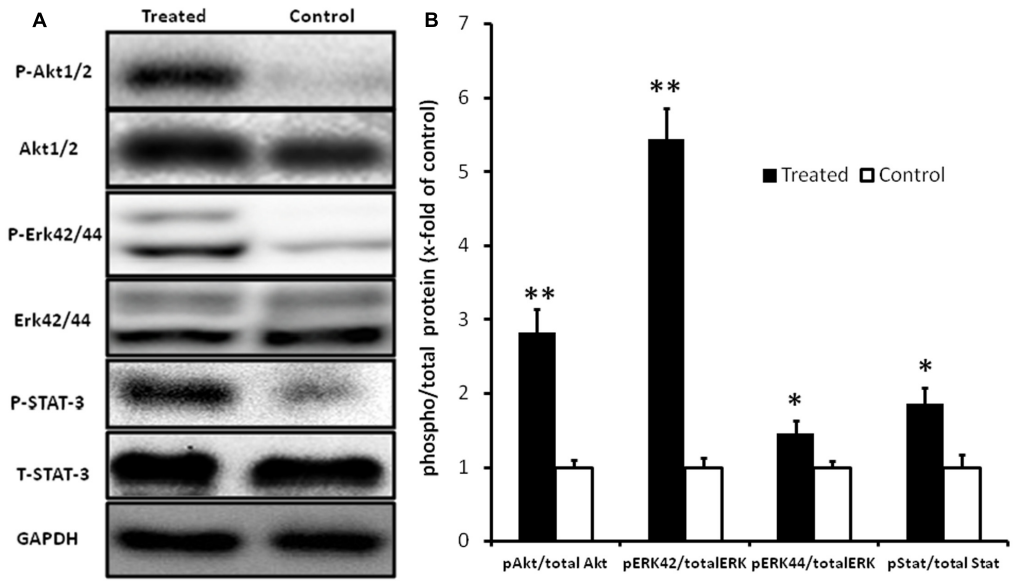

FIGURE 6 | Representative Western blot images **(A)** and graphical presentation of the ratio of pAkt1/2/Akt, pErk42/44/Erk, and pSTAT-3/STAT-3 **(B)**. Myocardial samples were collected at the end of 24 h reperfusion. Activation of Akt1/2, Erk42/44, and STAT-3 pathways in fingolimod treated group was measured by change in fold of phosphorylation with respect to control group. The GAPDH blot demonstrates that there was no change in the expression of total ERK1/2, total Akt1/2, and total STAT-3 in the treated group with respect to control group. Values are means ± SEM (*$p \leq 0.05$, **$p \leq 0.001$) ($n = 15$ each group).

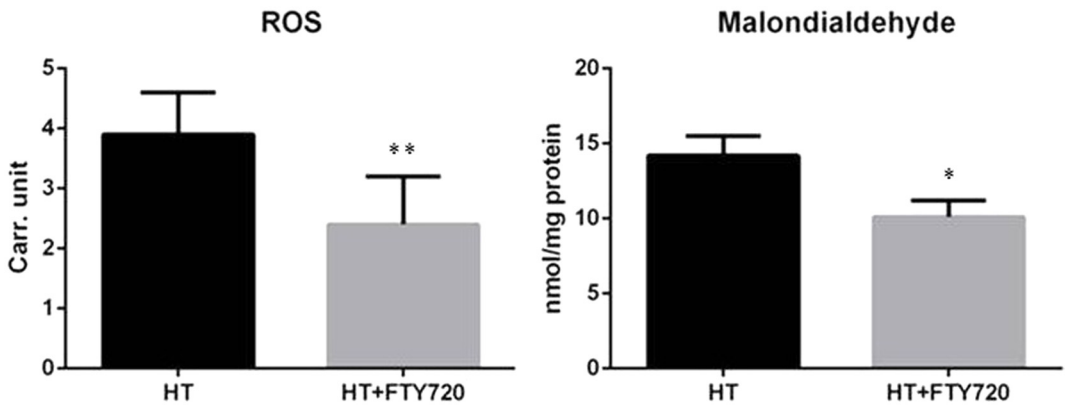

FIGURE 7 | Oxidative stress comparison of oxidative stress in heterotopic transplantation model with and without fingolimod treatment ($n = 15$ each group). ROS, reactive oxygen species; HT, heterotopic transplantation; FTY720, fingolimod; Carr. unit. Carratelli unit (**$p \leq 0.001$, *$p \leq 0.05$).

control. The inhibition of pro-apoptotic protein Bax enhanced immunostaining for anti-apoptotic protein Bcl-2 was found after 24 h of reperfusion in heterotopic transplanted heart tissue.

In this study, administration of fingolimod demonstrated inhibition of apoptosis by inhibiting inflammation and oxidative stress. In our investigations, on molecular and protein level both cardioprotection have been observed. According to previous reports, S1P and its agonist has important role in reduction of inflammatory mediators in I/R injury (Stone et al., 2015). Different models have been tested for immunosuppression including the porcine model of I/R, and spontaneous obstructive coronary atherosclerosis murine model showed the better myocardial protection and decrease inflammatory markers in the fingolimod-treated group. We measured inflammatory response in blood and heart tissue. In blood, we found the reduction of neutrophils and lymphocytes. Santos-Gallego et al. (2016) found improved myocardial salvage in animals using fingolimod and suggested an immunomodulatory role of fingolimod by activation of S1PRs. The inflammatory mechanism is one of the key factors in I/R injury. The ICAM-1, IL-6, and TNF-α contribute in as a pro-inflammatory cytokines to develop myocardial damage (Bonney et al., 2013; Li et al., 2013; Markowski et al., 2013). The correlation between anti-inflammatory effects of fingolimod with reduction in fibrosis is evident in our experiment.

It is well established that I/R causes myocardial injury due to oxidative stress (Ferrari et al., 2004; Ansley and Wang, 2013). Oxidative stress is also a major factor involved in apoptosis. Treatment with fingolimod reduces apoptosis in the I/R model (Chamorro et al., 2016; Santos-Gallego et al., 2016). This reduction in apoptosis is mediated via both RISK and SAFE pathways (Santos-Gallego et al., 2016). In our transplantation experiments, decreased level of malondialdehyde and ROS in the fingolimod-treated group was observed suggesting the reduction in oxidative stress is one of the cardioprotective mechanisms of S1PR modulators.

Myocardial I/R also produces nitric oxide synthase that releases nitric oxide which reacts with ROS as a result, forms toxic peroxynitrite leading to necrosis and apoptosis (Nour et al., 2013). Fingolimod treatment partially attenuates nitrative stress in transplanted myocardium (Colombo et al., 2014). Present findings suggest fingolimod can be efficiently used as a preconditioning agent to improve myocardial salvage.

Together, all these results are suggestive of the myocardial protective role having reduced fibrosis of fingolimod in global I–R. Fingolimod is the only available FDA-approved agent, containing S1PR agonist for prevention of multiple sclerosis relapses. According to our recent study on characterization and expression of S1PRs in human and rat (Ahmed et al., 2017) indicates potential translation of current study into clinical trials.

CONCLUSION

In conclusion, our study supports the cardioprotective role of S1PR modulator fingolimod for reduction in apoptosis, inflammation, oxidative stress, and fibrosis in an experimental model of heterotopic HT. This study provides insight for activation of cellular signaling pathways including RISK and SAFE pathways following significantly reduced cardiac fibrosis in long-term R.

AUTHOR CONTRIBUTIONS

NA, AR, GsF, GdF, and GL participated in research design; NA, DL, LSB, MG, and MA conducted experiments; AR and CC contributed new reagents; NA, NM, and MG performed data analysis; and NA, AR, and NM writing manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LM and handling Editor declared their shared affiliation.

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Targeting Adenosine Receptors for the Treatment of Cardiac Fibrosis

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Adenosine is a ubiquitous molecule with key regulatory and cytoprotective mechanisms at times of metabolic imbalance in the body. Among a plethora of physiological actions, adenosine has an important role in attenuating ischaemia-reperfusion injury and modulating the ensuing fibrosis and tissue remodeling following myocardial damage. Adenosine exerts these actions through interaction with four adenosine G protein-coupled receptors expressed in the heart. The adenosine A_{2B} receptor (A_{2B}AR) is the most abundant adenosine receptor (AR) in cardiac fibroblasts and is largely responsible for the influence of adenosine on cardiac fibrosis. *In vitro* and *in vivo* studies demonstrate that acute A_{2B}AR stimulation can decrease fibrosis through the inhibition of fibroblast proliferation and reduction in collagen synthesis. However, in contrast, there is also evidence that chronic A_{2B}AR antagonism reduces tissue fibrosis. This review explores the opposing pro- and anti-fibrotic activity attributed to the activation of cardiac ARs and investigates the therapeutic potential of targeting ARs for the treatment of cardiac fibrosis.

Keywords: adenosine, adenosine A_{2B} receptor, cardiac fibrosis, fibroblast, collagen synthesis, cAMP, myocardial infarction, heart failure

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INTRODUCTION

Cardiac fibroblasts form the largest population of interstitial cells in the adult mammalian heart (Chen and Frangogiannis, 2013). They have an essential role in the regulation of the extracellular matrix (ECM), which is crucial for maintaining the structural integrity of the myocardium and for electro-mechanical signal transduction (Camelliti et al., 2004; Souders et al., 2009). Cardiac fibroblasts are regulated by various mechanical and hormonal stimuli, in particular growth factors such as angiotensin II (ANGII) and the cytokine transforming growth factor β (TGFβ). ANGII and TGFβ can activate fibroblast cell-surface receptors to promote differentiation to myofibroblasts, the pro-fibrogenic phenotype that express the contractile protein α-smooth muscle actin (α-SMA) and exhibit enhanced secretory, migratory and proliferative properties (Schnee and Hsueh, 2000; Petrov et al., 2002; Leask, 2007; Porter and Turner, 2009; Lu and Insel, 2014). Following a myocardial infarction (MI), fibroblasts promote essential matrix deposition for proper tissue repair and scar formation to ensure structural integrity of the infarct zone. However, aberrant ECM deposition and excessive myofibroblast accumulation extending beyond the area of the original insult is responsible for maladaptive fibrosis leading to cardiac dysfunction, a hallmark feature of heart failure pathophysiology (See et al., 2005; Segura et al., 2012; Ferrari et al., 2016). Heart failure remains a major cause of mortality and morbidity in the western world with an estimated 50% 5 years survival rate after diagnosis (Mozaffarian et al., 2016). This highlights both the limitations

of current therapeutic management and the crucial need for new and innovative therapies for the treatment and prevention of heart failure. Extracellular nucleotides and nucleosides have recently been implicated as important mediators of fibroblast homeostasis and as such purinergic signaling has been investigated for its role in cardiac fibrosis. AMP catabolites, including inosine and oxypurines have also been shown to contribute to cardiac fibrosis and diastolic stiffening in some animal models of heart failure (Paolocci et al., 2006). The role of nucleotide (ATP, ADP, UTP) signaling in tissue fibrosis has been comprehensively reviewed previously (Lu and Insel, 2014; Ferrari et al., 2016; Novitskaya et al., 2016), therefore the current review will focus the modulation of cardiac fibrosis mediated by the nucleoside adenosine and adenosine receptors (ARs).

ADENOSINE SIGNALING IN THE HEART

Adenosine is a ubiquitous purine nucleoside that is an important regulator of cardiac function. Adenosine is described as a 'retaliatory metabolite' owing to its enhanced local release and ability to restore energy balance during times of cellular and metabolic stress (Newby, 1984; Shyrock and Belardinelli, 1997). The well-characterized cytoprotective actions have resulted in large clinical trials for adenosine and adenosine derivatives for the treatment of ischaemia-reperfusion injury post-MI (Kopecky et al., 2003; Ross et al., 2005; Forman et al., 2006). In addition to a clear role in cardioprotection, adenosine exerts a multitude of actions on the physiological regulation of the heart, including coronary vasodilation, heart rate control and AV nodal conduction, angiogenesis, myocardial hypertrophy and remodeling and fibrosis (Auchampach and Bolli, 1999; Peart and Headrick, 2007; Headrick et al., 2013). The myriad of cardiovascular effects stimulated by adenosine occur via activation of specific cell surface ARs. The AR family is comprised of four Class A G protein-coupled receptors (GPCRs), the A_1, A_{2A}, A_{2B} and A_3ARs. They exert distinct pharmacological actions through differential coupling to intracellular G proteins; the A_1AR and A_3AR preferentially activate $G_{i/o}$ proteins to inhibit adenylyl cyclase activity and subsequent cAMP production, while the A_{2A}AR and A_{2B}AR preferentially stimulate G_s proteins to activate adenylyl cyclase activity and increase cAMP accumulation (**Figure 1**) (Fredholm et al., 2001). The A_{2B}AR has also been shown to stimulate robust $G_{q/11}$ protein activation in some cell types (Feoktistov and Biaggioni, 1997; Linden et al., 1999). ARs, and the A_{2B}AR in particular, have also been shown to couple to additional transmembrane and intracellular proteins, which may influence downstream signal transduction (Mundell and Benovic, 2000; Fredholm et al., 2001; Sun and Huang, 2016). All four ARs are expressed in the heart and synchronous activation of multiple subtypes results in both complementary and opposing signal transduction for the fine-tuned regulation of cardiac function. Interestingly, both pro- and anti-fibrotic actions have been attributed to AR activation, which highlights both the complexity and ensuing challenges faced when targeting ARs for the treatment of cardiac fibrosis (Chan and Cronstein, 2009; Cronstein, 2011; Karmouty-Quintana et al., 2013). To date, the preponderance of evidence has implicated the A_{2B}AR in cardiac fibrosis (Epperson et al., 2009; Headrick et al., 2013; Novitskaya et al., 2016). Therefore, this review will explore the current understanding of the role of AR signaling in augmenting or attenuating cardiac fibrosis, with a focus on the predominant subtype implicated, the A_{2B}AR.

A_{2B}AR-MEDIATED ANTI-FIBROTIC SIGNAL TRANSDUCTION

Studies in isolated rat cardiac fibroblasts first proposed the A_{2B}AR as the subtype responsible for mediating adenosine's inhibitory actions on fetal calf serum-stimulated fibroblast proliferation (Dubey et al., 1997) and collagen and protein synthesis (Dubey et al., 1998). The role of the A_{2B}AR in adenosine-mediated anti-fibrotic signal transduction was later confirmed via antisense oligonucleotide A_{2B}AR silencing, which resulted in increased cell proliferation and basal collagen synthesis in cardiac fibroblasts (Dubey et al., 2001b). Similarly, A_{2B}AR overexpression had the opposite effect, significantly decreasing collagen and protein synthesis (Chen et al., 2004). The second messenger cAMP, has been shown to have a central role in inhibiting fibroblast and myofibroblast activity (Swaney et al., 2005; Lu et al., 2013). Accordingly, A_{2B}AR-mediated cAMP accumulation stimulated in fibroblasts by the non-selective AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) (Epperson et al., 2009) can reduce ANGII-stimulated collagen synthesis via an exchange factor directly activated by cAMP (Epac) and phosphoinositol-3 kinase (PI3K) dependent pathway (**Figure 1**) (Villarreal et al., 2009). In addition to effects on collagen synthesis, A_{2B}AR stimulation has been shown to decrease mRNA expression of pro-fibrotic gene markers including collagen I and connective tissue growth factor (CTGF) (Vecchio et al., 2016). Of specific importance to ARs, a positive feedback loop has been identified whereby β-adrenoceptor-stimulated cAMP can be secreted by fibroblasts or cardiac myocytes and metabolized in the extracellular space to adenosine to activate A_2ARs, thus exerting further inhibitory effects on fibroblast growth and function (Dubey et al., 2001a; Sassi et al., 2014).

Commensurate with the *in vitro* findings, an *in vivo* study in rats demonstrated chronic administration of the stable adenosine analog, 2-chloroadenosine (CADO) or the adenosine uptake inhibitor, dipyridamole, initiated 1 week after permanent ligation of the left anterior descending (LAD) coronary artery, protected against cardiac remodeling and reduced markers of fibrosis such as collagen volume fraction and matrix metalloproteinase gene expression (Wakeno et al., 2006). The effects of CADO on fibrotic and haemodynamic parameters were abolished in the presence of the selective A_{2B}AR antagonist MRS1754, but not selective antagonists for the other AR subtypes (Wakeno et al., 2006). Together, these studies suggest a salutary effect of A_{2B}AR activation on cardiac fibrosis, an effect which may be lost upon

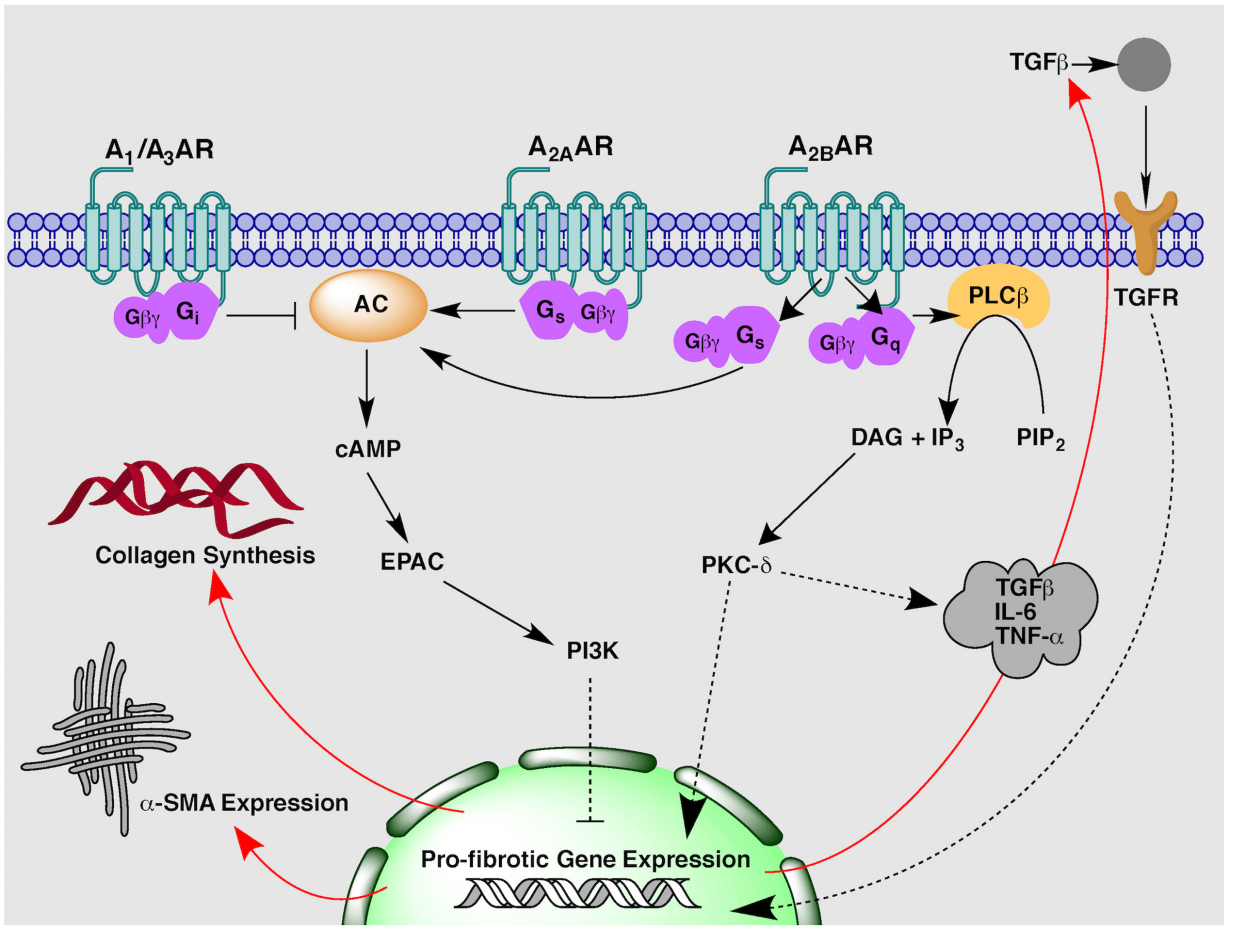

FIGURE 1 | An overview of proposed adenosine receptor-mediated intracellular signaling pathways implicated in the regulation of cardiac fibrosis.

$A_{2B}AR$ downregulation as observed in hearts taken from human patients with chronic heart failure (Asakura et al., 2007).

$A_{2B}AR$-MEDIATED PRO-FIBROTIC SIGNAL TRANSDUCTION

While the majority of *in vitro* studies have identified an anti-fibrotic role for the $A_{2B}AR$, recent studies have demonstrated $A_{2B}AR$ blockade appears to be beneficial within *in vivo* models of cardiac remodeling and fibrosis. In an *in vivo* mouse model of MI involving permanent coronary artery ligation, chronic administration of a novel, highly selective $A_{2B}AR$ antagonist, GS-6201, significantly reduced cardiac enlargement and dysfunction compared to vehicle-treated mice (Toldo et al., 2012). Similarly in an *in vivo* rat myocardial ischaemia-reperfusion model, GS-6201 improved ejection fraction and decreased fibrosis in the non-infarct and border zones with the greatest effect observed when GS-6201 was given 1 week rather 1 day after MI (Zhang et al., 2014). A pro-fibrotic role for the $A_{2B}AR$ has been supported by a study in $A_{2B}AR$ knock-out ($A_{2B}AR^{-/-}$) mice that demonstrate the $A_{2B}AR$ contributes to post-infarction heart failure (Maas et al., 2008). $A_{2B}AR^{-/-}$ mice had improved end diastolic pressure and reduced interstitial fibrosis when compared to wild-type mice 8 weeks after permanent left coronary ligation. Systolic blood pressure and infarct size remained the same between knock-out and wild-type animals suggesting the $A_{2B}AR$ contributes to heart failure pathology via post-infarction remodeling and reactive fibrosis rather than acute cardioprotection (Maas et al., 2008). The mechanism underlying the pro-fibrotic activity of the $A_{2B}AR$ may involve the pro-inflammatory effects mediated by this AR subtype. Blockade of the $A_{2B}AR$ inhibits caspase-1 activity and leukocyte infiltrate (Toldo et al., 2012), and attenuates secretion of pro-fibrotic and pro-inflammatory mediators such as TGFβ, tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) post-MI via a PKC-δ pathway (**Figure 1**) (Feng et al., 2009; Toldo et al., 2012; Zhang et al., 2014). A pro-inflammatory role of the $A_{2B}AR$ is reported by studies in other organ systems, in particular the lung where elevated adenosine concentrations and $A_{2B}AR$ activity promotes chronic fibrosis and inflammation in asthma and chronic obstructive pulmonary disease (Sun, 2006; Chan and Cronstein, 2009; Zhou et al., 2009; Karmouty-Quintana et al., 2013). Given the inflammatory response is intricately linked

to the regulation of tissue fibrosis, it is perhaps unsurprising therefore, that the A_{2B}AR has been implicated as a promoter of cardiac fibrosis *in vivo* (Ham and Rees, 2008; Kong et al., 2013; Stuart et al., 2016).

A_1AR MODULATION OF CARDIAC FIBROSIS

The protective role of A_1AR activation in cardiac remodeling appears to be largely attributed to the beneficial effects on cardiomyocyte hypertrophy rather than effects on fibrosis (Liao et al., 2003; Sassi et al., 2014; Chuo et al., 2016). A study using a non-selective adenosine analog (CADO) in mice subject to 4 weeks of chronic pressure overload via transverse aortic constriction (TAC), demonstrated reduced myocardial and perivascular fibrosis and hypertrophy compared to saline-treated mice (Liao et al., 2003). Attenuation of myocardial hypertrophy was A_1AR-mediated, as the anti-hypertrophic effects were reversed in the presence of an A_1AR-selective antagonist. As similar antagonist studies were not reported for measures of cardiac fibrosis (Liao et al., 2003), it cannot be ruled out that the anti-fibrotic effects were mediated by another AR subtype, in particular the A_{2B}AR. However, recent studies using more A_1AR-selective agonists do suggest an involvement of the A_1AR in cardiac fibrosis. A study of heart failure in dogs demonstrated capadenoson, an A_1AR partial agonist, decreased interstitial fibrosis (Sabbah et al., 2013). Similarly, activation of the A_1AR with a selective agonist N^6-cyclopentyladenosine (CPA), attenuated left ventricular collagen content and markers of fibrosis in response to α_1-adrenergic stimulation *in vivo* (Puhl et al., 2016).

Activation of the A_1AR has been recognized as central to the acute cardioprotective actions of adenosine (McIntosh and Lasley, 2012; Headrick et al., 2013). In agreement, overexpression of the A_1AR protects mice against acute ischaemic events, with cardiac infarct size markedly reduced in transgenic compared to wild-type animals (Yang et al., 2002). Paradoxically, however, chronic A_1AR cardiac overexpression in older mice (20 weeks) has been associated with enhanced baseline cardiac fibrosis and dilated cardiomyopathy (Funakoshi et al., 2006). Additionally, a study investigating myocardial fibrosis secondary to chronic renal failure demonstrated that an A_1AR-selective antagonist, SLV320, normalized cardiac collagen I and III content in the hearts of rats that had undergone a nephrectomy (Kalk et al., 2007). These studies may suggest chronic A_1AR stimulation reduces the cardiac resistance to non-ischaemic stress and may promote fibrosis, however, the conflicting evidence highlights the need for further studies to fully elucidate the role of this AR subtype in cardiac fibrosis.

A_{2A}AR MODULATION OF CARDIAC FIBROSIS

Separating the contribution of A_{2B}AR-mediated fibrotic signaling from that of A_{2A}AR activation has been difficult owing to the paucity of early subtype selective agonists and antagonists. Genetic alteration of the A_{2A}AR demonstrated that cardiac-specific overexpression of the A_{2A}AR in mice was protective against pressure-induced heart failure, attenuating fibrosis and improving cardiac function (Hamad et al., 2012). A more recent study demonstrated high A_{2A}AR expression in mouse cardiac fibroblasts stimulated the accumulation of the anti-fibrotic second messenger cAMP (Sassi et al., 2014), though perhaps to a lesser extent than the A_{2B}AR (Epperson et al., 2009). Combined with the known anti-inflammatory actions of the A_{2A}AR in the heart (Linden, 2001; Haskó et al., 2008), there is certainly valid grounds to suggest that A_{2A}AR signaling would attenuate cardiac fibrosis. However, further work is needed to clarify the exact role of A_{2A}AR, as stimulation of this receptor subtype has also been demonstrated to have pro-fibrotic effects in other organs such as the liver and skin (Chan et al., 2006a,b; Perez-Aso et al., 2014).

A_3AR MODULATION OF CARDIAC FIBROSIS

Comparatively few studies have investigated the role of the A_3AR in cardiac fibrosis, which is unsurprising given early studies examining the A_3AR (and A_1AR) expressed on isolated rat cardiac fibroblasts suggested these receptors to be of lesser functional importance than the A_2ARs (Chen et al., 2004). The A_3AR was investigated for its involvement in protecting against maladaptive cardiac hypertrophy and fibrosis on the basis that ecto-5′-nucleotidase (CD73; catalyzes the conversion of extracellular AMP to adenosine) deficiency exacerbated myocardial hypertrophy and heart failure in TAC mice (Xu et al., 2008). Contrary to hypothesis, A_3AR knock-out mice actually had reduced left ventricular hypertrophy, fibrosis and dysfunction after 5 weeks of TAC compared to wild-type animals. There was no effect of A_3AR deletion on parameters in the unstressed heart, suggesting the A_3AR has a deleterious role in cardiac fibrosis only in response to chronic pressure overload (Lu et al., 2008). In agreement, a recent study using a uninephrectomy and high salt-induced model of hypertension in mice, demonstrated that genetic abrogation of the A_3AR resulted in significantly less cardiac hypertrophy and fibrosis compared to wild-type animals (Yang et al., 2016). These studies suggest A_3AR antagonism may be a valid therapeutic approach to prevent chronic pressure overload-hypertrophy and fibrosis, however, further studies are warranted.

CONCLUSION AND FUTURE DIRECTIONS

Cardiac fibrosis is an important determinant of left ventricular dysfunction and remodeling following MI and is a hallmark of heart failure pathology, which is associated with an extremely high rate of mortality (See et al., 2005; Segura et al., 2012). It is therefore crucial to find new therapeutic approaches to prevent and ideally reverse underlying cardiac fibrosis in order

to modify the disease progression of heart failure. Purinergic signaling downstream of AR activation represents one such novel strategy to influence fibrosis homeostasis, however, much work is still needed to clarify the exact role of the receptor subtypes involved. A central question that remains is how the same receptor subtype can have both pro- and anti-fibrotic activity. The opposing effects as outlined in this review, may reflect differences in underlying disease pathology due to the type and duration of cardiac insult; whereby AR activation appears to be largely anti-fibrotic in acute ischaemic events but potentially pro-fibrotic under conditions of chronic myocardial stress. This supposition is supported by studies of adenosine's involvement in fibrosis of other organ systems (Karmouty-Quintana et al., 2013). In the lung, A_{2B}AR stimulation is protective in acute-bleomycin-induced lung injury but actually promotes fibrosis in chronic models of lung disease (Zhou et al., 2009, 2011). Similarly in the kidney, A_{2B}AR activation is beneficial in attenuating acute kidney injury (Grenz et al., 2012) but prolonged A_{2B}AR signaling increases interstitial fibrosis and collagen deposition in renal tissue (Roberts et al., 2014a,b). The exact mechanism behind these paradoxical effects requires further elucidation, but may reflect changes in differential receptor coupling with changes in cellular background as the disease progresses. Certainly, this idea is readily foreseeable for the A_{2B}AR with its high degree of plasticity and ability to couple to multiple G proteins and intracellular signaling cascades (**Figure 1**) (Cohen et al., 2010). In addition, it should be noted a great deal of our understanding of adenosine's role in cardiac fibrosis, in particular downstream of A_{2B}AR, has come from *in vitro* studies. This may not reflect the true course of disease progression *in vivo* due to the exclusion of the inflammatory response and loss of organ complexity including cross-talk with other cell types. Therefore, while AR signaling appears to be a promising target in cardiac fibrosis, further studies are needed to fully appreciate the potential of AR therapeutics in heart failure and underlying fibrosis.

AUTHOR CONTRIBUTIONS

EV drafted the manuscript. PW and LM made substantial contribution to the writing. EV, PW, and LM provided critical revision of the manuscript and approved it for publication.

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MINI REVIEW
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Cell-Based Therapies for Tissue Fibrosis

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The development of tissue fibrosis in the context of a wound-healing response to injury is common to many chronic diseases. Unregulated or persistent fibrogenesis may lead to structural and functional changes in organs that increase the risk of significant morbidity and mortality. We will explore the natural history, epidemiology, and pathogenesis of fibrotic disease affecting the lungs, kidneys, and liver as dysfunction of these organs is responsible for a substantial proportion of global mortality. For many patients with end-stage disease, organ transplantation is the only effective therapy to prolong life. However, not all patients are candidates for the major surgical interventions and life-long immunosuppression required for a successful outcome and donor organs may not be available to meet the clinical need. We will provide an overview of the latest treatment strategies for these conditions and will focus on stem or progenitor cell-based therapies for which there is substantial pre-clinical evidence based on animal models as well as early phase clinical trials of cell-based therapy in man.

Keywords: fibrosis, stem cells and regenerative medicine, cell therapy, mesenchymal stem cells, progenitor cells

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INTRODUCTION

An appropriate response to injury is required for homeostasis. While injury may take many forms, the repair response is typically generic. An understanding of aberrant wound repair has direct relevance to human disease given that organ fibrosis has been estimated to contribute to 45% of all-cause human mortality (Wynn, 2004). While large, this statistic should not be surprising given the significance of fibrosis in chronic diseases affecting multiple organs (**Table 1**). Despite an extensive understanding of fibrogenesis in response to injury, no effective anti-fibrotic therapies are currently available. The highly conserved wound healing response is also highly redundant with multiple overlapping pathways suggesting that inhibition of a single candidate molecule or pathway is insufficient and new approaches are required. Based on this notion, cell-based therapies with the potential to alter multiple therapeutic targets are gaining popularity. A broad discussion of all stem cell types is beyond the focus of this mini-review. We will concentrate on mesenchymal stem cells (MSCs), which form the largest experience in cell therapy, as well as our work with placental stem cells.

LUNG FIBROSIS

Epidemiology, Burden of Disease, and Natural History

Pulmonary fibrosis is a family of over 200 chronic lung diseases stemming from multiple underlying causes including autoimmune diseases such as scleroderma and rheumatoid arthritis. Pulmonary fibrosis may be a consequence of environmental exposure to inhaled dust, bacteria, or molds, but can also arise following exposure to cancer treatments such as radiation therapy or chemotherapy using bleomycin or methotrexate. However, idiopathic pulmonary fibrosis (IPF), a type of pulmonary fibrosis where the cause is unknown occurs in 3–9 per 100,000 people annually based on conservative estimates from Europe and North America (Hutchinson et al., 2015). The incidence of IPF is increasing globally, comparable to many cancers (Hutchinson et al., 2015). A low incidence of IPF in some countries may reflect exclusion of milder cases or inconsistent classification. The severity of reported disease appears to be greater in East Asia, where the majority of cases were recorded as "unspecified interstitial lung disease" rather than IPF (Munakata et al., 1994; Ohno et al., 2008; Lai et al., 2012; Han et al., 2013).

Current Clinical Management

The clinical progression of IPF is often slow and gradual but an accelerated decline has been reported in some patients, associated with episodes of acute respiratory exacerbations. The median survival rates are historically poor at 2–3 years, with 5-year survival ranging between 30 and 50% (Bjoraker et al., 1998; Mapel et al., 1998; Rudd et al., 2007; Raghu et al., 2011). To date, lung transplantation remains the only intervention with proven benefit. Corticosteroid use is discouraged due to the association between steroid use and survival rates following acute exacerbations (Papiris et al., 2015).

While drugs such as nintedanib and pirfenidone appear to reduce disease progression, widespread usage is unlikely due to their high cost and conflicting data surrounding clinical efficacy. Currently, the proposed use of pirfenidone is to bridge between diagnosis and lung transplantation (Delanote et al., 2016). Nintedanib has also been found to prevent disease progression, and both drugs are comparable in terms of their estimated costs and health-related quality of life benefits (Rinciog et al., 2017). However, neither is curative and their cost is high (£100,000 per QALY). Thus, there is a need to identify alternative therapies.

TABLE 1 | Fibrosis as a major component of chronic diseases.

Organ	Conditions
Skin	Systemic sclerosis (may involve lung and kidney)
	Keloids, burns
Lung	Idiopathic pulmonary fibrosis
	Interstitial lung disease (multiple aetiologies)
	Cystic fibrosis (may involve pancreas)
Heart, blood vessels	Congestive heart failure/cardiac fibrosis
	Atherosclerosis (affects multiple organs)
Liver	Cirrhosis (multiple aetiologies)
	Hepatorenal fibrocystic diseases
Intestine	Crohn's disease
	Post-operative adhesions
Pancreas	Chronic pancreatitis
Kidney	End-stage renal disease (diabetes or hypertension)
	Renal interstitial fibrosis
Immune system	Chronic graft vs. host disease
Musculo-skeletal system	Rheumatoid arthritis (may involve lung)
	Ankylosing spondylitis
	IgG4-related retroperitoneal fibrosis

Pathophysiology

Historically, IPF was believed to be an inflammatory disorder that progresses to fibrosis. The failure of anti-inflammatory and immunosuppressive therapeutic strategies triggered the need for reassessment (Selman et al., 2001; Raghu et al., 2012). The current consensus is that IPF is a consequence of multiple interacting genetic and environmental risk factors, with repeated damage and premature aging of alveolar epithelial cells (AECs) in genetically susceptible individuals (Wells and Maher, 2017). One robust genetic linkages to IPF is *MUC5B* polymorphism; however, the role of this gene in IPF pathogenesis remains undefined (Conti et al., 2016; Nakano et al., 2016). Unsurprisingly, the prototypic pro-fibrotic transforming growth factor-β (TGFβ) plays a central role in IPF, and while its function is well described, the source of excess TGFβ and activation of its latent form are poorly understood. A recent study by Froese et al. (2016) uncovered a role for mechanotransduction in TGFβ activation, unique to fibrotic lungs, suggesting that the physical stiffness of IPF lungs and mechanical forces applied to fibrotic lungs may contribute to disease perpetuation. Premature aging, telomere shortening, and alveolar senescence are also thought to contribute to IPF pathogenesis. Telomere dysfunction in AECs but not collagen-producing cells is responsible for age-related lung fibrosis (Naikawadi et al., 2016). When telomere dysfunction was conditionally induced in type 2 AECs (AEC2) in mice, an AEC2-induced cytokine response was detected and when challenged with bleomycin, a 100% mortality rate was observed, supporting the critical role of telomere function in AEC2 for alveolar repair (Alder et al., 2015). Given the role of AEC2 as alveolar progenitor cells, Adler et al. concluded that alveolar stem cell failure might contribute to lung fibrosis. These observations have led some to postulate that a regenerative approach is required (Chambers and Hopkins, 2013).

Cell Therapies for IPF

To date there are six Phase I/II clinical trials (ClinicalTrials.gov) using stem cells for IPF, predominantly allogeneic bone marrow-derived MSCs (NCT01919827, NCT02594839, and NCT02013700). However, placenta and adipose tissue-derived MSCs have also been tested (NCT01385644 and NCT02135380). Interest in MSC-based therapies is attributed to their reported immunomodulatory and anti-fibrotic properties exerted through paracrine mediators. For example, there is recent evidence that

MSC can reduce ER stress, thereby improving survival and function of AEC2 through the release of hepatocyte growth factor (Nita et al., 2017). One current clinical trial is aimed at a specific subset (p63+/Krt5+) of the patient's own lung stem cells (NCT02745184), with the purpose of encouraging cell engraftment and restoring the lost p63+/Krt5+ distal airway stem cells in the fibrotic lung (Zuo et al., 2015). The outcomes of two trials have been published. Allogeneic placental MSCs given at a dose of 1 or 2 $\times$ 10^6 cells/kg body weight were well tolerated in moderate to severe IPF (Chambers et al., 2014). Similarly, a single infusion of 20, 100, or 200 million allogeneic bone marrow MSCs was well tolerated by patients with mild to moderate IPF (Glassberg et al., 2016). While these safety outcomes are encouraging, clinical efficacy remains to be determined.

KIDNEY FIBROSIS

Epidemiology and Pathogenesis of Fibrosis in Kidney Disease

The epidemic of chronic kidney disease (CKD) and end-stage renal failure (ESRF) is a crisis for global healthcare. There is urgent need for new therapeutic options considering the high morbidity of dialysis, extensive healthcare costs, and donor-kidney shortages. Known risk factors for CKD include age, hypertension, obesity, and diabetes (McMahon et al., 2014).

Regardless of etiology, the common end-point of kidney injury is fibrosis leading to CKD development (Samarakoon et al., 2012). An excessive inflammatory and fibrotic response to injury results in decreased renal function as the renal tubules are damaged by scar tissue (Hewitson, 2009). Following initial renal injury, endogenous kidney cells release pro-inflammatory chemokines (Balasubramanian, 2013) that recruit inflammatory cells, activating fibroblasts, and causing tubular dilation (Meran and Steadman, 2011). The recruited immune cells release further inflammatory cytokines including those from the TGFβ superfamily and mitogen-activated protein kinases (MAPK/ERK) that activate fibrotic genes through SMAD signaling (Chevalier et al., 2010), leading to interstitial fibrosis and extracellular matrix accumulation. While inflammation and the TGFβ pathway are essential for normal kidney development and homeostasis, unopposed expression results in a harmful cycle of injury as seen in CKD (Schnaper et al., 2009).

Potential of Cell-Based Therapies for Kidney Disease

Stem or progenitor cell therapies offer a strategy for modulating CKD progression by suppressing multiple pathogenic pathways and promoting pro-regenerative mechanisms. MSCs are pursued as a therapeutic tool as they are immunomodulatory, easily obtainable from bone marrow, and can be expanded in culture for use in the clinic (Yagi et al., 2010). MSCs elicit endogenous repair through paracrine and/or endocrine mechanisms that modulate the immune response, ultimately allowing for cellular replacement. In pre-clinical studies we have demonstrated that MSCs have immunomodulatory properties, and secrete anti-inflammatory cytokines that promote inhibition of pro-inflammatory cytokines (Wise et al., 2014; Huuskes et al., 2015; Wise et al., 2016). MSCs have been used in experimental and clinical settings to improve diabetes and diabetic complications including kidney fibrosis. Recent clinical trials show that MSCs are safe and well tolerated in diabetes (Skyler et al., 2015); however, the diabetic microenvironment and/or comorbidities alter the quality or efficacy of MSCs following transplantation. Further mechanistic studies are needed to understand how MSCs protect against fibrotic injury and to improve efficacy following cell transplantation to overcome the transient clinical benefits that observed to date.

Endothelial progenitor cells (EPCs) also have therapeutic potential. EPCs can be mobilized from the bone marrow and adventitial tissue surrounding endothelial cells (ECs), and home toward sites of injury. There, they influence the release of vasoactive substances or directly differentiate into mature ECs to regenerate damaged endothelium. Diabetes-related EPC dysfunction is closely linked to the impaired healing response experienced by many patients with diabetic CKD. Circulating EPCs are low in type 2 diabetic patients and the loss of function of these cells may contribute to the vasodegenerative changes observed in diabetic micro- and macrovasculature disease (Schatteman et al., 2000). Therefore, harnessing the vascular reparative properties of EPCs represents a novel treatment for therapeutic revascularization and vascular repair for CKD patients with diabetes.

Challenges to Reverse Kidney Fibrosis to Promote Repair

A growing number of clinical trials show that MSCs are safe and well tolerated in diabetes (Skyler et al., 2015). The exogenous application of angiogenic-stimulating EPCs has shown promise for treatment of kidney failure, heart disease, and diabetes including retinopathy (Stitt et al., 2013). Both MSCs and EPCs mediate their effects largely through paracrine signaling and therefore require microenvironments that support optimal cell engraftment and proliferation. However, impediments in clinical translation occur due to low cell survival rates following transplantation that limit therapeutic efficacy (Chevalier et al., 2010). In particular, the fibrosis and chronic inflammation hamper cell survival and limit the cell integration into host tissue. Modulation and removal of the fibrotic lesion is therefore crucial to facilitate cell integration. In addition, the low number of transplanted cells retained at the site of injury also hampers stem cell efficacy.

To overcome these limitations, we recently reported a bimodal attack by combining MSC therapy and relaxin (RLX) to combat kidney fibrosis progression and aid in MSC survival (Huuskes et al., 2015). Combined MSCs and RLX administration in an obstructive nephropathy model significantly ameliorated kidney fibrosis, reduced macrophage infiltration, myofibroblast proliferation, and upregulated active MMP-2 compared to either therapy alone. This suggested that rather than inhibiting collagen accumulation, combination therapy induced significant collagen degradation. We provide evidence that RLX may influence

MSCs *in vivo* creating a more favorable environment for MSC-mediated repair (Huuskes et al., 2015). Targeting fibrosis resolution and limiting vascular damage may also be beneficial through combination therapy, as kidney function is dependent on adequate organ perfusion.

LIVER FIBROSIS

Epidemiology, Burden of Disease, and Natural History

Globally in 2013, cirrhosis was the 6th cause of life years lost in developed countries; ranging from 5th in Europe and central Asia, to 9th in southeast Asia and Oceania. In the United States, cirrhosis was the 12th leading cause of death overall and the 5th in adults aged 45–54 years (Heron, 2012). Common causes of chronic injury leading to cirrhosis include non-alcoholic steatohepatitis (NASH), alcohol use, and viral hepatitis.

Hepatic fibrosis will progress to cirrhosis in many patients unless the cause of injury is removed. Progressive hepatocyte loss and subsequent disruption of the hepatic vasculature by unregulated ECM expansion result in liver insufficiency characterized by jaundice, coagulopathy, and hypoalbuminemia. Portal hypertension leads to ascites, variceal hemorrhage, and hepatic encephalopathy. The onset of any of these conditions defines hepatic decompensation, which has a significantly higher 1-year mortality than compensated cirrhosis, 20% compared with 5% in one study of 700 patients (Zipprich et al., 2012). In these patients, the only treatment that alters long-term survival is liver transplantation. Unfortunately, not all patients are transplantation candidates and wait-list mortality remains a concern (Toniutto et al., 2016).

Pathogenesis

Hepatic fibrogenesis involves a dynamic interplay among hepatic stellate cells (HSCs), macrophages, and liver progenitor cells (LPCs). HSCs are pericytes that store vitamin A. During chronic liver injury, they transform to myofibroblasts, acquire a contractile phenotype, and accumulate at sites of injury where they secrete large amounts of ECM including collagen. TGFβ is a major fibrogenic cytokine that triggers HSC activation and ECM production and induces hepatocyte apoptosis (Gressner, 2002). Platelet-derived growth factor (PDGF) is the most potent mitogenic cytokine for HSC (Borkham-Kamphorst et al., 2007). These cytokines are logical targets for drug development. Blocking TGFβ and PDGF signaling has been effective in ameliorating experimental liver fibrosis (Yata et al., 2002; Liu et al., 2011), however, off-target effects hinder clinical development.

Kupffer cells (resident liver macrophages) and recruited circulating monocytes contribute to inflammation, fibrogenesis, and fibrosis resolution. Macrophages are capable of distinct activation states and functions, broadly classified as M1 (classical) or M2 (alternative) (Mantovani et al., 2004). M1 macrophages are classically pro-inflammatory, whereas M2 macrophages are responsible for immunomodulation and wound-healing responses. In addition a fibrolytic macrophage subset ($Ly6C^{lo}$) that produces high levels of matrix metalloproteinases that contribute to ECM degradation has been described (Ramachandran et al., 2012).

LPCs are rare in healthy tissue but proliferate and differentiate into cholangiocytes or hepatocytes during chronic liver injury. The LPC response corresponds with the degree of liver injury (Lowes et al., 1999; Roskams et al., 2003) because, unlike hepatocytes, LPC resist the anti-proliferative actions of TGFβ (Nguyen et al., 2007). LPC express surface markers representative of their primitive, undifferentiated state such as Thy-1 (CD90), prominin (CD133), and pan-cytokeratin. A close physical relationship exists between HSC and LPC suggesting that the two cell types proliferate in tandem as HSC depletion significantly dampens the LPC response (Roskams, 2008; Ruddell et al., 2009). HSC produce soluble factors that increase LPC proliferation and hepatocyte differentiation (Nagai et al., 2002; Lin et al., 2008) and ECM proteins produced by HSC, such as laminin, may activate the LPC response (Kallis et al., 2011). Conversely, LPC produce lymphotoxin (LT), which recruits HSC through paracrine signaling (Ruddell et al., 2009). LPC also recruit macrophages via CCL2 and CX3CL1. Macrophage-derived TNF and LT, in turn, influence LPC response (Viebahn et al., 2010).

Treatment of Hepatic Fibrosis

The concept that hepatic fibrosis develops from a wound-healing response to chronic injury provides a rational basis for treatment. Diminishing liver injury by inhibiting chronic hepatitis B replication results in significant fibrosis regression in cirrhotic patients (Marcellin et al., 2013). Similar outcomes occur in patients with chronic hepatitis C infection (Hoefs et al., 2011). In diseases without specific therapy, a general anti-fibrotic approach might be useful. However, a recent trial of a monoclonal antibody against lysl-oxidase-like 2, which mediates collagen cross-linkage, was not effective (Meissner et al., 2016). Considering the complex interactions involved in hepatic wound healing, cell-based therapy may provide a strategy to control inflammation, degrade collagen, and promote hepatic parenchymal regeneration. Human clinical trials have utilized MSC with variable cell doses, delivery routes, and administration frequency (**Table 2**). Trial endpoints commonly include liver tests, ascites volume, or clinical scores (Child–Pugh–Turcotte, model for end-stage liver disease). To date, outcomes have yet to translate into clinical practice. Furthermore, there is experimental evidence that bone marrow-derived MSC can contribute to hepatic fibrosis (Russo et al., 2006). MSCs as an anti-fibrotic therapy has been critically reviewed (Haldar et al., 2016).

We studied human amnion epithelial cells (hAECs), fetus-derived stem-like cells that arise prior to gastrulation and are easily isolated from the placenta, which is an abundant and ethically undisputed source. hAEC prevent and reverse inflammation and established fibrosis in immunocompetent animal models of liver injury (Manuelpillai et al., 2010), diminish myofibroblast activation, and skew hepatic macrophages toward a reparative phenotype (Manuelpillai et al., 2012). Similar effects are seen with cell-free conditioned media, suggesting that hAEC release factors responsible for the observed outcomes (Hodge

TABLE 2 | Summary of reports from clinical trials assessing safety and efficacy of cell therapies for lung and liver fibrosis.

Study	Number of patient treated/ control	Cell type	Route	Number of cells transfused/ number of injections	Functional benefit sustained to end of F/U period?	Safety
Clinical trials in lung fibrosis						
Tzouvelekis et al., 2013	14/0	Autologous adipose stromal cells	Endobronchial	0.5×10^6/kg body weight single injection	No, 12 months	No serious side-effects or complications
Chambers et al., 2014	8/0	Allogeneic placental MSC	Intravenous	1×10^6; 2×10^6 kg body weight single injection	No, 6 months	One chest infection; one IPF exacerbation
Glassberg et al., 2016	9/0	Allogeneic BM MSC	Intravenous	20, 100, or 200×10^6 single injection	Yes, 6 months	No serious side-effects or complications
Clinical trials in liver fibrosis						
Terai et al., 2006	9/0	Autologous BM	Peripheral IV	$2.21–8.05 \times 10^9$ Avg. 5.2×10^9	Significant decrease in average CPT at 4 and 24 weeks	All had fever (38°C) at 1 day post-therapy
Couto et al., 2011	8/0	Autologous BM MNC	HA	$2–15 \times 10^8$ single injection	Yes, 2 months No, 12 months	
Amer et al., 2011	20/20	Hepatocyte lineage from autologous BM MNC	Intrahepatic or intrasplenic	5 mL of cell suspension (2×10^6/mL) single injection	Yes, 6 months	Fever within 24 h after injection in 10 subjects (50%)
Peng et al., 2011	53/105	Autologous BM	HA	10^6/mL, number transfused not stated	Yes, 3 and 9 months No, 48 months	No serious side-effects or complications
El-Ansary et al., 2012	15/10	BM MNC nine undifferentiated six HC differentiated	Peripheral IV	10^6/kg (40% HLC, 60% MSC) single injection	Yes, 3 and 6 months	No safety evaluation
Zhang et al., 2012	31/15	Umbilical cord MSC	Peripheral IV	0.5×10^6/kg body weight	Yes, 48 weeks	Four had fever 38°C at 2–6 h
Mohamadnejad et al., 2013	15/12	BM MSC	Peripheral vein (30 min)	195 million (120–295 million) single injection	No difference between treated and control	
Lukashyk et al., 2014	6/0	BM MSC	Intrahepatic	5 mL suspension, 1×10^6/kg single injection	Yes, 1 and 6 months	No safety evaluation
Salama et al., 2014	20/20	G-CSF, autologous BM MSC	Peripheral IV	1×10^6/kg body weight	Yes, 6 months	
Mohamadnejad et al., 2016	18/9	Eight $CD133^+$ nine BM MNC	Portal vein	$4.7 \times 10^6–9.17 \times 10^8$ (averages) two injections	Yes, 3 months No, 6 months	No procedural complications

et al., 2014). Liver fibrosis reduction also occurs in hAEC-treated mice given a "Western diet" high in lipids and fructose to model fatty liver disease (unpublished). A phase 1 safety trial is planned in patients with compensated cirrhosis.

SUMMARY

The global burden of end-stage fibrotic disease can be seen in the impaired survival of patients with IPF, diabetic CKD, and cirrhosis. Fortunately, the pathogenesis of fibrosis in response to injury is relatively well understood and remarkably similar in different organs, suggesting that an integrated approach may be possible. Control or removal of the injury stimulus should be the primary focus in preventing disease progression, yet for many control is incomplete or unachievable, thus the need for a broadly effective anti-fibrotic therapy that targets multiple fibrogenic pathways remains. Cell-based approaches employing stem cells that are easy to isolate and upscale to sufficient quantities for clinical use have been successfully characterized in animal models of organ fibrosis. While the outcomes of early phase clinical trials indicate that cell-based (primarily MSC) therapies are safe, efficacy data remain scarce. Consequently, cell-based therapies remain largely experimental. The lack of robust efficacy data

may be due to the heterogeneity of MSC populations as well as limited agreement regarding differentiation state, doses, and administration regimens. Challenges remain in determining the goals of cell therapy – whether to supply sufficient cells to replace damaged parenchyma, to dampen inflammation with the aim of decreasing fibrosis, or to stimulate endogenous progenitor cells and repair processes. Furthermore, the ability to manufacture, transport, and store stem cells in a cost-effective manner must be considered. Clinical trials will continue to inform us about the most effective stem cell types on which to base therapy as well as the optimal dosages necessary to achieve a clinically meaningful reduction in fibrosis-related organ dysfunction.

AUTHOR CONTRIBUTIONS

WS contributed the liver fibrosis section; RL contributed the lung fibrosis section; SR contributed the kidney fibrosis section; and all authors reviewed the manuscript and provided critical intellectual input.

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The Human Amnion Epithelial Cell Secretome Decreases Hepatic Fibrosis in Mice with Chronic Liver Fibrosis

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Background: Hepatic stellate cells (HSCs) are the primary collagen-secreting cells in the liver. While HSCs are the major cell type involved in the pathogenesis of liver fibrosis, hepatic macrophages also play an important role in mediating fibrogenesis and fibrosis resolution. Previously, we observed a reduction in HSC activation, proliferation, and collagen synthesis following exposure to human amnion epithelial cells (hAEC) and hAEC-conditioned media (hAEC-CM). This suggested that specific factors secreted by hAEC might be effective in ameliorating liver fibrosis. hAEC-derived extracellular vesicles (hAEC-EVs), which are nanosized (40–100 nm) membrane bound vesicles, may act as novel cell–cell communicators. Accordingly, we evaluated the efficacy of hAEC-EV in modulating liver fibrosis in a mouse model of chronic liver fibrosis and in human HSC.

Methods: The hAEC-EVs were isolated and characterized. C57BL/6 mice with CCl_4-induced liver fibrosis were administered hAEC-EV, hAEC-CM, or hAEC-EV depleted medium (hAEC-EVDM). LX2 cells, a human HSC line, and bone marrow-derived mouse macrophages were exposed to hAEC-EV, hAEC-CM, and hAEC-EVDM. Mass spectrometry was used to examine the proteome profile of each preparation.

Results: The extent of liver fibrosis and number of activated HSCs were reduced significantly in CCl_4-treated mice given hAEC-EVs, hAEC-CM, and hAEC EVDM compared to untreated controls. Hepatic macrophages were significantly decreased in all treatment groups, where a predominant M2 phenotype was observed. Human HSCs cultured with hAEC-EV and hAEC-CM displayed a significant reduction in collagen synthesis and hAEC-EV, hAEC-CM, and hAEC-EVDM altered macrophage polarization in bone marrow-derived mouse macrophages. Proteome analysis showed that 164 proteins were unique to hAEC-EV in comparison to hAEC-CM and hAEC-EVDM, and 51 proteins were co-identified components with the hAEC-EV fraction.

Conclusion: This study provides novel data showing that hAEC-derived EVs significantly reduced liver fibrosis and macrophage infiltration to an extent similar to hAEC-EVDM and hAEC-CM. hAEC-EV-based therapy may be a potential therapeutic option for liver fibrosis.

Keywords: human amnion epithelial cells, extracellular vesicles, liver fibrosis, macrophages, anti-fibrotic therapy, secretome, soluble-factors

INTRODUCTION

Hepatic fibrosis results from chronic inflammatory liver injury attributed to many factors including steatohepatitis, viral hepatitis, alcohol, toxins, and autoimmune disease. Chronic liver injury may result in a dysregulated wound-healing response with persistent infiltration of inflammatory cells and accumulation of extracellular matrix (ECM) due to pathogenic activation of quiescent hepatic stellate cells (HSCs) with subsequent phenotypic transformation into collagen-secreting myofibroblasts. If this response is persistent, normal hepatic architecture is altered by extensive fibrosis and loss of functional hepatocyte mass leading to cirrhosis and portal hypertension. Patients with cirrhosis are at increased risk of liver failure and hepatocellular carcinoma (HCC) (Liedtke et al., 2013). Currently, the only effective therapy for patients with end-stage liver disease is liver transplantation, a complex surgical procedure reliant on donor availability (Francoz et al., 2007). The complexity of the procedure, an increase in patients requiring liver transplantation and donor shortages, demonstrates the urgent need for an alternate therapy.

Stem cells have been investigated as a potential therapy to treat end-stage liver disease. Mesenchymal stromal cells (MSC) have shown encouraging preclinical results in animal models of liver fibrosis (Chang et al., 2009; Cho et al., 2011; Huang et al., 2013) and in human clinical trials (Amin et al., 2013; Mohamadnejad et al., 2013). However, there are several arguments against the clinical use of MSC, including the possibility of malignant transformation and the requirement for *in vitro* expansion prior to clinical use (Baglio et al., 2012).

The human placenta contains several types of stem and stem-like cells including human amnion epithelial cells (hAEC). hAEC are non-tumorigenic and due to their low expression of HLA-Class IA and absent expression of Class II molecules do not induce host immune rejection (Wolbank et al., 2007; Pratama et al., 2011). hAEC are highly abundant – approximately 150×10^6 cells can be isolated from a single-term amniotic membrane, which is adequate for clinical use without the need for *in vitro* expansion (Murphy et al., 2010). We and others have shown the anti-fibrotic effect of hAEC in mouse models of bleomycin-induced lung fibrosis (Murphy et al., 2011), and in both acute (Manuelpillai et al., 2010) and chronic (Manuelpillai et al., 2012) liver fibrosis induced by carbon tetrachloride (CCl_4). Moreover, studies have shown that hAEC have low rates of engraftment in injured tissue (Vosdoganes et al., 2011, 2013), which suggests that hAEC mediate their effects through release of paracrine factors. We have shown that hAEC-conditioned media (hAEC-CM) contains soluble factors which suppress proliferation, activation, and collagen production and induce apoptosis of human HSC (Hodge et al., 2014).

Evidence that stem cell conditioned media alone could exert therapeutic effects has given rise to a new theory on the mechanisms of certain cell therapies. For example, the MSC secretome is thought to be responsible for many of its physiological functions (Makridakis et al., 2013; Lee S.K. et al., 2015). More recently, crucial evidence has come to light suggesting that extracellular vesicles (EVs) are the effectors of MSC paracrine actions (Baglio et al., 2012). EVs are complex membrane enclosed nanoparticles that carry a cargo of select proteins, RNAs, and lipids (Xu et al., 2016). They are categorized by their size and biogenesis and include microvesicles (100–1000 nm), apoptotic vesicles (20–1500 nm), ectosomes (50–200 nm), and exosomes (40–150 nm) (Xu et al., 2016). However, EVs derived from hAEC (hAEC-EV) have yet to be characterized and as such their ability to reduce fibrosis following transplantation remains unknown. In this study, we isolated hAEC-EV by serial ultracentrifugation, performed in-depth characterization of isolated EVs and their protein cargo, and investigated the therapeutic efficacy of hAEC-EV in reducing hepatic fibrosis.

MATERIALS AND METHODS

Isolation of hAEC

Human amnion epithelial cells (hAEC) were isolated from the placentas of 16 healthy women undergoing cesarean section at term (37–40 weeks gestation) as described previously (Murphy et al., 2010; Xu et al., 2016). Written informed consent was obtained from each woman. The study was approved by the Monash Health Human Research Ethics Committee (approval number: 01067B).

hAEC-Conditioned Media (CM)

The hAEC-CM was prepared by culturing 10 million hAEC in chemically defined, serum-free Ultraculture medium (Lonza, Walkersville, MD, United States). Cultures were maintained for 4 days at 37°C in a humidified chamber containing 5% CO_2 prior to harvesting conditioned media.

Isolation of hAEC-EV and Extracellular Vesicle Depleted Media (EVDM)

Serial centrifugation was used to obtain EV and EVDM from collected hAEC-CM (Ng et al., 2013). Briefly, hAEC-CM was centrifuged at 300 g for 10 min at 4°C and at 2000 g for 10 min at 4°C to remove cells and cellular debris. The supernatant then was

collected and centrifuged at 10,000 g for 30 min at 4°C to remove large shed microvesicles (Meldolesi, 2015). The supernatant was subjected to ultracentrifugation 110,000 g for 90 min at 4°C (KQ424, Optima L-90K Ultracentrifuge, Beckman, Australia). The supernatant (EV-depleted media, EVDM) was collected and the pellet was washed by resuspending in PBS and underwent a final ultracentrifugation step at 110,000 g for 90 min at 4°C. The washed EV pellet was then resuspended in PBS and stored in aliquots. All hAEC components, EV, CM, and EVDM, were stored at −80°C until required.

Immunoblotting

The EVDM was concentrated using a membrane with a 100 Kda molecular weight cutoff (UFC710008, Merck, Australia). Proteins were separated on a NuPAGE 4–12% Bis–Tris Precast gel (Thermo Fisher Scientific, Australia) and transferred to nitrocellulose membrane (Thermo Fisher Scientific). Membranes were incubated with mouse anti-human Alix (3A9, Abcam, Cambridge, MA, United States, 1/1000), mouse anti-human CD81 (M38, Thermo Fisher Scientific, 1/250), and mouse anti-human CD63 (TS63, Thermo Fisher Scientific, 1/250). Protein bands were detected using Odyssey imaging system (LI-Cor, Lincoln, NE, United States).

Transmission Electron Microscopy

Extracellular vesicles (EVs) suspended in PBS were placed on a formvar-carbon-coated electron microscope grid for 20 min then fixed in 1% glutaraldehyde for 5 min. Grids were then placed in uranyl-oxalate solution (UOA) followed by methylose–cellulose. These were then thoroughly dried before being subjected to a scanning electron microscope (H7500, Hitachi, Japan) at 70 kV.

Nanoparticle Tracking Analysis

The diameter and concentration of vesicles were determined using a NanoSight NS300 system (NanoSight technology, Malvern, United Kingdom) equipped with a blue laser (488 nm). Briefly, EVs and EVDM loaded into a flow-cell top plate using a syringe pump. Three videos (1 min) were recorded for each sample, merged and analyzed by NTA software (Build 3.1.45).

Animals

A male C57Bl/6J mice of 6- to 8-weeks-old were purchased from Monash Animal Services, Melbourne, VIC, Australia and maintained in pathogen-free conditions at the Monash Medical Centre Animal Facility. Twelve hourly dark–light cycles were maintained with food and water access provided ad libitum. The Monash University Animal Ethics Committee approved all animal experiments and mice received care under the Australian Code of Practice for the care and use of animals for scientific purposes.

CCl_4, CM, EVDM, and EV Administration

Mice were divided into 5 groups ($n = 6$–8) and, other than the healthy untreated group, each group received intraperitoneal injections (IP) of carbon tetrachloride (CCl_4) twice weekly for 12 weeks at 1 μL/g body weight, diluted 1:10 in olive oil as previously described (Manuelpillai et al., 2012). Eight weeks later, when bridging fibrosis was evident, mice were administered three intravenous doses of either 350 μL CM, 350 μL EVDM ($\sim 2 \times 10^6$ particles), or 1 μg EV ($\sim 24 \times 10^6$ particles) in 350 μL saline or saline only (as controls) weekly for the last 4 weeks. All mice were culled at week 12 after commencing CCl_4 administration and blood and liver tissue were collected.

Picrosirius Red Staining

Mouse livers were fixed in 10% neutral buffered formalin and were cut into 4-μm sections. The sections were dewaxed and rehydrated and incubated for 90 min in Picrosirius red (Direct Red 80, 0.1% wt/vol in saturated picric acid, Sigma–Aldrich, St. Louis, MO, United States), then washed with acetic acid and water (1:200) and mounted in DPX (Sigma–Aldrich, St. Louis, MO, United States). Five non-overlapping fields were acquired from untreated mice ($n = 8$), and mice treated with hAEC-CM ($n = 8$), EVDM ($n = 6$), and EV ($n = 8$). Fibrosis area was measured with ImageJ software package (NIH Image, Bethesda, MD, United States).

Immunohistochemistry

Four-micron-thick paraffin sections of liver tissue from untreated and treated mice as described above were dewaxed and rehydrated and heat-mediated antigen retrieval performed by incubation with 10 mM sodium citrate (pH 6). Sections were then incubated with 0.3% (v/v) H_2O_2 for 15 min and blocked with a universal protein blocking solution for 1 h. Primary antibodies F4/80 (MCA497, Bio-Rad, Puchheim, Germany, 1:600) and α-Smooth Muscle Actin (α-SMA) (A5228-200UL, Sigma–Aldrich, St. Louis, MO, United States, 1:1500) were applied and the tissue sections were incubated in a humidified chamber overnight at 4°C or 30 min at room temperature, respectively. The sections were then washed and biotinylated rabbit anti-mouse IgG2a (E0464, Dako, Carpinteria, CA, United States, 1:500,) and rabbit anti-rat IgG (E0468, Dako, Carpinteria, CA, United States, 1:150) were applied for 1 h at room temperature followed by visualization using the Vectastain ABC HRP kit (Vector Laboratories, Burlingame, CA, United States) and DAB substrate (Dako, Carpinteria, CA, United States).

Immunofluorescent Staining

Serial 4-μm paraffin-embedded sections from untreated and treated mice as described above were dewaxed, rehydrated, and incubated in 10 mM sodium citrate pH 6 for heat-mediated antigen retrieval. To remove auto-fluorescence, Sudan Black was applied to the tissue sections for 15 min. Prior to immunolabeling, tissues were blocked with a universal protein blocking solution for 1 h and incubated with primary antibodies Rat F4/80 (MCA497, Bio-Rad, Puchheim, Germany, 1:100) and rabbit CD86 (EP1158Y, Novus Biological, Littleton, CO, United States 1:300) or rabbit CD206 (Ab64693, Abcam, Cambridge, MA, United States 1:500). Tissue sections were then washed three times and incubated with secondary antibodies (Alexa Fluor conjugates, Life Technologies, Frederick, MD, United States) including goat anti-rat 488 (1:100), donkey

anti-rabbit 568 (1:100), and goat anti-rabbit 647 (1:500) for 90 min followed by incubation with DAPI (Sigma–Aldrich, St. Louis, MO, United States) for 10 min and mounted using fluorescent mounting medium (Dako, Carpinteria, CA, United States).

In Vitro Effects of hAEC-EV on Macrophage Polarization

Immortalized mouse bone marrow macrophages from wild-type mice (a gift from Associate Professor Ashley Mansell, Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research) were plated on 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (DMEM/F12-High Glucose, Life Technologies, Frederick, MD, United States), and antibiotics (50 U/ml Penicillin and 50 μg/ml streptomycin, Life Technologies, Frederick, MD, United States). On day 1, M1 activation was assessed following cell stimulation using lipopolysaccharide (LPS; 10 ng/ml) and interferon-γ (IFN-γ; 10 ng/ml) (Life Technologies, Frederick, MD, United States), while M2 activation was assessed using interleukins-4 and interleukins-13 (PeproTech, Rocky Hill, NJ, United States) (10 ng/ml each) (Bansal et al., 2015). To investigate their effect on macrophage polarization, 10 μg hAEC-EV, 50% hAEC-CM, or 50% hAEC-EVDM obtained from three donors ($n = 3$) were added the next day to M1, M2, and naïve macrophage cultures. After 24 h, cells were collected and then stained for the M1 marker CD86 (V450, 1:200, BD Biosciences, San Jose, CA, United States) and M2 marker CD206 (Alexa Fluor 647, 1:200, BioLegend, San Diego, CA, United States). Samples were analyzed by flow cytometry using a FACS Canto II machine (Becton-Dickinson). These experiments were performed in duplicate.

Collagen Synthesis

Hepatic stellate cell (HSC) collagen synthesis was analyzed as described earlier (Hodge et al., 2014). Briefly, human immortalized HSCs (LX2 cell line, a kind gift of Professor Scott Friedman, NY, United States) were serum starved in DMEM containing 5% FBS followed by culture in Ultraculture media overnight at 37°C. In the treatment groups, cells were cultured in 50% Ultraculture media and 50% hAEC-CM, 50% hAEC-EVDM, or 50% PBS with 10 μg EV. In the control groups, HSCs were cultured either in 100% Ultraculture media as a control for CM and EVDM or 50% Ultraculture media and 50% PBS as a control for EV. [^{3}H] Proline (1 μCi, PerkinElmer, Boston, MA, United States) was added to each sample.

Enzyme-Linked Immunosorbent Assay

Snap-frozen liver tissue was homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Tween-20, 0.1% SDS) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). TGF-β was measured by ELISA (R&D Systems, Minneapolis, MN, United States) according to manufacturer's instructions. The data were normalized against total protein concentration measured using a bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, United States).

Image Quantification and Analysis

Sirius red, F4/80, and α-SMA immunostaining were quantified in five non-overlapping fields of view per animal using a Olympus BX41 upright microscope at 10× magnification. A mean of means was calculated for each experimental group using the threshold function in the ImageJ software package (NIH Image, Bethesda, MD, United States). Data are represented as percentage (%) of positive area per field. M1 and M2 macrophages were identified as F4/80$^+$/CD86$^+$ and F4/80$^+$/CD206$^+$, respectively, in five non-overlapping fields of view and normalized to the number of DAPI$^+$ cells using an Olympus FV1200 confocal microscope at 10× magnification. We carried out negative controls in the absence of primary antibodies for all stains to indicate the level of background.

Proteomic Analysis

A total of 30 μg pooled EVs, CM, and EVDM isolated from 10 amnions were analyzed by mass-spectrometry-based proteomics using an in-solution digestion approach followed by nanoliquid chromatography (Ultimate 3000 RSLCnano) coupled directly to a Q-Exactive HF Orbitrap (Thermo Fisher Scientific) mass spectrometer (MS) operated in data-dependent acquisition mode with technical duplicates. Peptides were loaded (Acclaim PepMap100, 5 mm × 300 μm i.d., μ-Precolumn packed with 5 μm C18 beads, Thermo Fisher Scientific) and separated (BioSphere C18 1.9 μm 120Å, 360/75 μm × 400 mm, NanoSeparations) with a 120-min gradient from 2 to 100% (v/v) phase B, 0.1% (v/v) FA in 80% (v/v) acetonitrile (ACN), 2–100% 0.1% FA in ACN, 2–40% from 0 to 100 min, and 40–80% from 100 to 110 min at a flow rate of 250 nL/min operated at 55°C.

The mass spectrometer (MS) was operated in data-dependent mode where the top 10 most abundant precursor ions in the survey scan (350–1500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 60,000, with MS/MS resolution of 15,000. Unassigned precursor ion charge states and singly charged species were rejected, and peptide match disabled. The isolation window was set to 1.4 Th and selected precursors fragmented by HCD with normalized collision energies of 25 with a maximum ion injection time of 110 ms. Ion target values were set to 3e6 and 1e5 for survey and MS/MS scans, respectively. Dynamic exclusion was activated for 30 s. Data were acquired using Xcalibur software v4.0 (Thermo Fisher Scientific).

Database Searching and Protein Identification

Raw data were preprocessed as described (Gorshkov et al., 2015) and processed using MaxQuant (Cox and Mann, 2008) (v1.5.8.3) with Andromeda (v1.5.6) using a Human-only (UniProt #133,798 entries) sequence database (March 2017). Data were searched as described (Gopal et al., 2015; Greening et al., 2016b) with a parent tolerance of 10 ppm, fragment tolerance of 0.5 Da, and minimum peptide length 7, with false

discovery rate 1% at the peptide and protein levels, with peptide lists generated from a tryptic digestion with up to two missed cleavages, cysteine carbamidomethylation as fixed modification, and methionine oxidation and protein N-terminal acetylation as variable modifications (Luber et al., 2010). Contaminants and reverse identification were excluded from further data analysis. For pathway analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) and NIH Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) resources were utilized using recommended analytical parameters (Huang da et al., 2009). For gene ontology enrichment and network analyses, UniProt[1] database resource (biological process, molecular function) was utilized.

Statistics

Data were analyzed using GraphPad Prism version 6.0 software for Mac OSX (GraphPad Software, San Diego, CA, United States). Multiple comparisons between different groups were analyzed by one-way ANOVA with *post hoc* Bonferroni correction. An unpaired *t*-test was performed to compare between control and EV in the *in vitro* collagen synthesis experiment. Data are shown as mean ± SEM. Differences were considered significant at $P < 0.05$.

RESULTS

Characterization of hAEC-EV

Extracellular vesicles released by hAEC (hAEC-EV) were prepared using serial ultracentrifugation as described previously (Ng et al., 2013). Western blot analysis showed that hAEC-EV expressed specific exosome markers including Alix, CD81, and CD63, which were absent in hAEC-EVDM (**Figure 1A**). Transmission electron microscopy showed that hAEC-EV displayed cup-shaped morphology and had a size of approximately 40–100 nm (**Figure 1B**). Nanoparticle tracking analysis was used to determine size distribution of EVs, which displayed a mean 133.1 nm diameter (**Figure 1C**). These results showed that hAEC-EV displayed the minimal criteria of exosomes and were absent in hAEC-EVDM (Lötvall et al., 2014). However, the presence of EVs in the EVDM could not be excluded completely by nanoparticle tracking analysis (**Supplementary Figure S1**).

hAEC-CM, EVDM, and EV Suppress Hepatic Fibrosis and Reduce HSC Activation *in Vivo*

Mouse liver sections were stained with Picrosirius red to quantify the extent of liver fibrosis (**Figure 2A**). We observed a substantial reduction of liver fibrosis in mice given hAEC-EV (1.66 ± 0.06%, $P < 0.0001$), hAEC-CM (2.04 ± 0.27%, $P < 0.001$), and hAEC-EVDM (1.54 ± 0.30%, $P < 0.0001$), compared to mice given CCl_4 only (3.84 ± 0.48%) (**Figure 2B**). The activation and transformation of HSC to myofibroblasts that express α-SMA typically results in increased collagen production. Fittingly, the α-SMA staining of HSC was consistent with the Picrosirius staining showing reduced hepatic fibrosis (**Figure 3A**). We noted a significant reduction in HSC number in mice given hAEC-EV (1.97 ± 0.23%, $P < 0.0001$), hAEC-CM (2.20 ± 0.19%, $P < 0.0001$), and hAEC-EVDM (1.80 ± 0.15%, $P < 0.0001$) compared to those given CCl_4 only (5.46 ± 0.74%) (**Figure 3B**). TGF-β is a potent pro-fibrotic cytokine that initiates HSC activation (Pradere et al., 2013). We measured TGF-β1 in liver lysates by ELISA normalized to total protein content. A significant reduction in TGF-β1 was only observed in mice treated with hAEC-EV (14.22 ± 3.50 pg/mg, $P < 0.0001$), compared to control mice (63.00 ± 3.07 pg/mg). There was no significant reduction in liver lysate TGF-β1 following treatment with hAEC-CM (70.84 ± 6.59) and hAEC-EVDM (72.14 ± 11.14 pg/mg) (**Figure 3C**).

hAEC-CM and EV Inhibit Collagen Production *in Vitro*

To investigate whether hAEC-EV have a direct effect on HSC collagen production, we quantified collagen production by human stellate cells (LX2 cell line) using [^{3}H] proline incorporation. Collagen production was reduced in LX2 cells treated with hAEC-EV 10 μg (81.48 ± 2.06%, $P < 0.0001$) and hAEC-CM (88.12.57 ± 3.03%, $P < 0.01$) compared to 100% Ultraculture media controls (100 ± 2.08%) (**Figure 4A**) and 50% PBS and 50% Ultraculture media controls (100 ± 1.06) (**Figure 4B**).

hAEC-CM, EVDM, and EV Reduce Macrophage Infiltration and Induce a M2 Macrophage Phenotype

Hepatic macrophages contribute to fibrosis progression and to fibrosis resolution. A dramatic decrease in the percentage of F4/80+ liver infiltrating macrophages was seen in mice administered hAEC-EV (5.03 ± 0.17%, $P < 0.0001$), hAEC-CM (5.87 ± 0.61%, $P < 0.0001$), and hAEC-EVDM (5.18 ± 0.50%, $P < 0.0001$) compared to those given CCl_4 only (10.55 ± 0.46%) (**Figures 5A,B**). Polarization of macrophages toward an M2 phenotype is associated with fibrosis resolution (Manuelpillai et al., 2012). Thus, we analyzed the expression of M1 (CD86) and M2 (CD206) markers. Interestingly, the density of M1 polarized macrophages (identified by co-localization of F4/80 and CD86) was significantly higher in CCl_4 groups (0.122 ± 0.017%) while M2 macrophage density ($F4/80^+CD206^+$) increased dramatically in hAEC-EV (0.04 ± 0.01%, $P < 0.003$), hAEC-CM (0.029 ± 0.01%, $P < 0.002$), and hAEC-EVDM-treated mice (0.04 ± 0.004%, $P < 0.0001$) (**Figure 6**).

To confirm that hAEC-EV, hAEC-CM, and hAEC-EVDM have a direct effect on macrophage polarization, we co-cultured bone marrow-derived macrophages with each treatment and assessed the effects of co-culture on M1 and M2 activation. We found that M2 macrophage polarization (**Figure 7A**) significantly increased after co-culture with hAEC-CM (0.12 ± 0.01%, $P < 0.0001$), hAEC-EVDM (0.14 ± 0.01%, $P < 0.0001$), and hAEC-EV at 10 μg (0.12 ± 0.01%, $P < 0.0001$) compared to

[1] www.uniprot.org

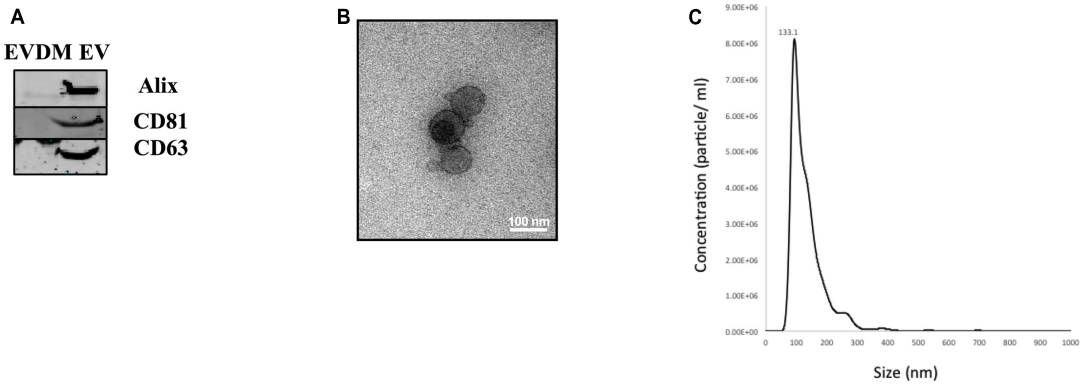

FIGURE 1 | Characteristics of amnion epithelial cell–derived exosomes. **(A)** Representative Western blot images of hAEC-EV and hAEC-EVDM showing presence and enrichment of Alix, CD81 and CD63 relative to EVDM. **(B)** Electron microscopy showing cup-shaped morphology of exosomes. **(C)** Size distribution of hAEC-EVs obtained by NTA.

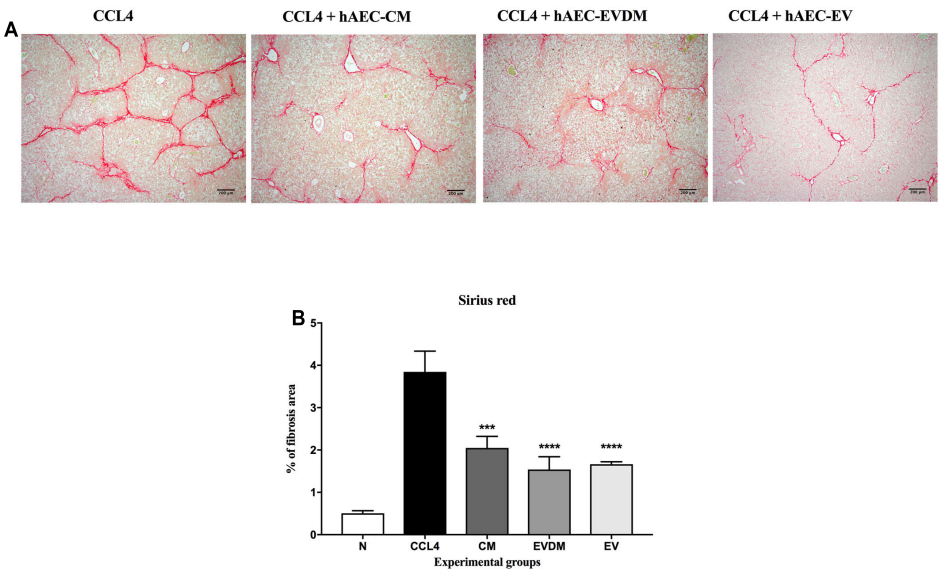

FIGURE 2 | (A) Representative images showing collagen staining with Sirius red in liver sections from control and treated groups. Scale bar = 200 μm, 10× magnification. **(B)** The CM-, EVDM-, and EV-treated mice had significantly reduced fibrosis area compare to CCl_4 only. Data are represented as mean ± SEM. n = 6–8 per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

M1 alone (0.05 ± 0.01) (**Figure 7B**). None of the treatments altered the phenotypes of naïve macrophages (**Figure 7C**) or M2 macrophages (**Figure 7D**).

Proteomics Analysis of hAEC-EV, hAEC-CM, and hAEC-EVDM

We next compared the proteome profiles of the hAEC-EV, hAEC-CM, and hAEC-EVDM using GeLC-MS/MS (Tauro et al., 2012a,b; Greening et al., 2016a, 2017). For purified EVs, this resulted in 231 proteins identified (Supplementary Table S1). We observed an abundance of typical exosome associated proteins such as tetraspanins CD9 and CD81, various Rab GTPases, and select components associated with vesicle sorting/trafficking including ARF1, LAMP1, and CLTC (Supplementary Table S1). We found 61 proteins identified in the exosome database ExoCarta (top 100 highly expressed proteins in exosomes)[2]. This supports the enrichment of select exosome marker proteins including HSPA8, CLTN, and integrins ITGA6 and ITGB1 (**Figure 8A** and Supplementary Table S2). In comparison with hAEC-CM (**Figure 8B**) and hAEC-EVDM (**Figure 8C**), we found 51 components in common with the isolated EV fraction (Supplementary Table S1). Additionally, 164 proteins were unique to hAEC-EV in comparison to hAEC-CM (Supplementary Table S3) and hAEC-EVDM (Supplementary Table S4). EV components included Milk fat globule epidermal growth factor–factor 8 (MFGE8), heat shock 72 kDa protein

[2]http://www.exocarta.org/

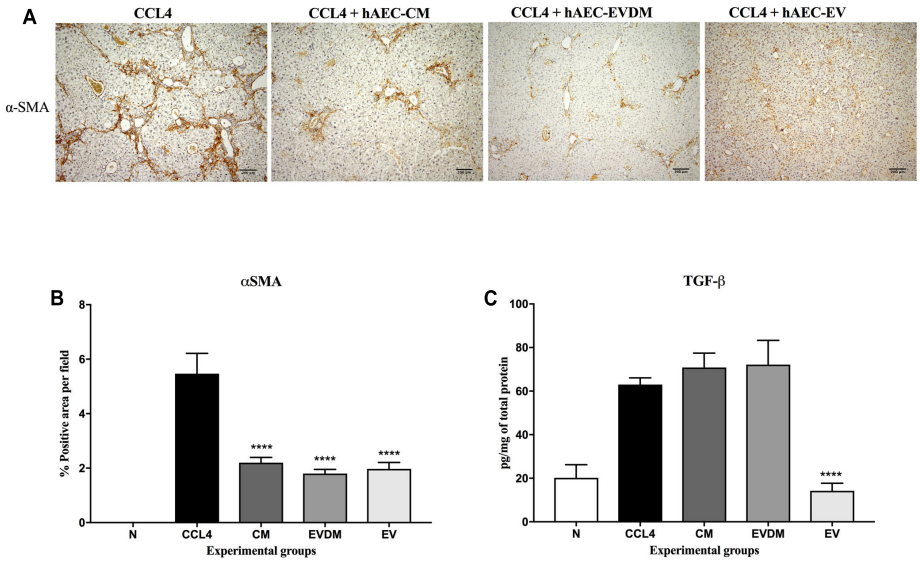

FIGURE 3 | (A) Representative images of activated HSC stained with α-SMA from control and treated groups. Scale bar = 200 μm, 10× magnification. **(B)** The percentage of HSC in the liver was significantly decreased in mice treated with CM, EVDM, and EV compared to CCl_4 only. **(C)** Concentration of TGF-β1 in liver was determined by ELISA and found to be significantly lower in mice treated with EV only. The data are represented as mean ± SEM. n = 6–8 per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

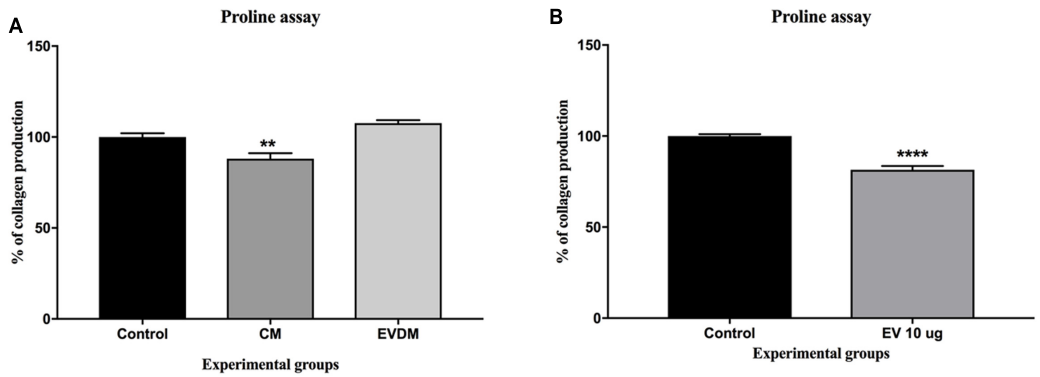

FIGURE 4 | Effect of hAEC-CM, EVDM, and EV on collagen synthesis. Confluent LX2 cells were cultured with hAEC-CM, EVDM, and EV with media containing 1μCi [3H] Proline. Collagen synthesis declined significantly in LX2 exposed to CM **(A)** and EV **(B)** compared to untreated control. Results are shown as mean ± SEM. Each analysis was based on biological replicates of EV, CM, and EVDM obtained from different hAEC, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

(HSP72), and Superoxide dismutase Cu-Zn SOD (SOD1). Furthermore, we found 61 proteins unique to hAEC-CM (Supplementary Table S5) and 65 proteins unique to hAEC-EVDM (Supplementary Table S6).

When subjected to a gene ontology analysis, we observed enrichment of biological processes associated with cell–cell adhesion, receptor-mediated endocytosis, protein transport, cell surface receptor signaling pathway, integrin-mediated signaling pathway, membrane organization, and wound healing (**Figure 8D** and Supplementary Table S7). Of note, we observed KEGG enrichment associated with the PI3K-Akt signaling pathway, focal adhesion, Rap1 signaling, ECM-receptor interaction, and antigen processing and presentation (**Figure 8E** and Supplementary Table S8).

DISCUSSION

This study has provides the first evidence that EVs secreted by hAEC have a therapeutic potential for the treatment of liver fibrosis. Evidence of therapeutic effects of the secretome of MSC and other stem cells gave rise to a new theory, specifically that outcomes of cell therapies may be mediated by EVs (Tolar et al., 2010; Fouraschen et al., 2012; Lee et al., 2016). These vesicles, which play an important role in cell-to-cell communication, can alter the phenotype and fate of target cells (Xu et al., 2016). There is increasing evidence that exosomes influence physiological processes such as cell transformation (Greening et al., 2015a), immunoregulation (Greening et al., 2015b; Nawaz et al., 2016), and importantly, cancer progression (Atay et al., 2014; Melo

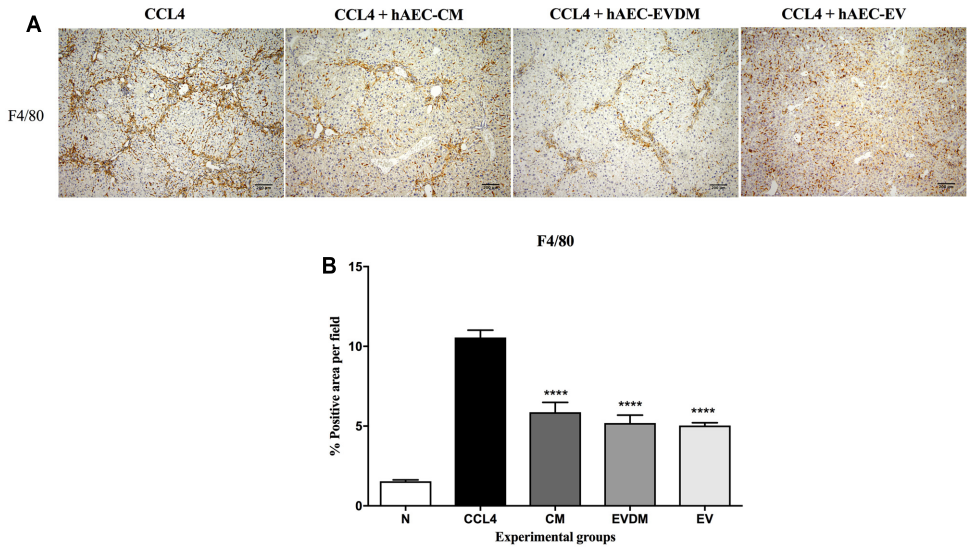

FIGURE 5 | (A) Hepatic macrophages were identified by F4/80 immunohistochemistry. Mice with established fibrosis treated with CM, EVDM, and EV had significantly lower percentage of F4/80 macrophages in the liver compare to CCl_4 only. Scale bar = 200 μm, 10× magnification. **(B)** Quantification of liver macrophage density using ImageJ software. The data are represented as mean ± SEM. n = 6–8 per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

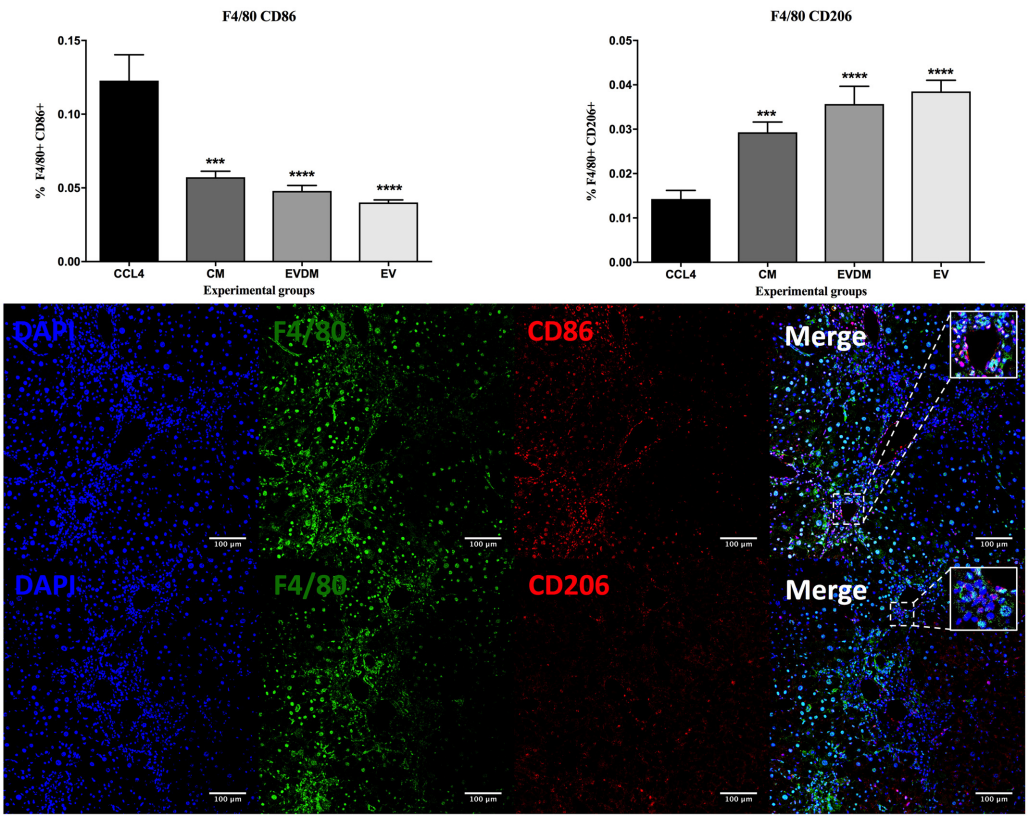

FIGURE 6 | Effect of hAEC-CM, EVDM, and EV on macrophage polarization in CCL_4-induced liver fibrosis. Quantification of $F4/80^{+}CD86^{+}$ and $F4/80^{+}$ $CD206^{+}$ double-labeled cells from CCl_4 groups. Representative images of $F4/80^{+}CD86^{+}$ and $F4/80^{+}$ $CD206^{+}$ double-labeled cells. Scale bar = 100 μm, 20× magnification. The data are represented as mean ± SEM. n = 6–8 per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

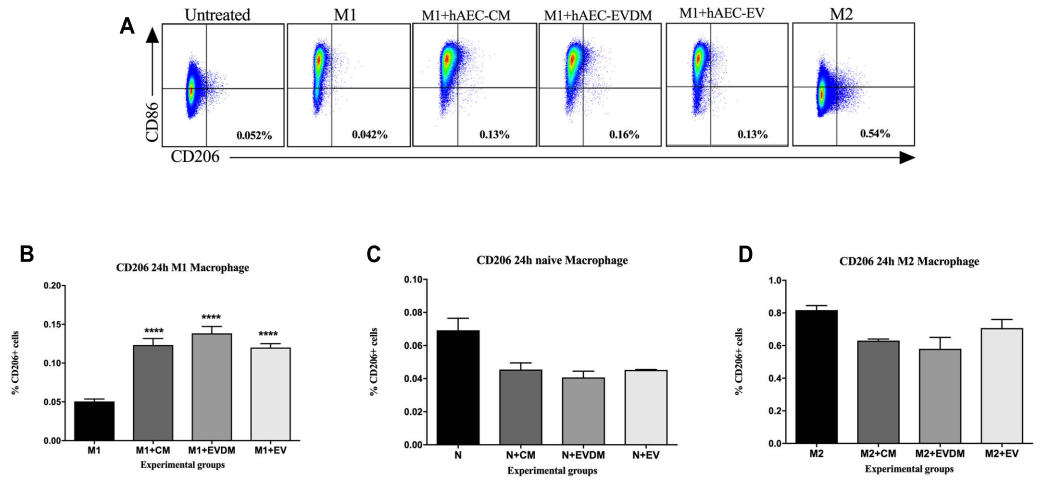

FIGURE 7 | (A) Representative FACS plot of macrophages at 24-h post treatment. Cells were pre-gated based on size and viability. Quantification of the percentage of total number of cells gated from the FACS plot of **(B)** untreated M1-differentiated (LPS/INF-γ) macrophages or treated with hAEC-CM, EVDM, and EV, **(C)** untreated macrophages or treated with hAEC-CM, EVDM, and EV and **(D)** untreated M2-differentiated macrophages (IL-4/IL-13) or treated with hAEC-CM, EVDM, and EV. M1 and M2 differentiations were determined by CD86 and CD206, respectively. The data are represented as mean ± SEM. Each analysis was based on biological replicates of hAEC-EV, CM, and EVDM obtained from different hAEC, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

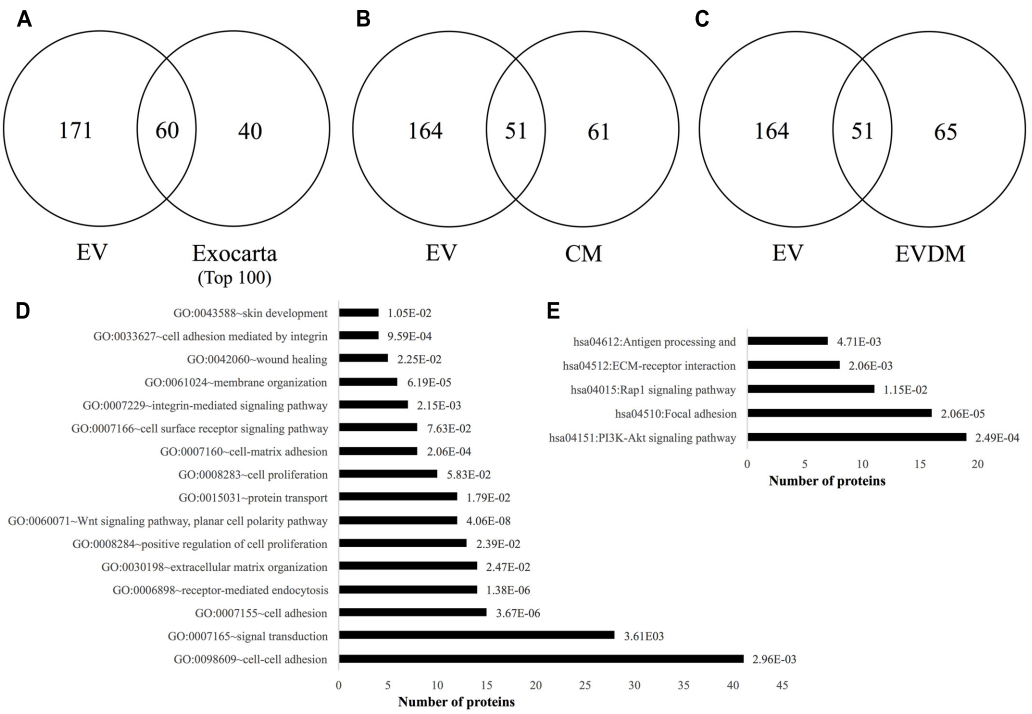

FIGURE 8 | Two-way Venn diagrams of proteins distributed between hAEC-EV cargo and **(A)** ExoCarta (top 100 highly expressed exosome marker proteins) (Supplementary Table S1), **(B)** hAEC-CM (Supplementary Table S5), and **(C)** hAEC-EVDM (Supplementary Table S6). Proteomic profiling of EV cargo reveals **(D)** Gene ontology (biological process)–related function analysis with *p*-values indicated (Supplementary Table S7) and **(E)** KEGG pathway analysis with *p*-values indicated (Supplementary Table S8).

et al., 2014), vaccination against infectious disease (Viaud et al., 2010), and vaccines for possible cancer treatments (Mignot et al., 2006; Gehrmann et al., 2013; Rak, 2013). These studies have led to several clinical and pre-clinical investigations of exosome/EV-based therapies (El Andaloussi et al., 2013; Buzas et al., 2014; Gyorgy et al., 2014; Vader et al., 2014; Xu et al., 2016). In the context of therapeutic applications, exosomes of selected cell types have been used as therapeutic agents in

immune therapy, vaccination trials, regenerative medicine, and drug delivery (Fais et al., 2016). Exosomes also provide a largely unexplored source of diagnostic, prognostic, and predictive biomarkers (Rak, 2013; Nawaz et al., 2014, 2016; Greening et al., 2017). Interestingly, administration of EV derived from MSC resulted in reduced liver fibrosis (Li et al., 2012) and promoted hepatic regeneration (Tan et al., 2014).

In this study, we investigated the therapeutic efficacy of hAEC-CM, EV, and EVDM in a mouse model of CCl_4-induced chronic liver fibrosis. We found that intravenous administration of CM, EV, and EVDM derived from hAEC significantly reduced HSC number and collagen production, reduced hepatic macrophage infiltration, and polarized macrophages to a pro-reparative phenotype *in vitro* and *in vivo*. Furthermore, only hAEC-EV significantly downregulated TGF-β1 signaling.

Carbon tetrachloride-induced liver fibrosis is a well-established model of liver injury (Manuelpillai et al., 2012). We commenced hAEC-CM, EV, and EVDM treatments while continuing CCl_4 administration to model the persistent injury that occurs in the clinical setting and where clinical management would be relevant. During liver fibrosis, activation of HSC and subsequent transformation into myofibroblasts leads to the production of collagen and ECM accumulation. Activated HSC are widely measured by the expression of α-SMA (Lee Y.A. et al., 2015). Treatment with hAEC-CM, EV, and EVDM significantly reduced liver fibrosis, as shown by a reduction in the number of activated HSC and collagen proportionate area, even in the presence of continued CCl_4 administration. We corroborated these findings *in vitro* using the human HSC cell line LX2, showing that hAEC-EV directly decreased collagen production in activated HSC. Interestingly, the therapeutic effect of hAEC-EV was similar to hAEC-CM, in regard to its reduction of liver fibrosis and HSC activation, which extends our previous findings showing that hAEC-CM contained soluble factors that have anti-fibrotic effects *in vitro* (Hodge et al., 2014). However, EVDM had a diminished ability to reduce collagen production *in vitro*, which is in contrast to our *in vivo* results. This could be a reflection of our use of an immortalized stellate cell line, which may not reflect an *in vivo* effect, as such the use of primary HSC should be the subject of further investigation. Alternatively, this may indicate that EVDM exerts its anti-fibrotic effects *in vivo* by acting on other cell types in the liver, rather than on stellate cells themselves.

TGF-β1 is well known to activate HSCs (Pradere et al., 2013). We found that hAEC-EV dramatically reduced the protein content of TGF-β1 in the livers of CCl_4 mice; however, this was not achieved with hAEC-CM or EVDM. TGF-β was identified in both CM and EVDM and the dose in our study was 350 μl 3 times weekly for 4 weeks. A study by Huang et al. (2016) reported that MSC-CM 250 μl twice weekly for 3 weeks promoted therapeutic effects in a chronic liver fibrosis model. The higher dose in our study may explain the impaired ability of CM and EVDM to reduce hepatic TGF-β and indicates the importance of investigating the dose efficacy of CM and EVDM.

Hepatic macrophages are a heterogeneous population of cells that have a wide range of functions during homeostasis and disease (Lee Y.A. et al., 2015). Chronic liver fibrosis is associated with recruitment of macrophages that co-localize with fibrotic regions (Manuelpillai et al., 2012). Macrophage depletion using a transgenic mouse (CD11b-DTR) resulted in decreased fibrosis and HSC in chronic liver fibrosis induced by CCl_4 (Duffield et al., 2005). In the present study, we found that CCl_4-treated mice exhibited a significant increase in F4/80 positive macrophage infiltration, which was significantly decreased by hAEC-EV exposure.

Experimental evidence suggests that macrophages exert dual functions during liver fibrosis. The activation of macrophages during the injury phase is associated with ECM accumulation and HSC activation. On the other hand, macrophages activated during recovery resulted in matrix degradation (Duffield et al., 2005; Ramachandran et al., 2012). Phenotypic polarization from classically activated macrophages (M1) to alternatively activated macrophages (M2) is dependent on signals received from the local environment (Martinez and Gordon, 2014). M1 macrophages produce high levels of pro-inflammatory cytokines and are induced by LPS and interferon-γ (IFN-γ) (Martinez and Gordon, 2014). On the other hand, M2 macrophages produce anti-inflammatory cytokines, collagen-degrading enzymes and are induced by IL-4 and IL-13 (Song et al., 2000; López-Navarrete et al., 2011). Interestingly, the mannose and scavengers receptors present in M2 macrophages are able to phagocytose ECM and apoptotic cells leading to fibrosis resolution (López-Guisa et al., 2012; Wynn and Ramalingam, 2012). In this study, we used CD86 to identify M1 macrophages and CD206 for M2 macrophages (Bility et al., 2016). However, CD206 is expressed in M2 liver macrophages as well as liver sinusoidal endothelial cells (DeLeve, 2015). We therefore identified M1 macrophages by co-localization of CD86 and F4/80 while M2 macrophages were identified by co-localization of F4/80 and CD206. We found that hAEC-EV increased liver M2 macrophages in CCl_4 mice, accompanied by a decrease in liver M1 macrophages, a similar effect seen with hAEC-CM and EVDM. This was corroborated *in vitro* using immortalized bone marrow macrophages. Taken together, our findings of reduced hepatic fibrosis area, reduced number of activated HSC and macrophages, reduced levels of TGF-β1 and polarization to M2 macrophages are consistent with those seen in our previous study, when we administered hAEC alone in the CCl_4 mouse model (Manuelpillai et al., 2012). Data from our current study indicate that both the vesicular fraction and whole hAEC-CM may mediate the anti-fibrotic effects observed in CCl4-induced chronic liver fibrosis.

While the field of EV research has grown exponentially in recent years, findings from our current study indicate that the *soluble fraction* of secreted or shed cellular products should not be entirely disregarded. The proteomic analysis of CM, EV, and EVDM indicates the presence of proteins enriched for Rap1 pathway and PI3K/Akt pathway. Rap1 is involved in the control of cell proliferation and cell adhesion (Bos et al., 2001), while PI3K/Akt is implicated in macrophage polarization, cell cycle progression, and prevention of apoptosis (Chang et al., 2003;

Vergadi et al., 2017). Therefore, our data suggest the PI3K/Akt pathway may modulate macrophage polarization. Interestingly, our proteomic analysis of hAEC-EV revealed the presence of proteins that target TGF-β signaling including MFGE8, HSP72, and SOD1. MFGE8 plays a critical role in reducing pulmonary fibrosis (Atabai et al., 2009). An et al. (2017) identified MFGE8 as an anti-fibrotic factor in the umbilical cord MSC secretome that inhibits TGF-β signaling and reduces liver fibrosis in mice. Moreover, HSP72 was found to attenuate renal tubulointerstitial fibrosis in obstructive nephropathy (Mao et al., 2008) and to inhibit epithelial-to-mesenchymal transition, which promotes collagen production, via effects on Smad2 activation (Zhou et al., 2010). Finally, the anti-fibrotic potential of SOD1 on radiation-induced fibrosis is mediated by downregulation of TGF-β signaling (Emerit et al., 2006). These proteins could play a role in the reduction of collagen production, fibrosis, and TGF-β expression observed in hAEC-EV-treated mice.

In summary, our findings suggest that the hAEC secretome, comprising soluble factors in hAEC-CM, both complete and EV depleted, in addition to hAEC-EV, had beneficial effects in reducing liver fibrosis in a murine model. This is the first study to provide evidence that hAEC-derived EVs can exert a therapeutic effect similar to what has been previously reported with hAEC in an experimental model of chronic liver fibrosis. Future studies could focus on identifying the specific anti-fibrotic factors that would support development of a clinically applicable therapy.

AUTHOR CONTRIBUTIONS

MA, DG, RL, and WS conceived and designed experiments. MA, JC, MZ, NK, RX, MS, AH, DG, RL, and WS performed experiments and analyzed data. MA, BL, DG, RL, and WS wrote the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2017.00748/full#supplementary-material

FIGURE S1 | Particle quantitation of hAEC-EVDM was performed by nanoparticle tracking analysis.

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REVIEW
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Novel Anti-fibrotic Therapies

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Fibrosis is a major player in cardiovascular disease, both as a contributor to the development of disease, as well as a post-injury response that drives progression. Despite the identification of many mechanisms responsible for cardiovascular fibrosis, to date no treatments have emerged that have effectively reduced the excess deposition of extracellular matrix associated with fibrotic conditions. Novel treatments have recently been identified that hold promise as potential therapeutic agents for cardiovascular diseases associated with fibrosis, as well as other fibrotic conditions. The purpose of this review is to provide an overview of emerging antifibrotic agents that have shown encouraging results in preclinical or early clinical studies, but have not yet been approved for use in human disease. One of these agents is bone morphogenetic protein-7 (BMP7), which has beneficial effects in multiple models of fibrotic disease. Another approach discussed involves altering the levels of micro-RNA (miR) species, including miR-29 and miR-101, which regulate the expression of fibrosis-related gene targets. Further, the antifibrotic potential of agonists of the peroxisome proliferator-activated receptors will be discussed. Finally, evidence will be reviewed in support of the polypeptide hormone relaxin. Relaxin is long known for its extracellular remodeling properties in pregnancy, and is rapidly emerging as an effective antifibrotic agent in a number of organ systems. Moreover, relaxin has potent vascular and renal effects that make it a particularly attractive approach for the treatment of cardiovascular diseases. In each case, the mechanism of action and the applicability to various fibrotic diseases will be discussed.

Keywords: fibrosis, cardiovascular disease, antifibrotic agents, bone morphogenic protein-7, micro-RNA, relaxin, peroxisome proliferator-activated receptors

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INTRODUCTION

Fibrosis is a critical stage of many chronic diseases that can lead to organ dysfunction, illness and death. The burden associated with fibrosis is staggering with nearly half of all deaths in the United States attributed to fibrotic diseases including liver, lung, kidney, and heart disorders (Wynn, 2008; Rosenbloom et al., 2010). Unfortunately, the impact of fibrosis on mortality and morbidity rates has not been countered by effective treatments. Decades of research have identified many potential targets to combat fibrotic tissue damage, yet there currently are no effective therapies or FDA approved antifibrotic agents. This review highlights the potential of four agents that have emerged from basic science testing that show promise as translatable options for the treatment of fibrotic diseases in humans.

The development of antifibrotic therapies relies on the comprehensive understanding of profibrogenic mechanisms in multiple organ systems as well as disease-specific locations. The global and foremost mechanism involved in fibrosis is the activation of myofibroblasts resulting in the excessive and often continual production of extracellular matrix (ECM) components, the foundation of scar formation (McAnulty, 2007; Herzog and Bucala, 2010). Myofibroblast activation is initiated in chronic inflammatory diseases where healthy wound healing in response to injury is not controlled, resolved, or is repetitively stimulated by an inciting factor. As a result, differentiated fibroblasts are stimulated to be in a proliferative, activated state producing ECM components. In the last 25 years, heightened research has identified potential targetable pathways and related individual factors that are involved in the differentiation of quiescent fibroblasts and the persistent activation of myofibroblasts during fibrogenesis. These include an array of cytokines (e.g., interleukins, IL-1, IL-6, IL-25, IL-33; and tumor necrosis factor-α, TNF-α) that are produced as a consequence of inflammation and epithelial and endothelial tissue damage (Rosenbloom et al., 2013). The burst of inflammatory mediators can directly stimulate fibroblast activation as well as influence downstream adaptive and innate immune mechanisms (Wynn and Ramalingam, 2012). For example, a stimulated adaptive immune system can affect a complex array of T and B cell activities resulting in the resolution as well as promotion of fibrosis via interferon gamma (IFN-γ) signaling (Gurujeyalakshmi and Giri, 1995). Innate immune activation is similarly complex involving responses of multiple cell types (e.g., macrophages, neutrophils and mast cells) along with their associated cytokines and growth factors that drive a fibrotic signaling cascade (Barrientos et al., 2008; Lech and Anders, 2013). Among the factors found to be associated with innate immune cells, TNF-α and IL-1β are noted profibrotic mediators along with transforming growth factor-beta (TGF-β), which has long been identified as a key factor in fibrosis (Roberts et al., 1988). Altogether, inflammatory and immune cell factors ultimately stimulate myofibroblasts to produce smooth muscle actin and matrix components such as collagen and fibronectin. The goal to inhibit profibrogenic factors or pathways has been the focus of research for years. Unfortunately, successful translation of effective antifibrotic treatments to humans has been limited due to inefficient and off-target effects. However, recent preclinical assessments of several agents show promise in achieving an attainable inhibition of fibroblast activation and ECM expression through mechanisms involving the blockade of signaling receptors, TGF-β stimulation, or translation of fibrogenic genes (Friedman et al., 2013; Gourdie et al., 2016).

Of the various mechanisms that have been studied to inhibit fibroproliferative events, strategies to target the activation of the myofibroblasts are emerging with high potential. Recent advances have been noted in the understanding and importance of particular factors in the inhibition and/or resolution of fibrosis. Specifically, excitement has been noted for the antifibrotic potential of bone morphogenetic protein-7 (BMP7); micro-RNAs; peroxisome proliferator-activated receptor (PPAR) signaling pathways; and the hormone relaxin. A strong indication for the use of these factors has been shown in studies of atrial fibrosis, heart failure, renal disease and cirrhosis of the liver. Acknowledging the importance and emergent nature of cardiovascular disease, this review will discuss the antifibrotic potential of the above agents in a disease-specific manner with emphasis on cardiac injury, as well their applicability as a broad intervention.

THE ANTIFIBROTIC THERAPEUTIC ROLE OF BMP7

Bone morphogenetic protein-7 was discovered nearly 30 years ago as a critical factor in development and bone formation (Ozkaynak et al., 1990). Interestingly, BMP7 was also determined to be part of the TGF-β family. Considering the association of BMP7 with the fibrogenic factor TGF-β, the intervening years since its identification has produced a wealth of information related to mechanisms and the targetability of BMP7 pathways in fibrotic disease. To date, basic science discoveries have detailed the importance of BMP7 in organ homeostasis and specifically as an opposing mechanism to the profibrogenic actions of TGF-β. However, the translation of BMP7 as an antifibrotic agent remains in development. The following overview highlights the functional significance of BMP7 and related signaling mechanisms that may lead to translational successes and clinically relevant treatments for fibrosis.

BMP7 is structurally and functionally similar to members of the TGF-β superfamily (Weiskirchen et al., 2009). Structurally, BMP7 has a related modular form and sequence to TGF family members including a C-terminal biologically active region that is highly homologous to TGF-β. Also, similar to other family members, BMP7 engages with serine/threonine kinase receptors leading to the initiation of signal transduction cascades (Massague et al., 2005). Details of BMP7-mediated signaling have been eloquently characterized describing a variety of gene responses that are induced as a result of BMP7 activity (Weiskirchen et al., 2009). Outcomes of BMP7 signaling were shown to include the regulation of genes associated with embryonic development including kidney, eye and skeleton formation. Moreover, BMP7 signaling significantly influences organ homeostasis and importantly, the regulation of antifibrotic mechanisms. A summary of the major and most recent reports on the effectiveness of BMP7 treatment is presented in **Table 1** and is discussed below.

In early studies using BMP7 deficient mice it was shown that a key factor in the antifibrotic effect of BMP7 involved the control of epithelial-to-mesenchymal transition (EMT) and TGF-β profibrogenic signaling in multiple organs (kidney, heart, liver, lung, and eye) (Luo et al., 1995; Zeisberg E.M. et al., 2007; Zeisberg M. et al., 2007; Myllarniemi et al., 2008). It is well known that TGF-β is a central inducer of myofibroblast activation and that TGF-β-mediated EMT is important in the transformation of fibroblasts involved in wound healing responses and fibrosis. Results from multiple fibrotic disease models demonstrated that BMP7 expression is downregulated

TABLE 1 | Antifibrotic therapeutic potential of targeting BMP7 signaling.

Fibrotic disease	Treatment	Findings	Study
Cardiac	rhBMP7	Inhibition of EMT	Zeisberg E.M. et al., 2007
	rhBMP7	Suppression of left ventricular remodeling	Merino et al., 2016
Renal	BMP7	Restoration of BMP7 levels; partial reversal of diabetic-induced kidney disease	Wang et al., 2003
	THR-123	Induction of BMP receptor activin-like kinase 3 signaling; suppression of inflammation, EMT	Sugimoto et al., 2012
Hepatic	AVV-BMP7	Suppression of carbon tetrachloride-induced fibrosis and promotion of hepatocyte regeneration	Hao et al., 2012
	rhBMP7	Reduction of hepato-schistosomiasis-associated fibrosis via antagonism of TGF-β signaling	Chen et al., 2013
	Cpd 861	Upregulation and activation of BMP7 signaling	Hou et al., 2016
Pulmonary	Tilorone	Enhancement of BMP7 expression and signaling in lung epithelial cells	Lepparanta et al., 2013
	rhBMP7	Reversal of TGF-β-mediated myofibroblast differentiation regulated by hyaluronan	Midgley et al., 2015
	BMP7	Attenuation of silica-induced fibrosis via regulation of BMP signaling	Liang et al., 2016
Corneal	ITF2357	Activation of Id3 and BMP7 levels	Lim et al., 2016

BMP7, Bone Morphogenetic Protein 7; rhBMP7, recombinant human BMP7; EMT, endothelial-mesenchymal transition; THR-123, small peptide BMP agonist, Thrasos Therapeutics, Canada; AVV-BMP7, adeno-associated virus carrying BMP7; Cpd 861, herbal compound 861; Tilorone, Tilorone dihydrochloride (CAS 27591-69-1); TGF-β, transforming growth factor beta; ITF2357, (diethyl-[6-(4-hydroxycarbamoyl-phenyl carbamoyloxymethyl)-naphthalen-2-yl methyl]-ammonium chloride (Givinostat).

during disease and that the restoration of BMP7 expression or treatment with recombinant protein resulted in the prevention or alleviation of fibrosis (Weiskirchen et al., 2009). The protective role of BMP7 was found to correlate with the inhibition of TGF-β-mediated profibrotic signaling. Despite the promising reports from various animal studies, the beneficial effects of exogenous BMP7 were found to be variable. This was evident from studies which failed to demonstrate an antifibrotic benefit of BMP7 as well as the indication that BMP7 expression in fact correlated with fibrotic disease (Ikeda et al., 2004; Tacke et al., 2007). However, the role of BMP7 as an opposing mechanism to the profibrogenic effects of TGF-β signaling remains clinically relevant, especially with the development of alternative strategies to alter BMP signaling through the use of small molecule inhibitors or agonists. Therefore, efforts continue to define the therapeutic effectiveness of BMP7 through more in-depth investigations including *in vivo* analyses, comparative studies and preliminary efficacy trials (Sugimoto et al., 2012; Lepparanta et al., 2013; Midgley et al., 2015; Lim et al., 2016).

Research efforts have determined that a key component of the balance between pro and antifibrotic signaling is the opposing action of BMP7 on TGF-β/Smad signaling (**Figure 1**). It was determined that BMP7 induces the antifibrotic phosphorylation of Smad1/5/8 that opposes TGF-β mediated phosphorylation of Smad2/3 and fibrogenic gene expression (Derynck and Zhang, 2003). Further evaluations into the role of BMP7/Smad signaling in various fibrotic diseases have been performed. In liver disease studies, models of hepato-schistosomiasis and carbon tetrachloride-induced fibrosis have been used to demonstrate the effectiveness of either exogenous BMP7 or adenovirus treatment in reducing key parameters of injury including TGF-β/Smad signaling and hepatic stellate cell activation (Hao et al., 2012; Chen et al., 2013). Additionally, in efforts to define regulators of BMP7/Smad signaling, it was demonstrated that a herbal compound was effective in alleviating hepatic fibrosis via enhancements in p-Smad1/5/8 levels and BMP7 antifibrotic signaling (Hou et al., 2016). In the context of chronic kidney disease, the negative regulation of TGF-β/Smad signaling by BMP7 has been shown to be involved in various nephropathies (Wang et al., 2003; Zeisberg et al., 2003; Chan et al., 2008). Further investigations have detailed parameters involved in the therapeutic restoration mediated by BMP7 overexpression which opposes Smad 3 signaling and protects against TGF-β-induced renal damage (Meng et al., 2013). In other works, the beneficial role of BMP7/Smad signaling has been shown in fibrotic diseases of the lung and heart. Of note, a recent evaluation provided correlative evidence of BMP7 antifibrotic effects in humans compared to animal models of cardiac disease. Particularly, it was determined that left ventricular LV remodeling in patients with aortic stenosis as well as mice with aortic constriction involved impaired BMP/Smad 1/5/8 signaling and increased TGF/Smad2/3 pathways; and that exogenous supplementation with BMP7 reduced LV disease in the mice (Merino et al., 2016). In models of lung disease, similar opposing actions of BMP7 on TGF-β/Smad signaling were indicated by reduced p-Smad 2/3 levels and attenuation of silica-induced pulmonary fibrosis in animals treated with recombinant BMP7 (Yang et al., 2013; Liang et al., 2016). Overall, the importance of BMP7 as an antagonist to profibrogenic TGF-β/Smad signaling has been demonstrated in multiple organ model systems providing support for further investigations into the clinical efficacy of BMP7 treatment strategies.

In addition to affecting Smad-dependent pathways, the antifibrotic role of BMP7 has been linked to several other mechanisms that hold promise for translation into human treatments. For example, DNA methylation changes may be important in BMP7 activity since the prevention of kidney fibrosis was linked to the reversal of Rasal1 promoter hypermethylation (Tampe et al., 2014). In another study, changes in the expression of specific receptors on tubular epithelial cells (e.g., CD44v3) resulted in enhanced BMP7 synthesis and an associated reduction of renal fibrotic damage (Rampanelli et al., 2013). Also, the protective role of BMP7 was shown to be related to changes in the expression of miRNAs as the suppression of miR-21 in rat kidney cells was found to associate with BMP7-mediated inhibition of fibronectin secretion and apoptosis (Yu et al., 2016). Another novel mechanism identified to be

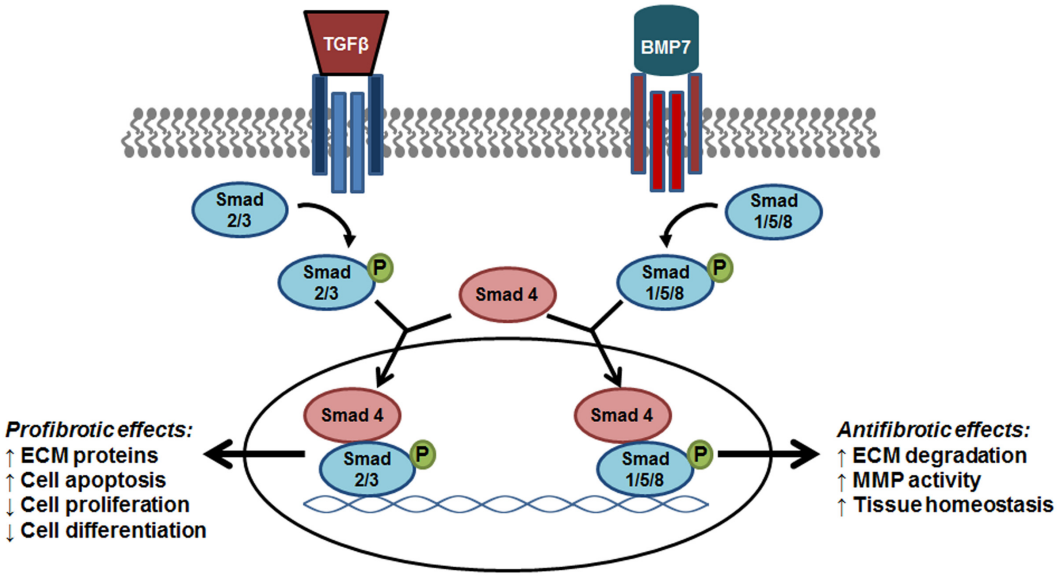

FIGURE 1 | The antifibrotic effect of BMP7/Smad signaling. As members of the same family, BMP7 and TGF-β trigger the phosphorylation of R-Smads that signal trafficking to the nucleus via Smad 4 for specific gene transcription. The phosphorylation of Smad 1/5/8 by BMP7 results in the transcription of target genes that oppose the fibrogenic effects induced by TGF-β-Smad2/3 signaling. BMP7, bone morphogenic protein; TGF-β, transforming growth factor- β1; ECM, extracellular matrix proteins; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1.

associated with BMP7 activity is the production and degradation of hyaluronan in human lung fibroblasts (Midgley et al., 2015). The role of antagonists and competing ligands in the inhibition of BMP7 activity is also part of current investigations which may lead to future translational advancements (Lin et al., 2005; Yanagita et al., 2006; Tanaka et al., 2010). In particular, research is underway to define the role of Activin A, a member of the TGF-β family that acts as an antagonist to BMP7 antifibrotic signaling (Abe et al., 2004; Aykul and Martinez-Hackert, 2016). To date, preclinical studies have shown the potential benefit of inhibiting Activin A signaling by disrupting receptor kinase activity or by blocking the receptor binding site of the antagonistic ligand (Laping et al., 2002; Pearsall et al., 2008; Aykul and Martinez-Hackert, 2016). In addition to the use of antagonists, mechanisms are being studied to enhance antifibrotic signaling through the action of BMP agonists. Notably, a recently developed synthetic peptide (THR-123, Thrasos Therapeutics, Canada) to the BMP receptor activin-like kinase 3 (ALK3), was found to be effective in controlling renal fibrosis in mice (Sugimoto et al., 2012). The successful preclinical work with THR-123 has led to the development of a similar analog, THR-184, which has been tested in a phase 2 trial for the resolution of acute kidney injury in cardiac patients (ClinicalTrials.gov ID NCT01830920). Although it is anticipated that forthcoming results will be clinically revealing, there are limitations in the global use of BMP agonists since the ALK3 receptor is expressed predominantly in the kidney (Sugimoto et al., 2012). Thus, the organ-specificity of BMP agonists needs to be considered and requires further characterization.

Overall, it is well established that BMP7 is an opposing mechanism to TGF-β-mediated profibrogenic signaling and that BMP7 activity is downregulated in fibrotic tissue injury. The potential therapeutic restoration of BMP7 through overexpression or exogenous administration has been demonstrated in multiple animal and organ models of fibrotic disease. However, the effectiveness of these strategies could be limiting due to potential off-target effects and low bioavailability of exogenous BMP7. It is known that systemically administered BMP7 has a short half-life resulting in the need to use of high doses to reach pharmacological effects (Vukicevic et al., 1998). Consequently, the stimulation of BMP signaling can occur in unwanted organ systems due to the ubiquitous expression of BMP receptors throughout the body (Wang et al., 2014). Therefore, current research efforts are focusing on the development and testing of alternative strategies to more specifically target BMP7 including the use of antagonist inhibitors and BMP agonists. Such investigations will likely contribute to translational clinical trials and the advancement of BMP7 as an antifibrotic agent.

MANIPULATION OF miRNA EXPRESSION AS AN ANTIFIBROTIC STRATEGY

MicroRNAs are short non-coding RNA molecules that regulate target messenger RNAs through post-transcriptional or translational repression mechanisms (Ambros, 2004). It has been shown that miRNA expression levels can be altered in disease states compared to normal conditions, highlighting the potential use of miRNAs as diagnostic or treatment targets. Studies to date have identified unique miRNA profiles

for disease states which are often found to be tissue and cellular specific. The dysregulation of miRNA expression during fibrotic disease can involve the aberrant overexpression as well as the downregulation of miRNAs (Bartel, 2009). Regardless of the change, altered miRNA levels can lead to the regulation of a multitude of protein coding genes and signaling mechanisms that promote disease. In fibrosis, miRNAs alterations can lead to disease-promoting changes in a variety of fibrotic mechanisms such as the action of signaling mediators such as TGF-β or the expression of tissue-remodeling ECM components (Jiang et al., 2010). Thus, because of the magnitude of their regulatory role, miRNAs have emerged as viable targets for therapeutic intervention of fibrotic pathways. The following discussion is a review of miRNAs that are promising candidates for the treatment of fibrotic disease.

Research into the role of miRNAs as an antifibrotic therapy in cardiovascular disease is a leading area of investigation. As in other organs, the transformation of cardiac tissue after injury can involve TGF-β signaling and fibroblast activation leading to detrimental scar formation (Khan and Sheppard, 2006). Studies have identified miRNAs in cardiac cells that contribute to the fibrotic cascade in various models of heart disease including atrial fibrosis, cardiac infarction and heart failure as summarized in **Table 2**. Several miRNAs have been noted to be upregulated in animal and human hearts including miR-214, miR-223, and miR-21 (Thum et al., 2008; van Rooij et al., 2008; Roy et al., 2009). Of note, the enhancement of miR-21 led to functional changes in cardiac cells through MAP kinase signaling, fibroblast growth factor 2 expression and the survival of cardiac fibroblasts. Further, a direct link to profibrotic mechanisms was confirmed when the inhibition of miR-21 via antogomir-21 treatments resulted in recovery of heart function in mice subjected to transverse aortic constriction (Thum et al., 2008). In other works, the downregulation of miRNAs was also observed in cardiac disease models including reductions in miR-133, miR-590, miR-30, miR155, miR-22, miR-29, and miR101 (van Rooij et al., 2008; Duisters et al., 2009; Shan et al., 2009; Pan et al., 2012; Kishore et al., 2013; Hong et al., 2016). In recent preclinical assessments, the functional significance of many of the downregulated miRNAs has been described using several models of cardiac fibrotic diseases. In a model of ischemia/reperfusion injury, the dysregulation of miR-29-30-133 were linked to the activation of TGF-β signaling which could be reversed by triiodothyronine (T3) treatment (Nicolini et al., 2015). Particularly, T3 treatment was found to be effective in countering the injury-related downregulation of miR-29c, miR-30c, and miR-133a resulting in the reduction of profibrogenic matrix metalloproteinase (MMP)-2 and CTGF expressions (Nicolini et al., 2015). In models of fibrosis induced by myocardial infarction (MI), the reduction of miRNAs has been shown to regulate many profibrotic proteins and signaling mediators. For example, the targeting of miR-22 shows clinical potential as this miRNA was found to be a negative regulator of cardiac fibrosis through the suppression of TGF-βR1 (Hong et al., 2016). The miR-29 and miR-101 families have also been identified as regulators of antifibrotic mechanisms and have been evaluated for their potential as therapeutic agents. In the case of the miR-29 family, miR-29a, miR-29b, and miR-29c were found to be reduced after MI and associated with the gene expression of ECM proteins and TGF-β signaling (van Rooij et al., 2008; Kriegel et al., 2012). Further, the synthetic overexpression of miR-29 reduced collagen production and fibrosis following MI (van Rooij et al., 2008). Interestingly, the antifibrotic effect of the cardioprotective drug, Tanshinone IIA, was found to involve the upregulation of miR-29b (Yang et al., 2015). Studies evaluating the miR-101 family have similarly demonstrated a protective role in healthy tissue and the importance of designing strategies to increase their expression following cardiac injury. As such, decreased levels of miR-101a/b after coronary artery ligation have been associated with the induction of profibrogenic signaling mediated by c-Fos and TGF (Pan et al., 2012). Importantly, the overexpression of miR-101a inhibited the signaling pathways and alleviated fibrosis in the injured heart. Overall, there is strong preclinical evidence in various injury models that miR-based therapy may be an effective antifibrotic strategy, and especially for the treatment of MI-related cardiac fibrosis. As discussed, the successful modulation of miRNAs by the use of inhibitors, genetic manipulation or pharmacological agents indicates the high therapeutic value of targeting miRNAs. Although the characterization of potential off-target effects of systemically delivered miRNA modulators remains to be determined, the future is bright for the effective and tissue-specific delivery of miRNA targeting treatments.

In addition to cardiac disease, the potential of targeting miRNAs in other fibrotic diseases is also of current clinical importance. In the last 10 years, research efforts have increased in the study of miRNAs in hepatic, renal, and pulmonary fibrosis. In liver fibrosis research, the role of hepatic stellate cells (HSCs) in the production of ECM components and fibrotic injury has been well-characterized (Iredale et al., 2013; Lemoinne et al., 2013). As with other fibrotic conditions, several miRNAs were found to be either upregulated or reduced with the altered expression level associating with HSC activation and liver fibrogenesis (Guo et al., 2009). The correlation of miRNA changes to functional outcomes has been shown for several of the miRNAs highlighting their translational potential (Schon et al., 2016). Examples include a noted reduction in HSC-produced collagens in response to the overexpression of miR-133a (Roderburg et al., 2013). In another study, the activation of HSCs was inhibited by treatment with salvianolic acid B and the induced expression of miR-152 (Yu et al., 2015). And as observed in cardiac disease, the downregulation of miR-29 is strongly associated with hepatic fibrosis. Notably, miR-29 mimics or overexpression has been shown to control ECM production by HSCs demonstrating therapeutic potential (Kwiecinski et al., 2011).

The role of miRNAs in renal fibrosis has also been associated with the regulation of signaling pathways that induce ECM factors such as collagens and fibronectin (Wang et al., 2008; Kato et al., 2009). Multiple works have defined the role of miRNAs in renal fibrosis including the effects of miR-148b and members of the miR-29 and miR-let7 families (Fang et al., 2013; Nagai et al., 2014; Szeto and Li, 2014; Srivastava et al., 2016). Additionally,

TABLE 2 | Notable miRNAs in cardiac fibrosis; targets and potential therapeutic benefits.

miRNAs	Expression	Model	Target	Effect on cardiac fibrosis	Reference
miR-21	↑	MI Cardiac fibroblasts	PTEN MAPK	↑ MMP2 expression, matrix remodeling, fibroblast survival, interstitial fibrosis	Roy et al., 2009; Thum et al., 2008
miR-29	↓	I/R, MI	TGF-β	↑ MMP2 expression, excessive reparative fibrosis	van Rooij et al., 2008; Kriegel et al., 2012; Nicolini et al., 2015; Yang et al., 2015
miR-30-133	↓	I/R	CTGF	↑ Collagen production	Duisters et al., 2009; Nicolini et al., 2015
miR-22	↓	MI	TGF-βRI	↑ Collagen deposition	Hong et al., 2016
miR-101	↓	MI	c-Fos TGF-β	↑ Collagens, fibronectin, MMP-2, MMP-9	Pan et al., 2012

MI, myocardial infarction; PTEN, phosphatase and tensin homolog; MAPK, mitogen-activated protein kinase; I/R, ischemia/reperfusion; TGF-β, transforming growth factor beta; CTGF, connective tissue growth factor; TGF-βRI, transforming growth factor beta receptor type I.

recent studies have demonstrated functional advancements as protection from fibrosis was gained from anti-miR-214 treatment (Denby et al., 2014). Further, the delivery of miR-let7c-expressing mesenchymal stem cells was found to be an effective method to target miRNAs in the damaged kidney (Wang B. et al., 2016). Altogether, research continues to define the therapeutic potential of miRNAs in renal fibrosis.

And lastly, investigation into the role of miRNAs in pulmonary fibrosis is emerging with potential targetable miRNAs identified including miR-29 (Cushing et al., 2015), miR-155 (Pottier et al., 2009), miR-21 (Zhou et al., 2015b; Liu et al., 2016), miR-26a (Liang et al., 2014), and miR-326 (Das et al., 2014). Interestingly, preclinical support of translational effects was indicated by the demonstration of pulmonary fibrosis that is induced as a result of miR-26a inhibition (Liang et al., 2014); and that pulmonary fibrosis could be effectively attenuated following the intranasal deliver of miR-326 mimics (Das et al., 2014). Overall, the increasing and emerging research in this field exemplify the power of targeting miRNAs for the treatment of fibrotic diseases and support the need of future evaluations.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

The PPARs are nuclear transcription factors that form obligate heterodimers with retinoid-X receptors to modulate transcription of target genes. Three PPAR subtypes have been identified, known as PPARα, PPARβ/δ, and PPARγ. Due to its role in the insulin-sensitizing effects of the thiazolidinedione (TZD) drugs, PPARγ is by far the most widely studied PPAR isoform. However, preclinical studies have implicated all three PPARs as potential targets for antifibrotic therapy (summarized in **Figure 2**). Recent studies supporting the use of PPAR agonists for this purpose are described below, along with discussion of recently completed or ongoing clinical trials.

PPARγ Agonists

Peroxisome proliferator-activated receptorsγ is involved in insulin sensitization and the promotion of adipogenesis (Gross et al., 2017). It is targeted primarily as a treatment for diabetes,

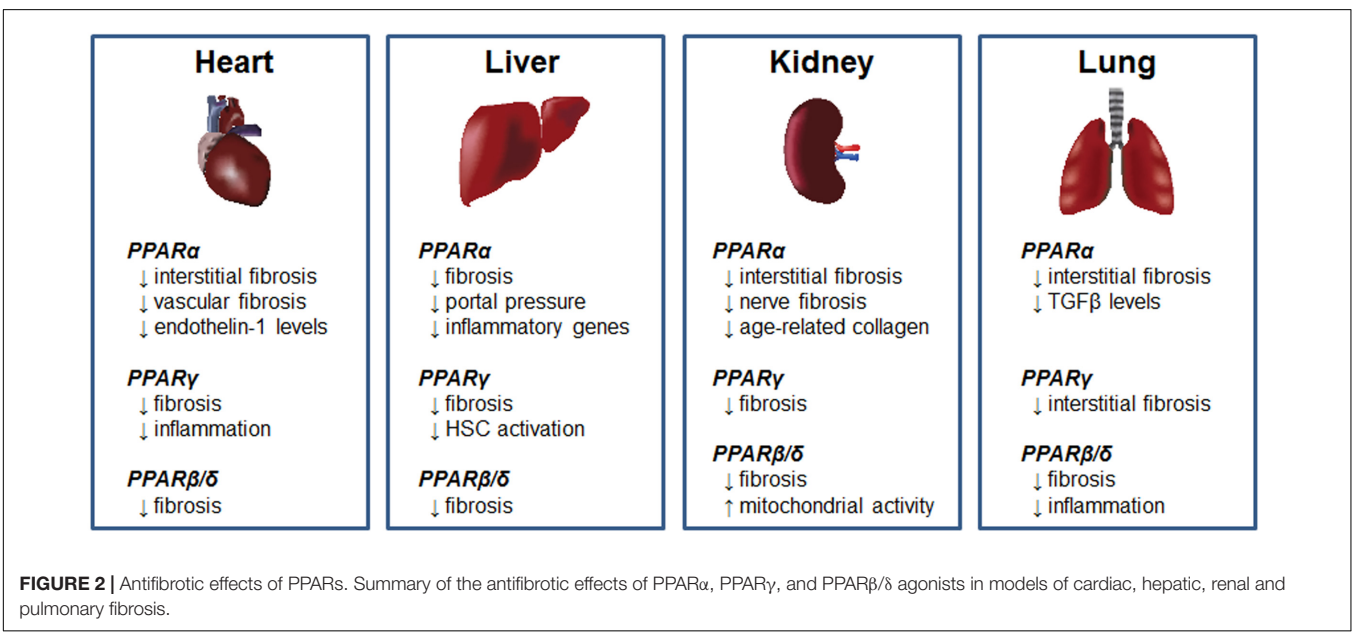

FIGURE 2 | Antifibrotic effects of PPARs. Summary of the antifibrotic effects of PPARα, PPARγ, and PPARβ/δ agonists in models of cardiac, hepatic, renal and pulmonary fibrosis.

where it enhances the insulin sensitivity of target tissues to promote glucose uptake. It also has potent lipid-lowering properties, and is anti-inflammatory. The endogenous ligands for PPARγ are thought to be prostaglandin and leukotriene derivatives, including 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PJ2). Synthetic ligands include the TZD drugs, such as rosiglitazone and pioglitazone, used clinically for diabetes treatment. The TZD have also shown promise in preclinical studies of established fibrosis, as well as in a limited number of clinical studies (summarized in **Table 3**).

A role for PPARγ in the control of fibrosis has long been apparent, with many of the studies focused on the liver. Early studies of liver myofibroblasts (hepatic stellate cells, or HSCs) revealed that PPARγ expression was present in quiescent HSC, but was reduced with activation to the myofibroblastic phenotype and fibrosis progression (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000). Overexpression of PPARγ in HSC, or treatment with the endogenous PPARγ ligand 15d-PGJ2 or TZDs, resulted in decreased myofibroblastic character of HSCs, with reduced collagen production and increased MMP activity (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000; Hazra et al., 2004). However, targeting PPARγ in preclinical animal models of liver fibrosis by treatment with TZDs has met with mixed results. In preclinical studies in rats, TZD treatment prevented acute CCl_4-induced liver damage (Kon et al., 2002), and chronic fibrosis induced by toxins, cholestasis, or choline-deficient diet (Galli et al., 2002; Kawaguchi et al., 2004; Marra et al., 2005; Bruck et al., 2009). Furthermore, recent studies using the endogenous PPARγ ligand 15d-PGJ2 prevented hepatic fibrosis induced by *Trypanosoma cruzi* infection and carbon-tetrachloride-induced fibrosis in mice (Jia et al., 2015; Penas et al., 2016). However, studies using PPARγ agonists in treatment models of established liver disease have met with very different results. In rat models of fibrosis, pioglitazone prevented toxin (CCl_4) and choline-deficient diet fibrosis, but was not effective when administered after the disease was established, and was ineffective against cholestasis-induced injury regardless of the length of treatment (Leclercq et al., 2006). Furthermore, pioglitazone was ineffective at reducing the activation of mouse

TABLE 3 | Selected preclinical and clinical studies using PPARγ agonists for established fibrosis.

Preclinical studies in animal models with established fibrosis

Fibrotic disease	Species	Model	Drug	Findings	Reference
Cardiac fibrosis	Mouse	AT-II	PGZ	Decreased fibrosis and inflammation	Caglayan et al., 2008
	Rat	Pressure overload	RGZ	Reduced fibrosis	Qi et al., 2015
	Rat	MCT	PGZ	Reduced right ventricular fibrosis and cardiomyocyte size	Behringer et al., 2016
	Mouse	T1DM (Akita)	CGZ	Reduced fibrosis, improved end diastolic diameter	Mishra et al., 2010
	Rat	T2DM (OLETF)	PGZ	Reduced fibrosis, increased MMP9 expression	Makino et al., 2009; Ihm et al., 2010
Hepatic fibrosis	Rat	CCl_4, BDL, CDAA	PGZ	Ineffective after fibrosis was established	Leclercq et al., 2006
	Mouse	CCl_4	PGZ	Ineffective after disease was established	Da Silva Morais et al., 2007

Clinical studies of fibrosis

Disease (*n*)	Study type	Drug	Dose	Duration	Findings	Reference
NASH *(25)*	P	RGZ	4 mg/day	48 weeks	Improved steatosis, inflammation, and fibrosis	Neuschwander-Tetri et al., 2003
NASH *(18)*	P	PGZ	30 mg/day	48 weeks	Improved steatosis, inflammation, and fibrosis	Promrat et al., 2004
NASH *(61)*	R,PC,DB	PGZ	30 mg/day	12 months	Improved steatosis, inflammation, and fibrosis	Aithal et al., 2008
NASH *(108)*	R,P,OL	RGZ	4 mg 2x/day	12 months	Improved steatosis, inflammation, and fibrosis	Torres et al., 2011
NASH *(63)*	R,PC,DB	RGZ	8 mg/day	12 months	Improved steatosis and liver function, no effect on fibrosis	Ratziu et al., 2008
NASH *(142)*	R,PC,DB	PGZ	30 mg/day	96 weeks	Improved steatosis and inflammation, no effect on fibrosis	Sanyal et al., 2010

AT-II, Angiotensin-II; BDL, bile duct ligation; CDAA, choline deficient L-amino acid defined diet; CGZ, ciglitazone; DB, double-blind; MCT, monocrotaline; n: number of patients who completed the study with pre- and post-treatment biopsies; NASH, non-alcoholic steatohepatitis; P, prospective; PC, placebo-controlled; PGZ, pioglitazone; R, Randomized; RGZ, rosiglitazone; T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus; OLETF, Otsuka Long-Evans Tokushima rats

HSC, and failed to prevent CCl_4-induced hepatic fibrosis (Da Silva Morais et al., 2007).

The reason for the discrepancies in the preclinical models is not currently known, but it has been speculated that effects of thiazolidinediones on other targets, such as alterations in adiponectin signaling or promotion of the fibropermissive (M2) phenotype of macrophages, may have counteracted the antifibrotic effects (Da Silva Morais et al., 2007; Sica et al., 2014). Furthermore, although long-term (12 months) treatment of NASH patients with rosiglitazone was associated with reduced liver fibrosis, there was evidence of increased liver inflammation (Lemoine et al., 2014), while similar studies using pioglitazone generally showed a decrease in inflammation (Mahady et al., 2011). The reason for this observation is not clear, but may be due to promotion of inflammatory cytokines by hepatocytes in response to rosiglitazone (Rogue et al., 2011; Lemoine et al., 2014). To circumvent these problems, targeted delivery of PPARγ agonists may be more efficacious. In support of this notion, nanoformulation of rosiglitazone into a biodegradable copolymer effectively reduced common bile duct ligation-induced hepatic fibrosis when administered early in the development of the disease (Kumar et al., 2014). Similarly, rosiglitazone packaged into liposomes with a HSC-targeting moiety effectively decreased established hepatic fibrosis in rats (Patel et al., 2012). Other approaches include the use of agents that activate PPARγ by alternate pathways. Recent studies suggested that treatment with rosmarinic acid and baicalin reduced hepatic fibrosis, by de-repression of PPARγ gene expression by the protein MeCP2, a process that could be reversed by inhibiting the Wnt signaling pathway (Yang et al., 2012; Kweon et al., 2016). Finally, it is possible that new, non-TZD activators of PPARγ, may prove more effective and with fewer side effects than TZDs. In support, a recent study showed that the non-TZD agonist GW570 prevented both cholestasis and CCl_4 induced fibrosis (Yang et al., 2010).

Peroxisome proliferator-activated receptorsγ also has a role in the progression and treatment of cardiac fibrosis. Many of the preclinical studies have focused on cardiac damage in association with rodent hypertensive models. Pioglitazone decreased cardiac fibrosis in spontaneously hypertensive rats (Nakamura et al., 2008). In rat pressure overload models, rosiglitazone prevented cardiac fibrosis, while a PPARγ antagonist worsened fibrosis (Gong et al., 2011). Using a similar model, ciglitazone prevented fibrosis, and promoted the expression of matrix-degrading enzymes (Henderson et al., 2007). In a mouse angiotensin-II treatment model, pioglitazone reduced cardiac fibrosis (Caglayan et al., 2008). Interestingly, this study used either macrophage- or cardiomyocyte-specific knockout of PPARγ, and concluded that PPARγ in both cell types contributed to decreased macrophage infiltration. However, pioglitazone decreased cardiomyocyte fibrosis and inflammation both wild-type and cardiomyocyte PPARγ-knockout mice, but not in macrophage PPARγ-knockout mice, suggesting that in this model, macrophage PPARγ activity is critical for the antifibrotic effect of pioglitazone. Treatment of established pressure overload-induced cardiac fibrosis with rosiglitazone reduced interstitial cardiac fibrosis and fibrosis-related gene expression (Qi et al., 2015). Using the cardiac pressure overload model in rats, TSG (2,3,4′,5-tetrahydroxystilibene-2-*O*-β-d-glucoside) administered early in the progression of the disease (3 days post-surgery) reduced cardiac fibrosis and expression of types I and II collagen through a pathway involving PPARγ (Peng et al., 2016). In a treatment model of established pulmonary artery hypertension-induced right ventricular cardiac fibrosis, pioglitazone treatment reduced right ventricular fibrosis and cardiomyocyte size (Behringer et al., 2016). The lipid-lowering drug atorvastatin prevented cardiac fibrosis induced by advanced glycation end-products (AGEs), by a mechanism that appeared to involve PPARγ (Chen et al., 2016). Similar findings were reported in a toxin-induced model of pulmonary fibrosis, where atorvastatin was found to be more effective that pioglitazone (Malekinejad et al., 2013). However, it is currently unclear whether atorvastatin directly or indirectly activates PPARγ.

Positive results have also been observed in models of diabetes-related cardiac fibrosis. In alloxan-induced diabetic rabbits, rosiglitazone prevented atrial remodeling and fibrosis (Liu et al., 2014). Similarly, ciglitazone treatment reduced cardiac fibrosis and improved end diastolic diameter in diabetic Akita mice (Mishra et al., 2010), while rosiglitazone reduced myocardial fibrosis in diabetic Otsuka Long-Evans rats (Makino et al., 2009; Ihm et al., 2010).

Finally, PPARγ has also been a target for treatment of fibrosis in several other organs. In relation to pulmonary fibrosis, bleomycin-induced lung injury in rodents was prevented by TZD or 15d-PGJ2 treatment (Genovese et al., 2005; Milam et al., 2008; Aoki et al., 2009; Samah et al., 2012). In a recent study, rosiglitazone prevented pulmonary fibrosis induced by radiation exposure (Mangoni et al., 2015). In a bleomycin-induced model of dermal fibrosis, rosiglitazone reduced inflammation and collagen deposition (Wu et al., 2009). The new PPARγ agonist GED-0507-34-Levo, when administered early in the progression of chronic colitis in mice, reduced intestinal fibrosis (Speca et al., 2016). This compound is currently in a phase 2 clinical trial as a treatment for ulcerative colitis (ClinicalTrials.gov ID NCT02808390). Finally, PPARγ has also been an effective treatment for some models of nephropathy-related fibrosis (Jia et al., 2014; Speeckaert et al., 2014).

Clinical trials assessing the treatment of fibrotic diseases with PPARγ agonists have largely been limited to patients with non-alcoholic steatohepatitis (NASH), and the results have been decidedly mixed. Early prospective studies of patients with NASH before and after 48 weeks treatment with either rosiglitazone or pioglitazone showed significant improvements in steatosis, inflammation and fibrosis (Neuschwander-Tetri et al., 2003; Promrat et al., 2004). Similar results were observed in prospective 12 months studies of rosiglitazone or pioglitazone treatment of NASH patients (Aithal et al., 2008; Torres et al., 2011). Conversely, a prospective 12 months study using rosiglitazone found no effect on fibrosis (Ratziu et al., 2008). A relatively short-term study (26 weeks) of insulin-resistant patients with NASH treated with pioglitazone or placebo in conjunction with a hypocaloric diet showed improvements in liver function, steatosis and inflammation, but no difference in fibrosis compared

with placebo (Belfort et al., 2006). A phase 3 randomized, multi-center placebo-controlled trial that was conducted to determine the effect of 96 weeks treatment of pioglitazone, vitamin E or placebo on patients with NASH revealed that, while pioglitazone treatment resulted in significantly improved liver function, steatosis, and inflammation, there was no significant effect on fibrosis (Sanyal et al., 2010). The reasons for the discrepancies in these studies are not clear, but may involve differences in patient population (e.g., inclusion or exclusion of diabetes patients, differences in medication) and study design between the individual trials. There may also be a difference in the relative effectiveness between thiazolidinedione, as a recent meta-analysis has concluded that pioglitazone, but not rosiglitazone, significantly decreased fibrosis in NASH (Musso et al., 2017). A phase 2 trial is currently underway to study pioglitazone in patients with non-alcoholic steatohepatitis, which includes liver inflammation and fibrosis as a secondary aim (ClinicalTrials.gov ID NCT01068444).

The use of the TZD drugs has waned considerably in recent years due to side effects such as weight gain, edema and bone density loss (Tahrani et al., 2016), as well as recent concerns regarding rosiglitazone and pioglitazone and the increased risk of cardiovascular disease and bladder cancer, respectively, although the latter associations remain controversial (Hoogwerf et al., 2016; Hampp and Pippins, 2017). Recent studies have focused on PPARγ partial agonists, also known as selective PPARγ modulators (SPPARMs), which retain the insulin-sensitizing effects of PPARγ activation, but lack the strong adipogenic effects. One of these synthetic PPARγ agonists, bardoxolone (also known as CDDO) effectively reduced collagen and antagonized TGF-β signaling in two models of dermal fibrosis, but the antifibrotic effects appeared to be independent from PPARγ (Wei et al., 2013). Another, INT131, showed promising results in reducing blood glucose in type 2 diabetes patients without weight gain or fluid retention (Dunn et al., 2011). To date, clinical studies of non-TZD PPARγ agonists on fibrotic diseases are lacking.

PPARα Agonists

Peroxisome proliferator-activated receptorsα is predominantly expressed in hepatocytes, cardiomyocytes, renal proximal tubule cells, and enterocytes. The main function of PPARα is to promote lipid β-oxidation, partially through promotion of peroxisomal enzymes, and thus is a target of lipid-lowering drugs such as the fibrates, the best known of which is fenofibrate. The endogenous ligands of PPARα are thought to be lipids, including fatty acids and arachidonic acid derivatives.

It has been long known that PPARα plays a role in tissue fibrosis. PPARα-null mice developed age-related myocardial fibrosis, which was detectable at 16 weeks of age, and pronounced by 32 weeks (Watanabe et al., 2000). In several different models of cardiac fibrosis, fenofibrate prevented interstitial and perivascular cardiac fibrosis, through a mechanism that involved reduced endothelin-1 levels (Ogata et al., 2002, 2004; Iglarz et al., 2003; Diep et al., 2004; LeBrasseur et al., 2007; Forcheron et al., 2009; Zhang et al., 2016). Similar results were observed using inducible overexpression of PPARα early in the progression of pressure overload-induced cardiac damage (Kaimoto et al., 2016). However, one study using fenofibrate treatment of chronic pressure overload in PPARα knockout mice showed that fenofibrate had profibrotic effects that were independent of PPARα (Duhaney et al., 2007).

Fenofibrate prevented interstitial renal fibrosis in a number of preclinical rodent models (Hou et al., 2010; Li et al., 2010; Tanaka et al., 2011; Balakumar et al., 2014). Similar results were observed using other PPARα agonists, gemfibrozil and BAY PP1 (Calkin et al., 2006; Boor et al., 2011). Fenofibrate prevented renal nerve fibrosis and injury in a type 2 diabetes mouse model (Cho et al., 2014), renal fibrosis in a rat type 1 diabetes model (Cheng et al., 2016), and age-related renal collagen accumulation in mice (Kim et al., 2016). In addition to the kidney, fenofibrate preventively decreased bleomycin-induced pulmonary fibrosis, and decreased lung TGFβ content (Samah et al., 2012).

In the liver, the strong PPARα agonist WY-14643 reduced fibrosis induced by a choline-deficient diet (Ip et al., 2004), and decreased fibrosis induced by a combination of ethanol feeding and CCl_4 injections (Nan et al., 2013). In a treatment model of established cirrhosis in rats, fenofibrate reduced portal pressure and fibrosis (Rodríguez-Vilarrupla et al., 2012). Fenofibrate prevented hepatic fibrosis induced by thioacetamide or concanavalin-A (Toyama et al., 2004; Mohamed et al., 2013). Interestingly, the ability of fenofibrate to attenuate hepatic steatosis and fibroses appears to be associated with its trans-repressive effects on inflammation- and fibrosis-related genes, rather than its ability to transcriptionally regulate genes associated with lipid metabolism (Pawlak et al., 2014).

Few clinical studies of PPARα agonists in fibrotic diseases have been conducted, and most of these focused on primary biliary cirrhosis, due largely to the lipid and bile acid modulating effects of the fibrate drugs, and their anti-inflammatory properties. However, most studies did not assess the effect on liver fibrosis directly. One study did show that fenofibrate reduced liver stiffness and serum hyaluronic acid, a marker of ECM accumulation (El-Haggar and Mostafa, 2015).

PPARβ/δ Agonists

Peroxisome proliferator-activated receptorsβ/δ, also known as PPARβ or PPARδ, is ubiquitously expressed. The physiological function of PPARβ/δ is not clear, but activation of the receptor results in modulation of lipid and glucose homeostasis, skeletal muscle function, and brown adipose tissue activity (Gross et al., 2017). The endogenous ligands for PPARβ/δ are thought to be lipid derivatives, and synthetic agonists have been produced for preclinical studies.

Treatment of fibrotic diseases with agonists of PPARβ/δ has been limited to preclinical animal studies. One synthetic agonist, GW0742, reduced bleomycin-induced lung fibrosis and inflammation in mice (Galuppo et al., 2010), and prevented pulmonary artery banding-induced cardiac hypertrophy and fibrosis in mice (Kojonazarov et al., 2013). In a rat model of type-1 diabetes-associated cardiac fibrosis, GW0742 reduced markers of cardiac fibrosis (Chang et al., 2016).

The PPARβ/δ agonist GW610742 was effective in preventing collagen content in a rat corneal wounding model (Gu et al.,

2014), while another agonist (GW501516) reduced peritoneal fibrosis and inflammation in rats (Su et al., 2014). However, in a rat model of MI, administration of GW610742 resulted in an early increase in cardiac fibrosis (7 days after MI), although there was no overall effect on collagen levels after 14 days (Park et al., 2016). Another PPARβ/δ agonist, HPP593, was effective in reducing renal fibrosis induced by chronic ischemia, by a mechanism that appeared to be due, at least in part, by a reduction in oxidative stress and preservation of mitochondrial function (Fedorova et al., 2013).

One study using the synthetic agonist KD3010 observed effective prevention of liver fibrosis induced by CCl_4 or chronic cholestasis (Iwaisako et al., 2012). Interestingly, the same study showed that GW501516 had no effect on fibrosis in the same models. One possible explanation is the observation that GW501516, but not KD3010, induced hepatic connective tissue growth factor, a profibrotic factor. Consistent with this observation, another study showed that GW501516, administered to mice concomitantly with CCl_4, enhanced the degree of fibrosis and inflammation, by a mechanism that involved enhanced proliferation of the hepatic stellate cells (Kostadinova et al., 2012).

Dual-, Pan-, and Mixed-PPAR Agonists

Many studies have attempted to use multi-specificity PPAR agonists, to varying degrees of success (Derosa et al., 2017). In the following section, recent studies using dual-, pan-, or mixed PPAR agonists will be discussed in terms of their potential for future treatment of fibrotic disease.

Dual-PPAR Agonists

A number of dual PPARα/γ activators, collectively known as glitazars, have been produced. One of these, tesaglitazar, reduced diabetic nephropathy in obese diabetic *db/db* mice (Cha et al., 2007). Similarly, another dual PPARα/γ activator, aleglitazar, was effective in promoting glucose regulation and renal function, and decreasing pancreatic islet fibrosis and degeneration in obese diabetic rats (Bénardeau et al., 2013). A clinical trial has been completed comparing saroglitazar with pioglitazone in the treatment of non-alcoholic fatty liver disease, but the results have yet to be published (ClinicalTrials.gov ID NCT02265276).

Elafibrinor (also known as GFT505), a recently developed dual PPARα/δ activator, was used in multiple rodent models of metabolic and fibrotic liver diseases. The effects were positive in all models, with elafibrinor reducing steatosis, inflammation and fibrosis in both preventative and treatment regimens (Staels et al., 2013). A placebo-controlled clinical study was recently performed with 1 year of elafibranor treatment of NASH patients (Ratziu et al., 2016). Elafibranor induced resolution of steatosis, and improved the fibrosis score in patients with decreased steatosis. A phase 3 trial is underway to determine the effect of elafibranor on NASH patients, with fibrosis evaluation included in the outcomes (ClinicalTrials.gov ID NCT02704403).

Pan-PPAR Agonists

A recently developed agonist that weakly activates all three PPARs (IVA337) was used in a mouse model of dermal fibrosis (Ruzehaji et al., 2016). Importantly, the drug was used in both prevention and treatment models, and furthermore was directly compared to the PPARγ agonist rosiglitazone. A phase 2 proof-of-concept study is underway to study IVA337 as a treatment for diffuse scleroderma (ClinicalTrials.gov ID NCT02503644), and a phase 2b trial for the treatment of NASH (ClinicalTrials.gov ID NCT03008070).

Mixed PPAR Agonists

Angiotensin 1 receptor antagonists, collectively knowns as the sartans, have long been used to modulate the activity of the renin-angiotensin system. Recently, it was discovered that several members of the sartans have weak PPARγ-activating properties (Michel et al., 2013). Of these, telmisartan appears to be the most potent at activating PPARγ. Telmisartan reduced fibrosis in response to MI in rats (Maejima et al., 2011; Nagashima et al., 2012). Similarly, irbesartan protected against cardiac and renal fibrosis and myocyte hypertrophy in a mouse model of salt-sensitive hypertension (Kusunoki et al., 2013). Interestingly, in both of these cases, most of the antifibrotic effects appeared to be independent of angiotensin receptor blockade, as they were blocked by PPARγ inhibition. Another study observed reduced myocardial fibrosis in angiotensin converting enzyme-2 null mice treated with irbesartan (Zhang et al., 2013). Similarly, irbesartan reduced bleomycin-induce pulmonary fibrosis and inflammation in mice (Tanaka et al., 2013). In a study supporting the concept of dual angiotensin 1 receptor blockage and PPARγ activation, candesartan combined with pioglitazone had increased efficacy in reducing fibrosis in spontaneous hypertensive rats compared with either agent alone (Nakamura et al., 2008). Interestingly, recent studies showed that telmisartan and elmisartan reduced cardiac fibrosis though a mechanism that involved activation of PPARβ/δ (Chang et al., 2016; Wei-Ting et al., 2016), suggesting that PPARγ may not be the only nuclear receptor activated by these agents.

Several cannabinoid compounds have been shown to have dual activity at the CB2 cannabinoid receptor as well as PPARγ and, in some cases, other PPAR isoforms (O'Sullivan, 2007). One of these, ajulemic acid, was effective in preventing and treating bleomycin-induced lung and skin fibrosis (Garcia-Gonzalez et al., 2012; Lucattelli et al., 2016). A new compound (VCE-004.8) that serves as a dual-agonist of PPARγ and the cannabinoid 2 receptor, reduced collagen deposition and dermal thickness in a mouse model of scleroderma (del Rio et al., 2016).

The cannabinoid oleoylethanolamide (OEA) is thought to be an endogenous PPARα agonist, although it can signal through other receptors (De Petrocellis and Di Marzo, 2010). When used to treat mice concurrently with a methionine-choline deficient diet to induce hepatic steatosis and mild fibrosis, OEA was found to reduce overall liver collagen content and decrease the gene expression of collagens type I and III, α-smooth muscle actin (SMA), TIMP1, MMP2, and MMP9 (Chen et al., 2015). Similar results were observed when more extensive hepatic fibrosis was induced using thioacetamide. Importantly, the protective effect of OEA was greatly reduced in PPARα-null mice, suggesting that the beneficial effects were primarily mediated via PPARα, although a role for other OEA receptors cannot be totally excluded.

RELAXIN

Relaxin is a polypeptide of the insulin/relaxin superfamily. It is produced by the corpus luteum of the ovary during pregnancy (Sherwood, 2004; Bathgate et al., 2013). Its roles during pregnancy involve widespread hemodynamic changes such as vasodilation, decreased systemic vascular resistance, and increased renal plasma flow and glomerular filtration rate (Conrad and Davison, 2014). Its roles outside of pregnancy were less clear until the generation of the relaxin knockout mouse, which developed widespread fibrosis with aging (Samuel et al., 2016a). Importantly, the male mice developed fibrosis as well, but the source of relaxin in males and non-pregnant females is unclear. Relaxin protects against fibrosis both by decreasing collagen production, and promoting collagen degradation by increasing the levels and activity of MMPs (Samuel et al., 2016b; Bennett, 2009). Furthermore, many of relaxin's effects involve antagonism of the effects of TGFβ. The relatively recent discovery of its receptor (RXFP1) has led to rapid development of new discoveries for the use of relaxin as an antifibrotic agent (Bathgate et al., 2013). Some of these findings are summarized in **Figure 3**.

The earliest human studies using relaxin to target fibrotic diseases began in the late 1950s, using partially purified porcine relaxin to treat scleroderma, with variable success (Samuel et al., 2016a). Preclinical animal studies have supported the use of relaxin for dermal fibrosis, (Unemori et al., 1993), and the relaxin knockout mouse spontaneously develops age-related skin fibrosis (Samuel et al., 2005). A peptide activator of RXFP1 prevented bleomycin-induced dermal fibrosis in mice (Pini et al., 2010). Finally, the development of recombinant human relaxin led to more recent clinical scleroderma trials. In a phase 2 study, beneficial effects of relaxin were observed in patients with moderate to severe diffuse systemic sclerosis after 24 weeks infusion with low relaxin (25 μg/kg/day), but not at a higher dose (100 μg/kg/day) (Seibold et al., 2000). However, in a subsequent phase 3 trial, relaxin had no clinically significant effects (Khanna et al., 2009). Part of the lack of success of this study may have been due to the relatively advanced degree of scleroderma in the patient population, a notion supported by the finding that relaxin treatment failed to reverse advanced scleroderma in the relaxin knockout mouse (Samuel et al., 2005), or the uncertainty in clinically meaningful outcomes measures in human systemic sclerosis trials (Seibold, 2002).

Relaxin has been extensively studied as a treatment for several preclinical fibrosis models (summarized in **Table 4**), including cardiac fibrosis. The relaxin knockout mouse serves as a model of age-related cardiac fibrosis and associated left ventricular dysfunction, but interestingly, only in the male mice, due to additional detrimental effects of testosterone (Du et al., 2003; Hewitson et al., 2012). The increased left ventricular collagen deposition was reversed by treatment with

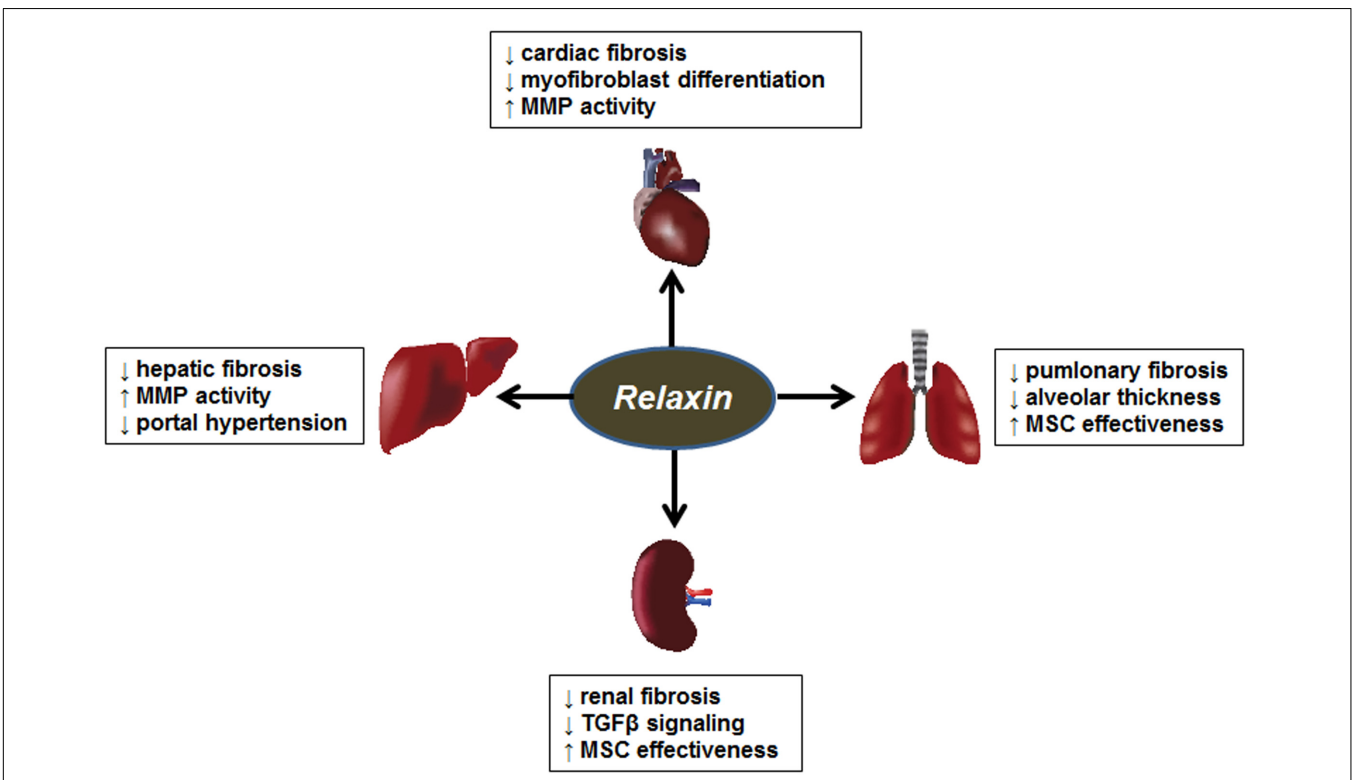

FIGURE 3 | Antifibrotic effects of relaxin. Summary of the antifibrotic effects of PPARα, PPARγ, and PPARβ/δ agonists in models of cardiac, hepatic, renal and pulmonary fibrosis. MMP, matrix metalloproteinase; MSC, mesenchymal stem cells.

TABLE 4 | Selected preclinical studies using relaxin for established fibrosis.

Preclinical studies in animal models with established fibrosis					
Fibrotic disease	**Species**	**Model**	**Dose**	**Findings**	**Reference**
Cardiac fibrosis	Mouse	TG- β2AR, Rln-KO	0.5 mg/kg/day	Decreased left ventricular fibrosis, inhibition of cardiac myofibroblast differentiation	Samuel et al., 2004a
	Mouse	TG-β2AR	Adenovirus-delivered	Decreased left ventricular collagen content	Bathgate et al., 2008
	Mouse	Iso-prenaline	0.5 mg/kg/day	Decreased cardiac fibrosis, suppressed TGFβ expression and signaling, increased MMP13	Samuel et al., 2014
	Rat	SHR	0.5 mg/kg/day	Decreased cardiac and renal collagen, suppressed myofibroblast differentiation, increased MM2	Lekgabe et al., 2005
	Rat	SHR	0.4 mg/kg/day	Suppressed atrial fibrillation, decreased fibrosis and hypertrophy	Parikh et al., 2013
	Mouse	STZ-mRen2	0.5 mg/kg/day	Decreased fibrosis and left ventricular stiffness, increased MMP13 and reduced TIMP1	Samuel et al., 2008
	Rat	MI	1 μg/day	Decreased fibrosis and myocardial apoptosis	Bonacchi et al., 2009
Hepatic fibrosis	Mouse	CCl_4	25–75 μg/kg/day	Reduced fibrosis, increased MMP13 expression, increased collagen degrading activity	Bennett et al., 2014
	Mouse	CCl_4, BDL	0.5 mg/kg/day	Reduced markers of fibrosis, reduced portal pressure	Fallowfield et al., 2014
Pulmonary fibrosis	Mouse	Rln-KO	0.5 mg/kg/day	Reduced lung collagen content and restored alveolar structure	Samuel et al., 2003
	Mouse	OVA-AAD	0.5 mg/kg/day	Decreased collagen deposition and epithelial thickening, but no effect on inflammation	Royce et al., 2009; Royce et al., 2015
	Mouse	OVA-AAD	0.8 mg/ml IN	Decreased lung collagen and epithelial thickening	Royce et al., 2014
Renal fibrosis	Mouse	Rln-KO	0.5 mg/kg/day	Decreased kidney collagen	Samuel et al., 2004b
	Rat	Aging	96 μg/day	Reduced renal collagen, improved renal function	Danielson et al., 2006
	Rat	DS	12 μg/day	Decreased collagen and TGFβ signaling, improved systolic blood pressure	Yoshida et al., 2011
	Mouse	UUO	0.5 mg/kg/day	Reduced renal collagen and myofibroblast differentiation	Huuskes et al., 2015
	Mouse	STZ-eNOS-KO	32 or 320 μg/kg/day	No effect on renal fibrosis	Dschietzig et al., 2015

DS, salt-sensitive rat model; IN, intranasal; MI, myocardial infarction; MMP, matrix metalloproteinase; OVA-AAD, ovalbumin-induced allergic airway disease; Rln-KO, Relaxin knockout mouse; SHR, spontaneously hypertensive rats; STZ-mRen2 (streptozotocin-treated mRen2 transgenic mice); STZ-eNOS-KO, streptozotocin-treated eNOS knockout mice; TG-β2AR, transgenic β2-adrenergic receptor overexpressing mice; TIMP, tissue inhibitor of metalloproteinase; UUO, unilateral ureteral obstruction

relaxin, by a mechanism that appeared to involve inhibition of cardiac myofibroblast differentiation (Samuel et al., 2004a). In preclinical animal models, relaxin treatment, or relaxin delivered by adenovirus, effectively reduced cardiac fibrosis induced by β-adrenergic stimulation in rodents (Samuel et al., 2004a; Zhang et al., 2005; Bathgate et al., 2008). More recently, relaxin was more effective than the angiotensin converting enzyme inhibitor enalapril in treating isoproterenol-induced cardiac injury, while the combination of relaxin and enalapril was more efficacious than either treatment alone, in both prevention and treatment approaches (Samuel et al., 2014). Furthermore, the effect of relaxin treatment on isoproterenol-induced cardiac fibrosis may involve inhibition of endothelial to mesenchymal transition (Zhou et al., 2015a). Relaxin was also effective in reversing cardiac fibrosis in the spontaneously hypertensive rat model (Lekgabe et al., 2005). Furthermore, relaxin reduced both fibrosis and atrial fibrillation in spontaneously hypertensive rats (Parikh et al., 2013). Relaxin reduced collagen content in a rat model of angiotensin II-induced fibrosis, and furthermore, berberine was used to induce relaxin expression, with similar results (Gu et al., 2012). Relaxin was also successful in reducing collagen in a diabetic cardiomyopathy model (Samuel et al., 2008). Conversely, in a chronic pressure overload model, relaxin knockout mice had no more collagen deposition in the heart than wild-type mice (Xu et al., 2008), suggesting that the effectiveness of relaxin may depend on the nature of cardiac injury.

In rats subjected to MI, relaxin reduced cardiac fibrosis, inhibited cardiac myofibroblast differentiation, and promoted induction of MMPs (Bonacchi et al., 2009; Samuel et al., 2011). Furthermore, a recent study suggested that in addition to reduced post-MI-induced fibrosis, relaxin reduced tachyarrhythmia and cardiac dysfunction in rats (Wang D. et al., 2016). Recombinant human relaxin has been used in clinical studies of acute heart failure, with some promising results in regard to dyspnea and hemodynamic properties, as well as cardiovascular death and all-cause mortality in a phase III study (Teerlink et al., 2009). A second phase III trial is currently underway to study the effect of relaxin on acute heart failure (ClinicalTrials.gov ID#NCT02064868).

The fibrotic lung is also a therapeutic target of relaxin. The relaxin knockout mouse developed age-related pulmonary fibrosis, and as with cardiac fibrosis, the effect was more pronounced in male mice, and was reversed after relaxin treatment (Samuel et al., 2003). Similarly, the relaxin knockout mouse developed more severe fibrosis in studies using ovalbumin-induced allergic airway disease (Mookerjee

et al., 2006; Samuel et al., 2007). In rodent models of pulmonary fibrosis induced by bleomycin or ovalbumin-induced allergic airway disease, lung collagen content and alveolar thickness were reduced with relaxin (Unemori et al., 1996; Royce et al., 2009). Recent studies also showed efficacy of intranasal relaxin administration in allergic airway disease and cigarette smoke induced lung damage (Royce et al., 2014; Pini et al., 2016). In an exciting development, relaxin and bone marrow-derived mesenchymal stem cells or amnionic epithelial stem cells were found to be synergistic in the treatment of established airway disease (Royce et al., 2015, 2016). The mechanism for this effect is currently under investigation, but appears to be related to the matrix remodeling and anti-TGFβ effects of relaxin promoting a favorable environment for the establishment of stem cell residency in the damaged tissue (Samuel et al., 2016b).

Treatment of experimental renal fibrosis has also supported relaxin as a novel antifibrotic agent. As with the heart, skin and lungs, the relaxin knockout mouse develops renal fibrosis with age, which can be reversed with relaxin treatment (Samuel et al., 2004b). In several animal models of renal fibrosis, relaxin was effective in reducing collagen deposition (Garber et al., 2001, 2003; McDonald et al., 2003; Lekgabe et al., 2005; Danielson et al., 2006; Yoshida et al., 2011; Yoshida et al., 2014). Relaxin reduced unilateral uteretic obstruction-induced renal disease by antagonizing the effect of TGF-β, and that this effect was enhanced by coadministration of mesenchymal stem cells (Hewitson et al., 2010; Huuskes et al., 2015). On the other hand, relaxin was not effective in a diabetes-related renal disease model (Wong et al., 2013; Dschietzig et al., 2015). However, unlike the unilateral uteretic obstruction models, TGFβ was not increased in the diabetic model. Therefore, given relaxin's well-known role in opposing the profibrotic effects of TGFβ, the lack of effect in the diabetes model is perhaps not surprising.

There is also evidence that relaxin can treat liver fibrosis. In prevention models, relaxin reduced collagen production and promoted MMP in CCl_4-induced hepatic fibrosis (Williams et al., 2001; Bennett et al., 2009). In a more clinically relevant model, relaxin treatment also reduced established liver fibrosis (Bennett et al., 2014). Finally, relaxin reduced portal hypertension in animal models of liver disease (Fallowfield et al., 2014), and a phase 2 trial is currently underway to study the effect of relaxin on portal hypertension in cirrhotic patients (ClinicalTrials.gov ID# NCT02669875).

There is evidence of interplay between relaxin and other signaling pathways (**Figure 4**). It was recently shown that the relaxin receptor RXFP1 can form heterodimers with the

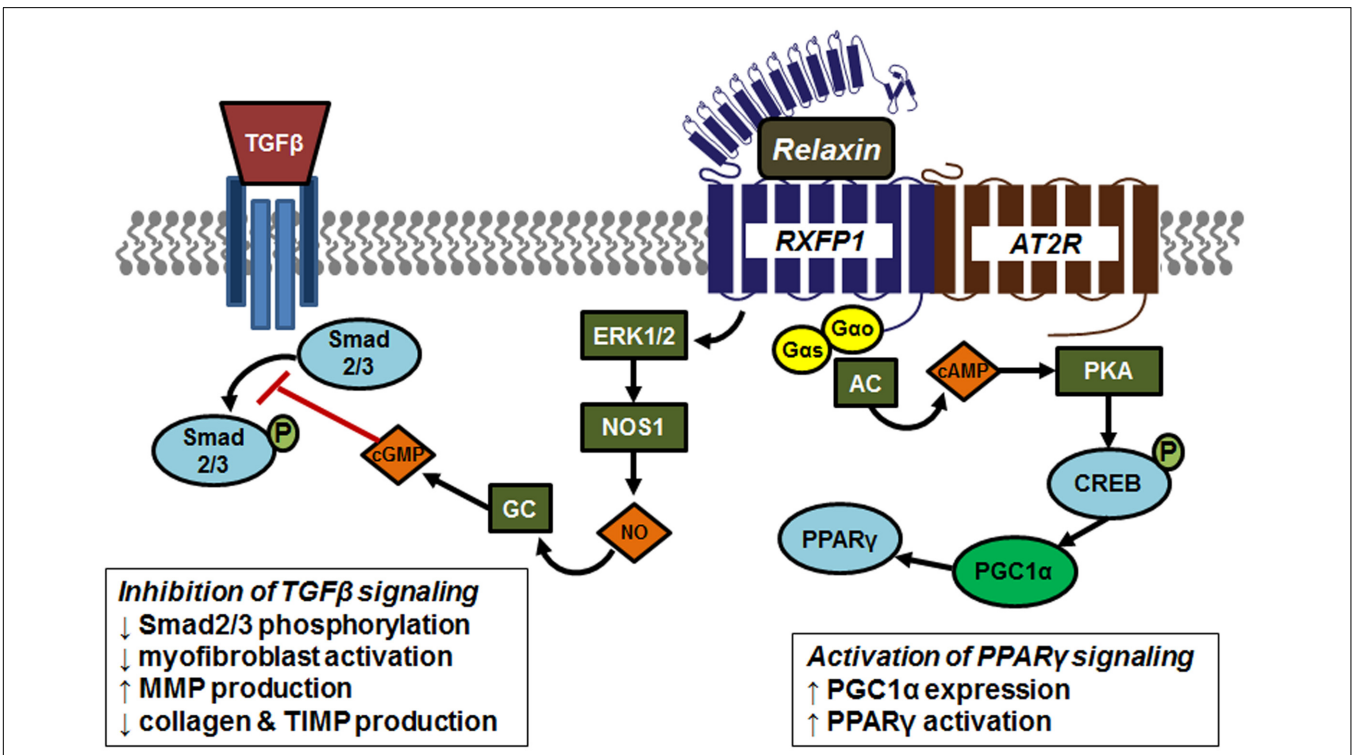

FIGURE 4 | Signaling and crosstalk associated with relaxin activation of its receptor RXFP1. The relaxin receptor RXFP1 can form heterodimers with the angiotensin II type 2 (AT2R), contribute to tissue-protective effects of relaxin. Relaxin antagonizes TGFβ signaling through activation of the protein kinase ERK1, with downstream activation of endothelial nitric oxide synthase (NOS1) resulting in increased nitric oxide (NO) production. This in turn activates soluble guanylyl cyclase (GC) and cGMP production. This pathway inhibits phosphorylation of Smad2/3, resulting in decreased TGFβ signaling. The RXFP1 signaling pathway also involves coupling to Gαs to promote activation of adenylyl cyclase (AC), cAMP production and activation of protein kinase A (PKA). Activated PKA phosphorylates and activates the transcription factor cAMP response element binding protein (CREB) to induce expression of the coactivator protein PPARγ coactivator 1α (PGC1α), which serves to increase PPARγ activity.

angiotensin II type 2 (AT2R), and that renal-protective effects of relaxin required the presence and activity of AT2R (Chow et al., 2014). One major mechanism for relaxin's antifibrotic effects is by antagonism of TGFβ signaling (Samuel et al., 2016a,b). Relaxin binding to RXFP1 results in activation of the protein kinase ERK1, with downstream activation of endothelial nitric oxide synthase (eNOS, or NOS1) and increased nitric oxide production. This in turn activates soluble guanylyl cyclase and cGMP production. This pathway has been shown to inhibit the phosphorylation of Smad2/3, resulting in decreased TGFβ signaling (Chow et al., 2012). An additional RXFP1 signaling pathway results from coupling to Gαs to promote activation of adenylyl cyclase, cAMP production and activation of protein kinase A (PKA). It was recently shown that this pathway, through phosphorylation of the transcription factor cAMP response element binding protein (CREB), promotes expression of the coactivator protein PPARγ coactivator 1α (PGC1α), which serves to increase PPARγ activity, providing potential cross-talk between these two antifibrotic pathways (Singh et al., 2015; Singh and Bennett, 2010). Relaxin activation of PPARγ activity has also been detected in brain arterioles (Chan and Cipolla, 2011; Chan et al., 2013). In support of this concept, a recent study showed that relaxin enhanced the response of other airway dilators, including the PPARγ agonist rosiglitazone (Lam et al., 2016).

SUMMARY AND PERSPECTIVE

The current options for the treatment of fibrotic diseases are extremely limited, and to date no effective drug has emerged that successfully targets established fibrosis. The four avenues of potential treatments discussed here show considerable progress, but to date have not translated to clinical treatment. Useful antifibrotic therapies must be effective against not only against reducing the excess collagen accumulation, but also accommodate collagen degradation. Ideally, the agents should have oral bioavailability. This presents challenges to peptide-based treatments, such as BMP7 or relaxin, and novel small molecule receptor agonists may hold the key to future therapeutics in these areas. In addition, and perhaps most importantly, off-target effects should be minimized, which might be overcome with nanoformulated preparations of the drugs. The novel agents provided in this review show promise as potential future treatments for fibroses, but more work is needed to determine if they will be ultimately translated to human disease.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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A Clinical Perspective of Anti-Fibrotic Therapies for Cardiovascular Disease

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Cardiac fibrosis are central to various cardiovascular diseases. Research on the mechanisms and therapeutic targets for cardiac fibrosis has advanced greatly in recent years. However, while many anti-fibrotic treatments have been studied in animal models and seem promising, translation of experimental findings into human patients has been rather limited. Thus, several potential new treatments which have shown to reduce cardiac fibrosis in animal models have either not been tested in humans or proved to be disappointing in clinical trials. A majority of clinical studies are of small size or have not been maintained for long enough periods. In addition, although some conventional therapies, such as renin-angiotensin-aldosterone system (RAAS) inhibitors, have been shown to reduce cardiac fibrosis in humans, cardiac fibrosis persists in patients with heart failure even when treated with these conventional therapies, indicating a need to develop novel and effective anti-fibrotic therapies in cardiovascular disease. In this review article, we summarize anti-fibrotic therapies for cardiovascular disease in humans, discuss the limitations of currently used therapies, along with possible reasons for the failure of so many anti-fibrotic drugs at the clinical level. We will then explore the future directions of anti-fibrotic therapies on cardiovascular disease, and this will include emerging anti-fibrotics that show promise, such as relaxin. A better understanding of the differences between animal models and human pathology, and improved insight into carefully designed trials on appropriate end-points and appropriate dosing need to be considered to identify more effective anti-fibrotics for treating cardiovascular fibrosis in human patients.

Keywords: cardiac fibrosis, anti-fibrotic therapies, clinical trials, diffuse fibrosis, cardiac magnetic resonance imaging, collagen turnover markers, diastolic function

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INTRODUCTION

Cardiac fibrosis is a hallmark of various cardiovascular disease such as hypertension, myocardial infarction (MI), and ischemic, dilated, and hypertrophic cardiomyopathies. Cardiac fibrosis not only leads to cardiac diastolic dysfunction, but is also a major determinant of malignant arrhythmias and end-stage systolic heart failure and consequently, increases the risk of cardiac death. There are two types of fibrosis: regional fibrosis (reparative fibrosis, scarring from MI) and diffuse fibrosis (reactive fibrosis, interstitial fibrosis in response to different stimuli). Cardiac fibrosis can be definitively diagnosed by endomyocardial biopsies, but they are an invasive evaluation only representative in diffuse fibrosis. Circulating biomarkers, particularly collagen turnover markers, are widely used to noninvasively assess cardiac fibrosis, however they are not reliable and unable to differentiate regional fibrosis from diffuse fibrosis. Cardiac magnetic

resonance imaging (CMR) is an emerging technique to accurately and noninvasively evaluate regional and diffuse cardiac fibrosis by late gadolinium enhancement and post-contrast myocardial longitudinal relaxation time (T1) mapping, respectively, but CMR is expensive and not easily accessible. Functional consequences of cardiac fibrosis, particularly impaired left ventricular (LV) relaxation and heart failure, are also potential, albeit nonspecific, markers of fibrosis.

It is known that a complex interaction involving a network of growth factors/cytokines/hormones and fibroblasts and other cell types (such as cardiomyocytes, monocytes, lymphocytes) is responsible for initiating and maintaining fibrotic response (Kong et al., 2014). In addition to resident fibroblasts, fibroblasts originate from circulating pecursors, sometimes termed fibrocytes (Fang et al., 2013), endothelial cells or epithelial cells (Kong et al., 2014). Renin-angiotensin-aldosterone system (RAAS), growth factors [such as transforming growth factor (TGF)-β], endothelin, matricellular proteins (such as connective tissue growth factor (CTGF) and proinflammatory factors (such as tumor necrosis factor (TNF-α), interleukin (IL)-6 and IL-1) are some of the best studied mediators implicated in cardiac fibrosis (Kong et al., 2014). Recently, new mediators with therapeutic potential of cardiac fibrosis have been emerging such as cardiotrophin-1, galectin, and miRNAs etc. (Fang et al., 2015; Heymans et al., 2015). Although many anti-fibrotic therapies on cardiac fibrosis seem promising in experimental models, clinical data are limited and mixed. Most of new anti-fibrotic therapies have not been evaluated in patients. Some clinical data have demonstrated benefits on cardiac fibrosis mainly with RAAS inhibitors, but most clinical trials on anti-fibrotic drugs are disappointing. This review will summarize findings from clinical trials of anti-fibrotic therapies on cardiac fibrosis (**Table 1**) and discuss the discrepancy between animal research and clinical trials as well as future directions.

ANTI-FIBROTIC THERAPIES ON CARDIAC FIBROSIS IN CLINICAL TRIALS

RAAS Inhibitors

The first family of anti-fibrotic drugs are inhibitors of angiotensin II. Angiotensin II interacts with angiotensin II type I receptors, which stimulates fibroblast proliferation, and increases collagen synthesis (Kong et al., 2014). Several clinical studies have shown that both angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers reduce cardiac fibrosis in patients independent of their antihypertensive effects. In hypertensive patients, endomyocardial biopsies at baseline and 6 months revealed a decrease of collagen volume fraction (CVF) only in the group treated with lisinopril ($n = 18$), but not with hydrochlorothiazide ($n = 17$) (Brilla et al., 2000). A comparison between losartan ($n = 21$) and amlodipine ($n = 16$) given for 1 year in hypertensive patients revealed that only losartan significant decreased both CVF (by endomyocardial biopsies) and the carboxy-terminal peptide of procollagen type I (PICP) (López et al., 2001). Another study demonstrated that in patients with hypertensive heart disease, losartan treatment for 12 months decreased CVF (by endomyocardial biopsies) and LV chamber stiffness in patients with severe fibrosis ($n = 7$), but not in those with nonsevere fibrosis ($n = 12$) (Díez et al., 2002). In patients with end-stage renal disease, losartan ($n = 13$) more effectively suppressed cardiac fibrosis than did enalapril ($n = 13$) or amlodipine ($n = 13$) (Shibasaki et al., 2005). Another small study showed attenuation of progression of cardiac fibrosis with losartan in patients with nonobstructive hypertrophic cardiomyopathy (Shimada et al., 2013). Treatment with candesartan for 24 months also reduced the amino-terminal peptide of type III procollagen (PIIINP) in patients with atrial fibrillation (Kawamura et al., 2010).

The mineralo-corticoid receptor antagonists, spironolactone and eplerenone, also have anti-fibrotic effects in humans. Additional treatment of spironolactone for 6 months improved LV diastolic function and decreased PICP and PIIINP in 80 patients with metabolic syndrome treated with angiotensin II inhibition (Kosmala et al., 2011). In another study of 113 patients with obesity and mild LV diastolic dysfunction, spironolactone treatment for 6 months improved myocardial deformation and decreased PICP and PIIINP (Kosmala et al., 2013). In 44 patients with diastolic heart failure, eplerenone reduced PIIINP at 12 months after treatment, associated with modest improvement of diastolic function (Mak et al., 2009). Similar findings were made in another study showing that eplerenone reduced the amino-terminal peptide of type I procollagen (PINP) and PICP in 44 patients with heart failure with preserved ejection fraction (Deswal et al., 2011).

Although the above clinical studies have shown that RAAS inhibitors reduces cardiac fibrosis in humans, the study population in these studies is rather small. Furthermore, inhibition of RAAS only modestly regresses cardiac fibrosis. Cardiac fibrosis persists in heart failure patients even when treated as recommended by the official guidelines (Querejeta et al., 2004). Thus, there is a compelling need to develop novel and effective anti-fibrotic therapies in cardiovascular disease.

Inflammation Modulators

Inflammatory modulation might have beneficial effects on cardiac fibrosis and heart failure since inflammation is involved in the formation and progression of cardiac fibrosis. TNF-α plays an important role in cardiac fibrosis. However, the RENEWAL study (Mann et al., 2004) which examined the effect of TNF-α antagonist etanercept in patients with heart failure was negative. Additionally, the ATTACH trial was stopped prematurely as the high dose of the TNF-α antagonist infliximab increased all-cause mortality in patients with moderate-to-severe chronic heart failure (Chung et al., 2003). The finding that TNF receptor 1 and 2 exert opposing effects on cardiac remodeling may partly explain that direct blockade of one inflammatory actor could cause these unexpected clinical results (Hamid et al., 2009).

Statins possess potent anti-inflammatory effects and are widely used in cardiovascular disease. Rosuvastatin attenuated cardiac fibrosis in animal models (Chang et al., 2009), which is supported by a small clinical study showing that statin therapy for 6 months reduced PIIINP in heart failure population ($n = 56$) (Abulhul et al., 2012). However, two large-scale clinical

TABLE 1 | Anti-fibrotic therapies on cardiac fibrosis in clinical trials.

Study	Agent	Length of treatment	Patient included (*n*)	Main findings
RAAS INHIBITORS				
Brilla et al., 2000	Lisinopril	6 months	35	Lisinopril but not hydrochlorothiazide decreased CVF in hypertensive patients.
López et al., 2001	Losartan	12 months	37	Losartan but not amlodipine decreased CVF and PICP in hypertensive patients.
Díez et al., 2002	Losartan	12 months	19	Losartan decreased CVF and LV chamber stiffness in hypertensive patients with severe fibrosis, but not in those with nonsevere fibrosis.
Shibasaki et al., 2005	Losartan	6 months	39	Losartan more effectively suppressed cardiac fibrosis than enalapril or amlodipine in patients with end-stage renal disease.
Shimada et al., 2013	Losartan	12 months	20	Losartan attenuated the progression of cardiac fibrosis in patients with nonobstructive hypertrophic cardiomyopathy.
Kawamura et al., 2010	Candesartan	24 months	153	Candesartan reduced PIIINP in patients with atrial fibrillation.
Kosmala et al., 2011	Spironolactone	6 months	80	Additional spironolactone decreased PICP and PIIINP in patients with metabolic syndrome.
Kosmala et al., 2013	Spironolactone	6 months	113	Spironolactone improved myocardial deformation and decreased PICP and PIIINP in patients with obesity and mild LV diastolic dysfunction.
Mak et al., 2009	Eplerenone	12 months	44	Eplerenone reduced PIIINP and modestly improved diastolic function in patients with diastolic heart failure.
Deswal et al., 2011	Eplerenone	6 months	44	Eplerenone reduced PINP and PICP in patients with heart failure with preserved ejection fraction.
INFLAMMATION MODULATORS				
RENEWAL	Etanercept	24 weeks	2,048	The study ruled out a clinically relevant benefit of etanercept on the rate of death or hospitalization due to chronic heart failure in patients with heart failure.
ATTACH	Infliximab	At 0, 2, 6 weeks	150	High dose of infliximab increased all-cause mortality in patients with moderate-severe heart failure.
Abulhul et al., 2012	Atorvastatin	6 months	56	Atorvastatin reduced PIIINP in heart failure patients.
CORONA	Rosuvastatin	32.8 months	5,011	Rosuvastatin did not reduce the primary outcome or the number of deaths from any cause in older patients with systolic heart failure.
GISSIF-HF	Rosuvastatin	3.9 years	4,574	Rosuvastatin daily did not affect clinical outcomes in patients with chronic heart failure of any cause.
UNIVERSAL	Rosuvastatin	6 months	86	Rosuvastatin did not beneficially alter parameters of LV remodeling in patients with chronic systolic heart failure.
TGF-β INHIBITORS				
PRESTO	Tranilast	1, or 3 months	11,484	Tranilast did not improve the quantitative measures of restenosis (angiographic and intravascular ultrasound) or its clinical sequelae in patients receiving successful percutaneous coronary intervention.
ENDOTHELIN INHIBITORS				
Sütsch et al., 1998	Bosentan	2 weeks	36	Bosentan improved systemic and pulmonary hemodynamics in heart failure patients who were symptomatic with standard triple-drug therapy.
EARTH	Darusentan	24 weeks	642	Darusentan did not improve cardiac remodeling or clinical outcomes in patients with chronic heart failure.
Prasad et al., 2006	Enrasentan	6 months	72	In asymptomatic patients with LV dysfunction, LVEDVI increased over 6 months with enrasentan compared with enalapril treatment.
SELECTIVE HEART RATE-REDUCING DRUG				
SHIFT	Ivabradine	22.9 month follow up	6,558	Ivabradine improved clinical outcomes in patients with symptomatic heart failure.
SHIFT substudy	Ivabradine	8 month follow up	411	Ivabradine reversed cardiac remodeling in patients with heart failure.
LOOP DIURETICS				
López et al., 2004	Torsemide	8 months	36	Torsemide but not furosemide reduced PICP and CVF in hypertensive patients with symptomatic heart failure.
López et al., 2007	Torsemide	8 months	22	Torsemide but not furosemide decreased PCP in patients with chronic heart failure.
López et al., 2009	Torsemide	8 months	24	Torsemide corrected both lysyl oxidase overexpression and enhanced collagen cross-linking leading to normalization of LV chamber stiffness in patients with heart failure.

(Continued)

TABLE 1 | Continued

Study	Agent	Length of treatment	Patient included (*n*)	Main findings
TORAFIC	Torsemide	8 months	155	In hypertensive patients with chronic heart failure randomized to torsemide or furosemide, there were no difference in PICP levels between the two groups.
CYCLIC GMP-SPECIFIC PHOSPHODIESTERASE TYPE-5A INHIBITOR				
Giannetta et al., 2012	Sildenafil	3 months	59	Sildenafil improved LV contraction parameters and reduced TGF-β and MCP-1 in patients with diabetic cardiomyopathy.
Redfield et al., 2013	Sildenafil	24 weeks	216	Sildenafil did not improve exercise activity in patients with heart failure with preserved ejection fraction.
MATRIX METALLOPROTEINASE INHIBITOR				
PREMIER	PG-116800	90 days	253	PG-11680 did not prevent LV remodeling or improve clinical outcomes 90 days after myocardial infarction.
RELAXIN				
Pre-RELAX-AHF	Relaxin	48 h	234	Relaxin improved dyspnoea and lowered cardiovascular deaths or readmissions due to heart or renal failure at day 60 in patients with acute heart failure.
RELAX-AHF	Serelaxin	48 h	1,161	Serelaxin improved dyspnoea and reduced cardiovascular deaths and all-cause mortality through day 180 in patients with acute heart failure.

CVF, collagen volume fraction; PICP, the carboxy-terminal peptide of procollagen type I; LV, left ventricular; PIIINP, the amino-terminal peptide of type III procollagen; PINP, the amino-terminal peptide of type I procollagen (PINP); PCP, procollagen type I carboxy-terminal proteinase; TGF-β, transforming growth factor-β; MCP-1, monocyte chemoattractant protein-1; LVEDVI, LV end diastolic volume index.

trials, the CORONA(Kjekshus et al., 2007) and GISSIF-HF (Tavazzi et al., 2008) observed a neutral effect of rosuvastatin compared to placebo on major clinical outcomes in heart failure. Beneficial effects of statin on cardiac remodeling were not observed in universal trial either (Krum et al., 2007). A sub-study of universal trial actually showed reduced coenzyme-10 and increased serum collagen markers in the statin-treated group (Ashton et al., 2011). So, statin's effect on cardiac fibrosis in human are generally disappointing. Peroxisome proliferator-activated receptor (PPAR) agonists have anti-inflammation properties. Preclinical data showed that PPAR-α agonist inhibited cardiac fibrosis and improved cardiac function (Ogata et al., 2004). However, considerable controversy exists on the cardiac safety profile of PPAR agonists (Sarma, 2012). Overall, there is lack of effective inflammatory modulators to inhibit cardiac fibrosis in patients. However, the negative results of inflammatory modulators do not necessarily mean the end of inflammatory modulators in cardiac fibrosis. Future studies should identify the crucial actors and their mechanisms of action in the immunopathogenesis of cardiac fibrosis, which is a prerequisite for the development of new inflammatory modulators in patients with cardiac fibrosis. Selective p38 MAPK inhibitors blocking the secretion of TNF-α and decreasing cardiac fibrosis in mice (Westermann et al., 2006) may be a new treatment modality in humans.

TGF-β inhibitors

TGF-β plays a central role in activating cardiac fibrosis and it activates both canonical (ALK/Smad2/3/Smad4) and noncanonical (TAK/p-38/JNK and NOX4/ROS) signaling pathways. Anti-TGF-β antibodies and ALK5 inhibitors attenuated cardiac fibrosis in animal models, but they were associated with adverse cardiovascular effects (Frantz et al., 2008; Engebretsen et al., 2014), suggesting that targeting canonical TGF-β signaling pathway might not be applicable clinically. While TGF-β promotes fibrogenesis, it also inhibits inflammation, suggesting that broad targeting of TGF-β may be problematic. Alternatively, targeting TAK or NOX4 downstream of TGF-β might be viable anti-fibrotic approaches. Clinically, two agents, pirfenidone and tranilast, which inhibit TGF-β and other growth factors (Edgley et al., 2012), have been available. Both pirfenidone and tranilast have been shown to reduce cardiac fibrosis in animal studies (Edgley et al., 2012). However, tranilast was disappointing in the PRESTO study for post-percutaneous transluminal coronary angioplasty restenosis prevention (Holmes et al., 2002). The use of pirfenidone and tranilast also have adverse effects such as liver dysfunction. Now research is being conducted to search for new compounds that could overcome these potential safety concerns. A new compound called FT011 displays improved activity and reduced toxicity compared to tranilast (Zammit et al., 2009), which needs to be investigated in clinical studies.

Endothelin Inhibitors

Endothelin is another important contributor of fibrotic process and bosentan, a dual endothelin receptor subtype A and B antagonist, prevents fibrosis of various organs in animal models (Clozel and Salloukh, 2005). Dual endothelin subtype A and B inhibitors bosentan and macitentan and the ETA inhibitor ambrisentan are approved in the U.S. for the treatment of pulmonary hypertension. An initial small study in human showed that additional administration of bosentan improved systemic and pulmonary hemodynamics in severe heart failure patients receiving conventional treatments including ACE inhibitors (Sütsch et al., 1998). However, most of subsequent clinical trials of endothelin receptor antagonists were negative or neutral (Anand et al., 2004; Prasad et al., 2006). The harmful effects of endothelin receptor antagonists were generally

attributable to enhanced fluid retention, which could be alleviated by early diuretic therapy. However, in general, additional blockade of endothelin may not be beneficial in patients with heart failure or cardiac fibrosis receiving angiotensin inhibitors.

β-Blockers

β-blockers have been demonstrated to prevent cardiac fibrosis and improve survival in a rat model (Kobayashi et al., 2004). A meta-analysis showed that the β-blockers treatment for the patients with heart failure with preserved ejection fraction was associated with a lower risk of all-cause mortality (Liu et al., 2014). However, the mechanisms of β-blockers' benefit on mortality have not been precisely clarified and whether β-blockers attenuate cardiac fibrosis in human remains unknown.

Selective Heart Rate-Reducing Treatment: Ivabradine

Ivabradine is an oral medication that provides selective heart rate reduction by inhibiting the f-channel. A large trial SHIFT showed that over a median follow-up of 22.9 months, ivabradine significantly reduced cardiovascular death or hospital admission for worsening heart failure in patients with symptomatic heart failure with an LV ejection fraction $\leq$35%, and in sinus rhythm with a heart rate of $\geq$70 bpm (Swedberg et al., 2010). An echocardiographic sub-study of SHIFT further found that ivabradine improved both LV end-systolic and end-diastolic volume indexes compared with placebo from baseline to the 8-month follow-up (Tardif et al., 2011). Thus, ivabradine has been introduced in the treatment guidelines for chronic heart failure in patients (McMurray et al., 2012). However, evidence on whether ivabradine attenuates cardiac fibrosis in patients with heart failure is still lacking although ivabradine effectively reduced fibrosis and circulating angiotensin II and aldosterone levels in animal models (Busseuil et al., 2010). Ivabradine could also reduce fibrosis through its inhibitory effects on inflammatory responses and cardiac apoptosis (Becher et al., 2012).

Loop Diuretics: Torsemide

There are three loop diuretics utilized in heart failure patients: furosemide, torsemide, and bumetanide. López et al. reported that torasemide ($n = 19$), but not furosemide ($n = 17$), reduced circulating PICP and myocardial collagen in hypertensive patients with symptomatic heart failure (López et al., 2004). They then found that activation of the enzyme responsible for the cleavage of PICP, procollagen type I carboxy-terminal proteinase (PCP), was also decreased in 22 patients with chronic heart failure taking torasemide (López et al., 2007). They further reported the ability of torsemide to correct both lysyl oxidase overexpression and enhanced collagen cross-linking leading to normalization of LV chamber stiffness in patients with heart failure (López et al., 2009). This was supported by preclinical data showing torsemide's effect on RAAS inhibition including decreasing aldosterone secretion, inhibiting aldosterone receptor and Ang II effects (Buggey et al., 2015). However, in the TORAFIC study, a multi-center study of 155 hypertensive patients with chronic heart failure randomized to torsemide or furosemide, investigators did not find significant differences between the two groups in changes of PICP (Group, 2011). The TORAFIC study's patient population had less severe heart failure and lower baseline serum PICP compared to those in the studies by Lopez and colleagues, which possibly explains the divergent results among these studies. So, it is important to select patients who may benefit from torsemide treatment.

Sildenafil

Sildenafil inhibits cyclic GMP-specific phosphodiesterase type-5A and it has been used to treat idiopathic pulmonary fibrosis. In the first proof-of-concept human study, 59 patients with isolated diabetic cardiomyopathy randomly treated 3 months with sildenafil showed improved LV contraction parameters and reduced TGF-β and monocyte chemoattractant protein-1, when compared with controls (Giannetta et al., 2012). However, among patients with heart failure with preserved ejection fraction sildenafil for 24 weeks ($n = 113$), compared with placebo ($n = 103$), did not improve exercise capacity or clinical status (Redfield et al., 2013). Notably, fibrosis parameters were not measured in these two studies. The discordant results indicate that the same treatment does not exert similar effects in various cardiovascular disease.

Matrix Metalloproteinase (MMP) Inhibitors

Cardiac fibrosis is associated with activation of MMPs. It has been shown that MMP inhibition attenuates cardiac fibrosis and LV remodeling in experimental models (Heymans et al., 2005; Matsusaka et al., 2006). However, the PREMIER study of an orally active MMP inhibitor, PG-116800, in 253 patients after M failed to prevent LV remodeling or improve clinical outcomes 90 days after MI (Hudson et al., 2006), although it should be noted that no fibrosis parameters were measured in this study.

Relaxin

Relaxin is an intriguing endogenous hormone that is a potent vasodilator with a number of pleiotropic effects. Relaxin inhibits fibrosis through various mechanisms including inhibiting TGF-β and Smad, regulating the balance between MMPs and tissue inhibitors of metalloproteinases, and inhibiting inflammatory response (Samuel et al., 2016). Relaxin has been shown to have anti-fibrotic effects in a range of experimental models of cardiovascular disease including MI (Samuel et al., 2011), fibrotic cardiomyopathy (Samuel et al., 2014), hypertension (Lekgabe et al., 2005), diabetes (Samuel et al., 2008), and atrial fibrillation (Henry et al., 2016). Furthermore, relaxin more effectively ameliorated cardiac fibrosis than enalapril in an experimental model of fibrotic cardiomyopathy and relaxin in combination with enalapril augmented the anti-fibrotic efficacy of enalapril (Samuel et al., 2014). However, relaxin is not universally beneficial in cardiac fibrosis since relaxin did not affect pressure overload-induced cardiac fibrosis that was associated with biochemical wall stress rather than elevated TGF-β1 levels (Xu et al., 2008). The beneficial effects of a single 48-h infusion of relaxin in the acute heart failure trials (Teerlink et al., 2009, 2013) has led to great interest in its clinical application in in human disease. Although anti-fibrotic effects of relaxin

is well characterized in various experimental models, clinical trials have failed in patients with other fibrotic conditions such as scleroderma (Khanna et al., 2009). The negative results of clinical trials of relaxin have pointed to the challenges previously underscored. First, relaxin has short *in vivo* half-life and it is costly to produce. The relatively short duration of relaxin treatment have partly contributed to the failed clinical trials since fibrosis is a slow process in human. Second, it is important to know the expression of relaxin receptors in different tissues and organs and to understand tissue competence to respond to relaxin along with signaling pathways. Nevertheless, relaxin still holds great potential as a therapy for cardiac fibrosis associated with various cardiovascular disease.

CHALLENGES AND FUTURE DIRECTIONS

The failure of many clinical trials on anti-fibrotic drugs indicates that extrapolating research data from animal models to human requires caution since there are significant species differences in physiology and genetics between animals and human. Compared to mice, fibrosis is a slower condition in humans, which takes decades to develop and require long-term treatment to diminish its progression. Furthermore, many animal models of diseases do not mimic various clinical settings, thus controversial results are likely to be obtained since there are different signaling pathways and mechanisms in cardiac fibrotic processes in various diseases. In addition, the animals used are normally young while patients with cardiac fibrosis are at a more advanced age. In order to improve clinical translation, it is important to design, conduct and analyse animal experiments properly and to summarize data from animal research adequately before conducting clinical trials. Moreover, the failure of previous trials also emphasizes the need for optimal design of future clinical trials including a selection of suitable patients, appropriate dose, and route and timing and length of administration.

There are some important areas for future research in this field. First, although CMR is too expensive to be used in large populations, it should be used to investigate potential new treatments with relatively small group sizes since it allows accurate assessment of regional and diffuse fibrosis. Second, anti-fibrotic therapies targeting downstream signaling pathways may improve safety and efficacy of current treatments such as TGF-β inhibitors. In addition to their role in fibrosis, many proteins are involved in other biological processes. Thus, developing more specific agents targeting fibrotic signaling pathways is likely to be beneficial to minimize potential side effects. Third, combined anti-fibrotic therapies seem more effective than single drug treatment. It is shown that spironolactone or relaxin in combination with angiotensin II inhibitors augmented anti-fibrotic efficacy (Kosmala et al., 2011; Samuel et al., 2014). Combined anti-fibrotic agents with different mechanisms of actions is likely to exert better effects on cardiac fibrosis.

CONCLUSION

Although many fibrotic therapies on cardiac fibrosis are promising in preclinical models, clinical translation is limited. There is still a lack of effective treatments to regress cardiac fibrosis in patients with various cardiovascular disease. Future optimally designed clinical studies are required to test new potential treatments and currently available drugs with improved safety and efficacy after adequate analysis of evidence from animal research.

AUTHOR CONTRIBUTIONS

LF was responsible for assembling and drafting of the manuscript. AM and AD contributed to the drafting of the manuscript.

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