ADVANCES IN MECHANISMS OF RENAL FIBROSIS

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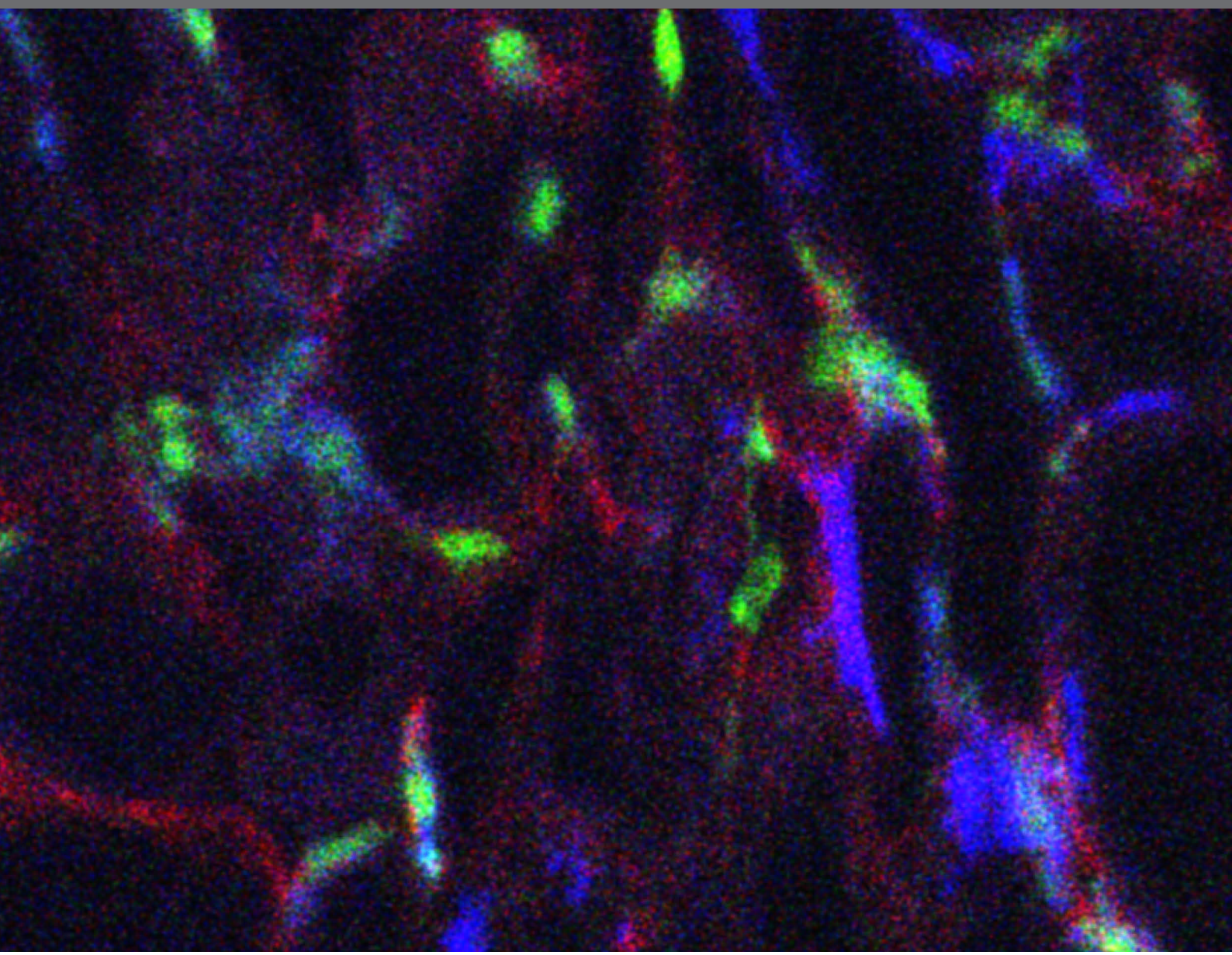

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ADVANCES IN MECHANISMS OF RENAL FIBROSIS

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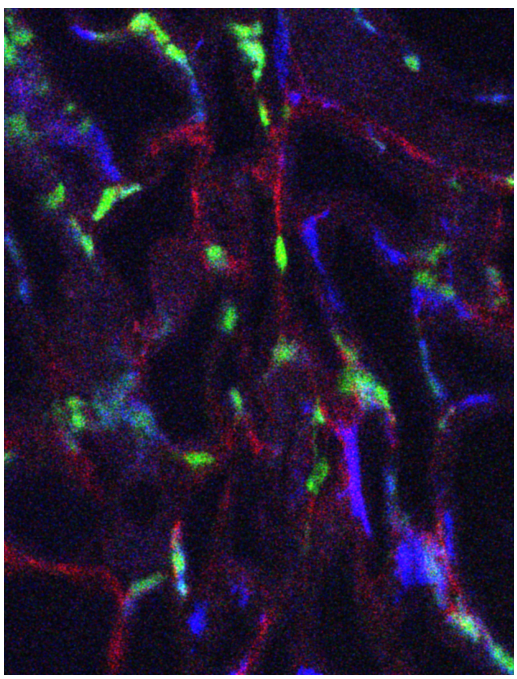

Alpha-smooth muscle-possitive myofibroblasts (red) are derived from bone-marrow macrophages identified by co-expressing GFP (green) and F4/80 (blue) antigens via the process of macrophage-to-myofibroblast transition (MMT) in the fibrotic kidney of obstructive nephropathy induced in GFP chimeric mice.

Image credit: Professor Hui Yao Lan, MD, PhD, Chinese University of Hong Kong

Scarring of the glomerular and tubulointerstitial compartments is a hallmark of progressive kidney disease. Renal fibrosis involves a complex interplay between kidney cells, leukocytes and fibroblasts in which transforming growth factor-ß (TGF-ß) plays a key role. This eBook provides a comprehensive update on TGF-ß signalling pathways and introduces a range of cellular and molecular mechanisms involved in renal fibrosis both upstream and downstream of TGF-ß. The wide variety of potential new targets described herein bodes well for the future development of effective therapies to tackle the major clinical problem of progressive renal fibrosis.

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EDITORIAL
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Editorial: Advances in Mechanisms of Renal Fibrosis

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Keywords: BMP7, fibroblast, HIPK2, JNK, miRNA, Smad, TGF-beta

Editorial on the Research Topic

Advances in Mechanisms of Renal Fibrosis

Scarring of the glomerular and tubulointerstitial compartments is a hallmark of progressive kidney disease and is considered a common pathway leading to end-stage of renal failure. Renal fibrosis involves a complex interplay between intrinsic kidney cells, leukocytes, and fibroblasts in which transforming growth factor-β (TGF-β) plays a key role. Inhibition of TGF-β1 suppresses renal fibrosis in a number of animal models; however, TGF-β1 is also a negative regulator of the immune response so that targeting this key factor has been a difficult proposition. Much effort has focused on how TGF-β1 promotes renal fibrosis to identify other steps that can be targeted safely. The articles in this eBook describe advances in our understanding in the mechanisms of renal fibrosis that operate upstream and downstream of TGF-β/Smad signaling.

TGF-β signals via canonical (Smad-based) and non-canonical (non-Smad based) pathways (Meng et al.). An important development is the demonstration that Smad2 and Smad3 exert opposing roles in renal fibrosis, with Smad2 being anti-fibrotic and Smad3 pro-fibrotic. This provides potential avenues for manipulating the Smad2/3 balance within Smad2/3/4 complexes to alter the outcome of TGF-β/Smad signaling. Two other proteins can suppress canonical TGF-β/Smad signaling. First, Smad7 has a negative feed-back role in TGF-β/Smad signaling. Genetic strategies to over-express Smad7 in mice inhibits renal fibrosis with the challenge being how to translate these proof of principle studies into the clinic (Meng et al.). Second, bone morphogenetic protein 7 (BMP7) is a member of the TGF-β super family which exerts anti-fibrotic effects in many models of renal fibrosis (Li et al.). This is attributed to counterbalancing the pro-fibrotic effects of TGF-β such as reducing collagen formation, increasing matrix degradation and inactivating matrix-producing cells. However, the promise of recombinant BMP7 as an anti-fibrotic therapy has yet to be established in the clinic (Li et al.).

MECHANISMS UPSTREAM OF TGF-β SIGNALING

Several mechanisms are described in this eBook which operate upstream of TGF-β/Smad signaling. Homeodomain-interacting-protein kinase 2 (HIPK2) was identified as an important regulator of inflammation and fibrosis in a mouse model of HIV-associated nephropathy acting upstream of both NF-kB and TGF-β signaling (Nugent et al.). *Hipk2* deficient mice are viable and show protection against renal fibrosis in animal models. However, the clinical use of HIPK2 inhibitors may be problematic since HIPK2 also functions as a tumor suppressor and loss of HIPK2 may lead to neurodegenerative disease.

A mainstay of therapy for chronic kidney disease is inhibition of the production or action of angiotensin II (Ang II). Indeed, Ang II is an inducer of TGF-β production and activation. Further

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opportunities for targeting renal fibrosis are evident in the non-classical renin-angiotensin system (RAS), including the ACE2/Ang(1–7)/Mas receptor axis, the (pro)renin receptor, and the Ang A/alamandine/MrgD axis (Lv and Liu). The challenge is to understanding the precise role of these non-classical members of the RAS in renal fibrosis and in selecting the most effective therapeutic strategies.

Stress-induced activation of the c-Jun amino terminal kinase (JNK) pathway in cells of the glomerular and tubulointerstitial compartments is a common feature of chronic kidney disease (Grynberg et al.). Pharmacologic inhibition of JNK suppresses inflammation, fibrosis, and apoptosis in several models of renal fibrosis. JNK signaling acts to increase TGF-β1 expression, to promote activation of latent TGF-β1, and to promote transcription of pro-fibrotic molecules via direct phosphorylation of the linker region of Smad3. However, a lack of efficacy of JNK inhibitors in models of diabetic nephropathy and the recent failure of JNK inhibition in a trial of idiopathic pulmonary fibrosis have raised questions regarding how best to target this pro-fibrotic mechanism (Grynberg et al.).

Histone deacetylases (HDAC) are a group of enzymes that induce deacetylation of both histone and non-histone proteins and thereby modify many cellular functions. Pan- or class-specific HDAC inhibitors can suppress the activation and proliferation of cultured renal fibroblasts and attenuate renal fibrosis in animal models (Liu and Zhuang). While clinical trials of HDAC inhibitors are progressing in cancer, this has yet to be reported for kidney disease. A major challenge is whether chronic inhibition of one or more HDAC enzymes can suppress renal fibrosis without significant side-effects of enzyme inhibition.

MECHANISMS DOWNSTREAM OF TGF-β SIGNALING

Substantial progress has been made in identifying miRNA molecules which regulate TGF-β/Smad3 induced fibrosis. In particular, TGF-β promotes fibrosis by increasing levels of miR-21, miR-433, and miR-192 which amplify TGF-β signaling and promote de-differentiation of tubular epithelial cells (Chung and Lan). In addition, TGF-β signaling reduces levels of the miR-29 and miR-200 families which protect against renal fibrosis. The delivery of modified oligonucleotides or plasmid-based expression constructs to up- or down-regulate miRNA levels in tissues is an active area of clinical research, particularly in cancer, although many issues will need to be addressed before chronic administration of such reagents can be performed in fibrotic kidney disease (Chung and Lan).

Studies by Yan et al. have described the recruitment of bone marrow-derived fibroblasts in models of tubulointerstitial fibrosis. In response to injury, tubular epithelial cells release chemokines (CCL21/CXCL16/CCL2) and TGF-β1. These chemokines recruit monocytes and fibrocytes from the circulation while TGF-β1 plus other factors, such as adiponectin and Jak3/STAT6 signaling, induce transition of these cells into collagen producing myofibroblasts thereby promoting renal fibrosis (Yan et al.). These findings identify several potential therapeutic targets to inhibit the recruitment and activation of bone marrow cells in the injured kidney.

Finally, an exciting new finding based on careful lineage tracing studies is the identification of a population of renal erythyropoietin-producing (REP) cells as a significant source of collagen producing interstitial myofibroblasts (Souma et al.). Hypoxia activates fibroblasts in culture and hypoxia is a common feature in renal fibrosis. REP cells represent a direct mechanism by which hypoxia induces myofibroblast transition of intrinsic renal fibroblast-like cells. What is particularly interesting is the finding that such transformed REP cells can recover their original physiological properties upon resolution of hypoxia.

In conclusion, these articles provide a detailed description of both the key TGF-β/Smad signaling pathway as well as other mechanisms involved in renal fibrosis both upstream and downstream of TGF-β. The wide variety of potential new targets described herein bodes well for the future development of effective therapies to tackle the major clinical problem of progressive renal fibrosis.

AUTHOR CONTRIBUTIONS

DN-P and HL reviewed all the papers included in the Research Topic of Frontiers in Renal and Epithelial Physiology and summarized in the Editorial their main findings, together with a commentary on the current knowledge about renal fibrosis.

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REVIEW
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TGF-β/Smad signaling in renal fibrosis

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TGF-β (transforming growth factor-β) is well identified as a central mediator in renal fibrosis. TGF-β initiates canonical and non-canonical pathways to exert multiple biological effects. Among them, Smad signaling is recognized as a major pathway of TGF-β signaling in progressive renal fibrosis. During fibrogenesis, Smad3 is highly activated, which is associated with the down-regulation of an inhibitory Smad7 via an ubiquitin E3-ligases-dependent degradation mechanism. The equilibrium shift between Smad3 and Smad7 leads to accumulation and activation of myofibroblasts, overproduction of ECM (extracellular matrix), and reduction in ECM degradation in the diseased kidney. Therefore, overexpression of Smad7 has been shown to be a therapeutic agent for renal fibrosis in various models of kidney diseases. In contrast, another downstream effecter of TGF-β/Smad signaling pathway, Smad2, exerts its renal protective role by counter-regulating the Smad3. Furthermore, recent studies demonstrated that Smad3 mediates renal fibrosis by down-regulating miR-29 and miR-200 but up-regulating miR-21 and miR-192. Thus, overexpression of miR-29 and miR-200 or down-regulation of miR-21 and miR-192 is capable of attenuating Smad3-mediated renal fibrosis in various mouse models of chronic kidney diseases (CKD). Taken together, TGF-β/Smad signaling plays an important role in renal fibrosis. Targeting TGF-β/Smad3 signaling may represent a specific and effective therapy for CKD associated with renal fibrosis.

Keywords: TGF-ß, Smads mediators, renal fibrosis, therapeutics, mechanisms

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Introduction

The TGF-β superfamily consists of highly pleiotropic molecules including activins, inhibins, BMPs (Bone morphogenic proteins), GDFs (Growth differentiation factors) and GDNFs (Glial-derived neurotrophic factors), and exerts multiple biological functions in renal inflammation, fibrosis, cell apoptosis, and proliferation (Massague and Wotton, 2000). Among them, TGF-β is known as a key pro-fibrotic mediator in fibrotic diseases. Three isoforms of TGF-β have been identified in mammals, termed TGF-β1, 2, and 3, of which TGF-β1 is the most abundant isoform and can be produced by all types of renal resident cells. After synthesis, TGF-β1 is released in association with LAP (latency-associated peptide) as a latent form of TGF-β1 which binds to LTBP (Latent TGF-β-binding protein) in the target tissues. When exposed to multiple types of stimuli, including ROS (Reactive oxygen species), plasmin and acid (Lyons et al., 1990; Munger et al., 1999; Meng et al., 2013), TGF-β1 can be released from the LAP and LTBP and becomes active. The active TGF-β1 then binds to TβRII (Type II TGF-β receptor), a constitutively active kinase, which recruits TβRI

(Type I TGF-β receptor) and phosphorylates the downstream receptor-associated Smads (R-Smads) i.e., Smad2 and Smad3 (Wrana et al., 1994). Then the phosphorylated Smad2 and Smad3 form an oligomeric complex with a common Smad, Smad4, and translocates into the nucleus to regulate the transcription of target genes in collaboration with various co-activators and co-repressors. It is interesting that an inhibitory Smad, Smad7, can be induced in a Smad3-dependent manner. Smad7 consequently competes with the R-Smads for binding to the activated receptors, in order to exert its negative effect on TGF-β/Smad signaling (Shi and Massague, 2003). Additionally, TGF-β1 is able to function through the Smad-independent pathways, including p38, ERK (Extracellular-signal-regulated kinase), MAPK, Rho-GTPases, Rac, Cdc42, ILK (Integrin linked kinase) (Attisano and Wrana, 2002; Derynck and Zhang, 2003; Li et al., 2009; Loeffler and Wolf, 2014). In this review, we focus on the pathological roles of TGF-β/Smad signaling in renal fibrosis.

Role of TGF-β1 in Renal Fibrosis

Renal fibrosis, characterized by excessive deposition of ECM (Extracellular matrix), is recognized as a common pathological feature of CKD (Chronic kidney diseases) which leads to the development of ESRD (End-stage renal disease), accompanied by a progression of renal malfunctions (Eddy and Neilson, 2006). Although effective therapy for renal fibrosis is still lacking, a number of studies demonstrated that TGF-β is the key mediator in CKD associated with progressive renal fibrosis. It is well documented that TGF-β1 has multiple biological properties including cell proliferation, differentiation, apoptosis, autophagy, production of ECM, etc. (Meng et al., 2013). Considerable evidence revealed that TGF-β is substantially upregulated in the injured kidney on both patients and animal disease models (Yamamoto et al., 1996; Bottinger and Bitzer, 2002). It is also showed that the urinary levels of TGF-β are significantly increased in patients with various renal diseases, which is positively correlated with the degree of renal fibrosis (Murakami et al., 1997). Moreover, the importance of TGF-β1 in renal fibrosis is further supported by the findings that overexpression of active TGF-β1 in rodent liver is capable of inducing the fibrotic response in kidney; whereas blocking TGF-β with neutralizing antibody, antisense oligonucleotides, inhibitors, or genetic deletion of receptors can attenuate kidney fibrosis *in vivo* and *in vitro* (Sanderson et al., 1995; Kopp et al., 1996; Border and Noble, 1998; Moon et al., 2006; Petersen et al., 2008; Meng et al., 2012a). In contrast to the active form of TGF-β1, the latent form of TGF-β1 can protect the kidney against fibrosis and inflammation by upregulating Smad7 that is observed in the latent TGF-β transgenic mice received with UUO-induced nephropathy or anti-GBM-induced glomerulonephritis (Huang et al., 2008a,b). Taken together, TGF-β exerts profibrotic effects on the kidney through several possible mechanisms: (1) TGF-β1 directly induces the production of ECM, including collagen I and fibronectin, through the Smad3-dependent or -independent mechanisms (Samarakoon et al., 2012); (2) TGF-β1 suppresses the degradation of ECM by inhibiting MMPs (Matrix metalloproteinases) but inducing TIMPs (Tissue inhibitor of metalloproteinase) and the natural inhibitor of MMPs; (3) TGF-β1 is believed to play critical roles in the transdifferentiation toward myofibroblast of several types of cells, including epithelial cells, endothelial cells, and pericytes, although the origin of myofibroblast is still undefined (Meng et al., 2013; Wu et al., 2013); (4) TGF-β1 acts directly on different types of renal resident cells, for example: it can promote the proliferation of mesangial cells in order to increase matrix production, or induce the elimination of tubular epithelial cells and podocytes which may lead to a deterioration of renal injury and incur more severe renal fibrosis (Bottinger and Bitzer, 2002; Lopez-Hernandez and Lopez-Novoa, 2012) (**Figure 1**).

Role of Smads in Renal Fibrosis

Smad2 and Smad3

It is consistently demonstrated that Smad2 and Smad3 are extensively activated in the fibrotic kidney in patients and animal models with CKD (Meng et al., 2013). Although Smad2 and Smad3 share more than 90% similarity in their amino acid sequences, their functional roles in renal fibrosis are distinct. It is well documented that Smad3 is pathogenic since knockout of Smad3 gene inhibits fibrosis in obstructive nephropathy (Sato et al., 2003), diabetic nephropathy (Fujimoto et al., 2003), hypertensive nephropathy (Liu et al., 2012), and drug-toxicity-related nephropathy (Zhou et al., 2010). Of note, Smad3 promotes renal fibrosis by directly binding to the promoter region of collagens to trigger their production (Vindevoghel et al., 1998; Chen et al., 1999), and inhibiting the ECM degradation via induction of TIMP-1 while reducing MMP-1 activities in fibroblasts (Yuan and Varga, 2001). In contrast to Smad3, Smad2 is unable to directly bind to the genomic DNA (Dennler et al., 1998). Previous study suggested that roles of Smad2 and Smad3 might be different in fibrotic diseases (Piek et al., 2001; Yang et al., 2003b; Phanish et al., 2006). Consistent with the finding that the endogenous ratio of Smad2 and Smad3 may ultimately influence the cytostatic function of Smad3 (Kim et al., 2005), results from our recent study demonstrated that conditional knockout of Smad2 from tubular epithelial cells enhances Smad3-mediated renal fibrosis *in vivo* and *in vitro,* which is associated with the increase in phosphorylation and nuclear translocation of Smad3, promotion of the Smad3 responsive promoter activity, and binding of Smad3 to Col1A2 promoter (Meng et al., 2010).

Smad4

As a common Smad for TGF-β/BMP signaling, Smad4 plays a critical role in nucleocytoplasmic shuttling of Smad2/3 and Smad1/5/8 complexes (Massague and Wotton, 2000). It has been demonstrated that loss of Smad4 in mesangial cells inhibits TGF-β1-induced ECM deposition (Tsuchida et al., 2003), which is further confirmed by our recent finding that specific deletion of Smad4 from renal tubular epithelial cells attenuates the UUO-induced renal fibrosis by suppressing Smad3 responsive promoter activity and decreasing the binding of Smad3 to the target genes independent of its phosphorylation and nuclear translocation (Meng et al., 2012b).

Smad7

As an inhibitory regulator in the TGF-β/Smad signaling pathway, Smad7 can be induced by a Smad3-dependent mechanism,

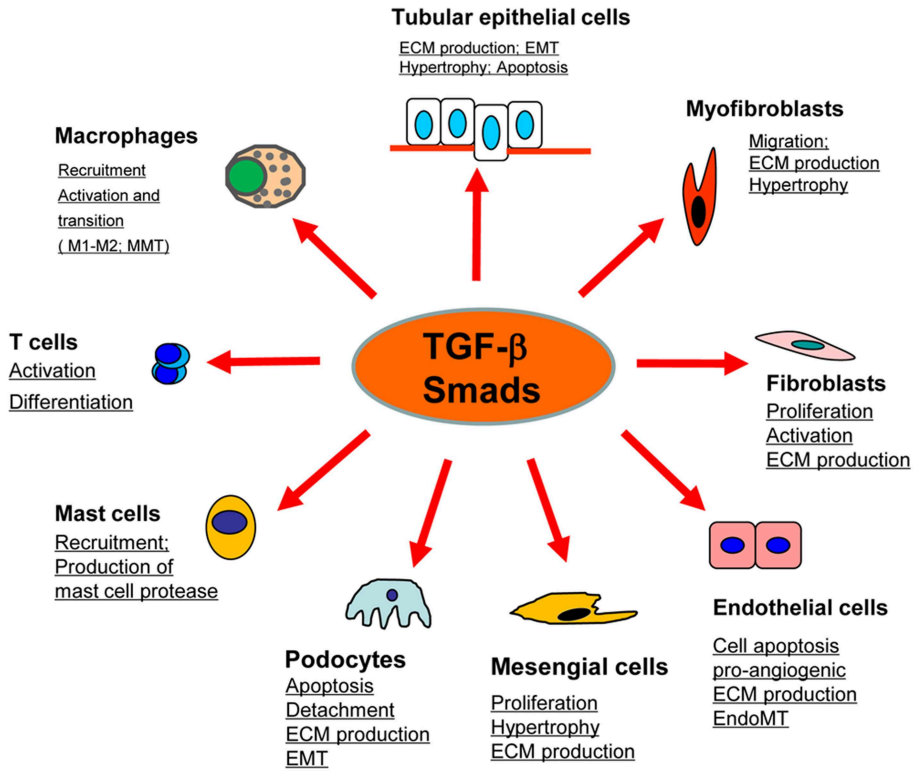

FIGURE 1 | Role of TGF-β/Smad signaling in kidney disease. TGF-β1 signals through the downstream mediators to exert its biological activities on different cell types of kidney cells during renal inflammation and fibrosis.

which in turn blocks the signal transduction of TGF-β1 via its negative feedback loop (Afrakhte et al., 1998; Zhu et al., 1999; Kavsak et al., 2000; Ebisawa et al., 2001). Moreover, the regulatory mechanism of Smad7 on TGF-β signaling occurs in an elegant manner, i.e., TGF-β not only induces Smad7 transcription, but also promotes the degradation of Smad7 by activating the Smad3-dependent Smurfs/arkadia-mediated ubiquitin–proteasome degradation pathway (Kavsak et al., 2000; Ebisawa et al., 2001; Fukasawa et al., 2004; Liu et al., 2008). In this setting, the level of Smad7 protein is significantly reduced in response to the high level of active TGF-β1 in CKD. Most importantly, the functional role of Smad7 is further defined by the findings that deletion of Smad7 accelerates renal fibrogenesis in obstructive nephropathy, diabetic nephropathy as well as hypertensive nephropathy (Chung et al., 2009; Chen et al., 2011; Liu et al., 2013), suggesting Smad7 as a therapeutic agent for treatment of CKD (Lan et al., 2003; Hou et al., 2005; Ka et al., 2007, 2012; Chen et al., 2011; Liu et al., 2014).

Collectively, compelling evidence indicates that hyperactivation of Smad3 associated with progressive degradation of Smad7, is a key feature of renal fibrotic diseases. More importantly, the imbalance of Smad3 and Smad7 was proved to be one of the major mechanisms in mediating the fibrotic response. In this regard, rebalancing the disturbed Smad3/Smad7 ratio, through downregulating Smad3 and upregulating Smad7 simultaneously, seems to be an effective strategy for treatment of renal fibrosis.

Role of TGF-β in Transdifferentiation of Myofibroblasts

Emerging evidence suggests that the accumulation of myofibroblasts, a predominant source for ECM production, is a critical step in the progression of renal fibrosis (Wynn and Ramalingam, 2012). However, the origin of myofibroblast is still controversial. It has been reported that myofibroblasts may be derived from the resident fibroblasts, pericytes, bone marrow cells (e.g., fibrocytes), epithelial cells (Epithelial–mesenchymal transition, EMT), and endothelial cells (Endothelial–mesenchymal transition, EndMT) (Allison, 2013; LeBleu et al., 2013; Meng et al., 2014). Our latest data also revealed that bone marrow-derived macrophages were capable of becoming myofibroblast phenotype via a process of macrophage-myofibroblast transition (MMT) in patients and UUO model with active renal fibrosis (Nikolic-Paterson et al., 2014). In addition, it is generally accepted that local fibroblasts can differentiate into myofibroblast under the stimulation of TGF-β (Evans et al., 2003; Midgley et al., 2013). Increasing evidence indicates that fibrocytes can produce a large amount of collagens directly in response to the stimulus such as TGF-β (Hong et al., 2007; Wada et al., 2007). Administration of TGF-β promotes the transdifferentiation of epithelial cells and endothelial cells toward myofibroblast-like cells, whereas, blockade of TGF-β/Smad signaling with inhibitors or antagonists attenuates or reverses the process of EMT or EndMT (Fan et al.,

1999; Zeisberg et al., 2003, 2008; Li et al., 2010; Liu, 2010; Yang et al., 2010; Xavier et al., 2014). In addition, TGF-β1 can promote renal fibrosis via the cell-cell interaction mechanism as TGF-β1 released from the injured epithelium is able to activate pericyte-myofibroblast transition (Wu et al., 2013). Moreover, we also identify that advanced glycation end products (AGEs) and angiotensin II are capable of activating Smad3 to mediate the process of EMT under diabetes and hypertension conditions (Li et al., 2003, 2004; Wang et al., 2006b; Yang et al., 2009, 2010; Chung et al., 2010b).

Role of TGF-β1/Smad-dependent miRNAs in Renal Fibrosis

Increasing evidence demonstrates that TGF-β1 can also regulate several miRNAs to facilitate renal fibrogenesis. As illustrated in **Figure 2**, TGF-β1 up-regulates miR-21, miR192, miR-377, miR-382, and miR-491-5p, but down-regulates miR-29 and miR-200 families during renal fibrosis (Kantharidis et al., 2011; Kriegel et al., 2012; Lan and Chung, 2012; Chung et al., 2013a,b). In fibrotic kidneys, the level of miR-21 is highly induced (Godwin et al., 2010; Zhong et al., 2011, 2013; Chau et al., 2012; Xu et al., 2012; Wang et al., 2013), whereas inhibition of miR-21 attenuates deposition of ECM and halts the progression of renal fibrosis (Zhong et al., 2011, 2013; Chau et al., 2012). Role of miR-192 in fibrosis is still controversial. It is reported that miR-192 is elevated in fibrotic mouse models and TGF-β1-treated murine cells (Kato et al., 2007; Chung et al., 2010a; Putta et al., 2012). Knockout or knockdown of miR-192 largely attenuated renal fibrosis possibly through induction of ZEB1/2 *in vivo* and *in vitro*.

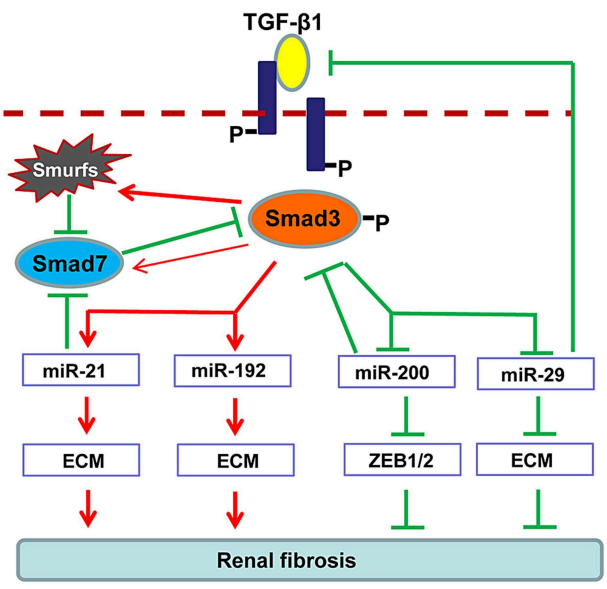

FIGURE 2 | Regulation of TGF-β/Smad3 in fibrosis-related microRNAs during renal fibrosis. TGF-β1 activates Smad3 that binds directly to a number of microRNAs to either negatively or positively regulate their expression and function in renal fibrosis.

However, a recent study indicated that TGF-β1 reduces miR-192 expression in human TECs and deficiency of miR-192 accelerates renal fibrosis in diabetic nephropathy (Krupa et al., 2010), which is further evident by the results from the renal biopsy of diabetic patients with lower level of miR-192 (Wang et al., 2010). The discrepancy in these studies suggests the complexity of miR-192 in renal fibrogenesis. The miR-29 and miR-200 are TGF-β1-dependent anti-fibrotic miRNAs that are extensively suppressed in the diseased kidneys (Qin et al., 2011). Of note, more than 20 ECM-related genes, including collagens, are potential targets for miR-29 where some of them are regulated by the TGF-β signaling (van Rooij et al., 2008; Xiao et al., 2012). Overexpression of miR-29 attenuates renal fibrosis *in vivo* in obstructive and diabetic nephropathies and suppresses the fibrotic genes *in vitro* in response to various stimuli including TGF-β1, high glucose or salt-induced hypertensive conditions (Du et al., 2010; Liu et al., 2010; Qin et al., 2011; Chen et al., 2014). The miR-200 family contains miR-200a, miR-200b, miR-200c, miR-429, and miR-141 (Howe et al., 2012). Downregulation of miR-200a and miR-141 are detected in the fibrotic kidneys of obstructive and diabetic nephropathies (Wang et al., 2011; Xiong et al., 2012). As miR-200 has a major role in maintaining the epithelial differentiation, delivery of miR-200b significantly reduces renal fibrotic response by suppressing the transcriptional repressors of E-cadherin ZEB1 and ZEB2 (Korpal et al., 2008; Oba et al., 2010).

TGF-β/Smad Signaling as a Therapeutic Potential for Renal Fibrosis

General Blockade of TGF-β Signaling

The therapeutic potential of anti-TGF-β1 therapy has been widely tested according to the pathogenic role of TGF-β1 in fibrogenesis. It has been shown that TGF-β neutralizing antibodies, antisense TGF-β oligodeoxynucleotides, soluble human TβRII (sTβRII.Fc) and specific inhibitors to TβR kinases (such as GW788388 and IN-1130) can effectively halt the progression of renal fibrosis in a number of experimental kidney disease models. A recent study also demonstrated that blockade of TGF-β1 receptor posttranslational core fucosylation can attenuate renal interstitial fibrosis (Shen et al., 2013). In addition, some TGF-β inhibitors have been further tested in preclinical or clinical trials (Tampe and Zeisberg, 2014). For an instance, treatment with Pirfenidone, a small molecule that blocks TGF-β1 promoter, can prevent the decline of eGFR (estimated glomerular filtration rate) in patients with focal segmental glomerulosclerosis (FSGS) or diabetic nephropathy (Cho et al., 2007; Sharma et al., 2011). In addition, Fresolimumab and LY2382770, neutralizers for TGF-β1 activity, are also tested in FSGS and diabetic kidney diseases in human (Trachtman et al., 2011; Choi et al., 2012; Tampe and Zeisberg, 2014). However, the major obstacle and risk for these potential therapies by generally blocking TGF-β signaling may be related to the abrogation of its anti-inflammatory and anti-tumorigenesis property. Nevertheless, it should be mentioned that TGF-β1 may also serve as a potential biomarker for renal fibrosis, since significant upregulation of urine TGF-β1 have been detected in progressive renal diseases (Tsakas and Goumenos, 2006).

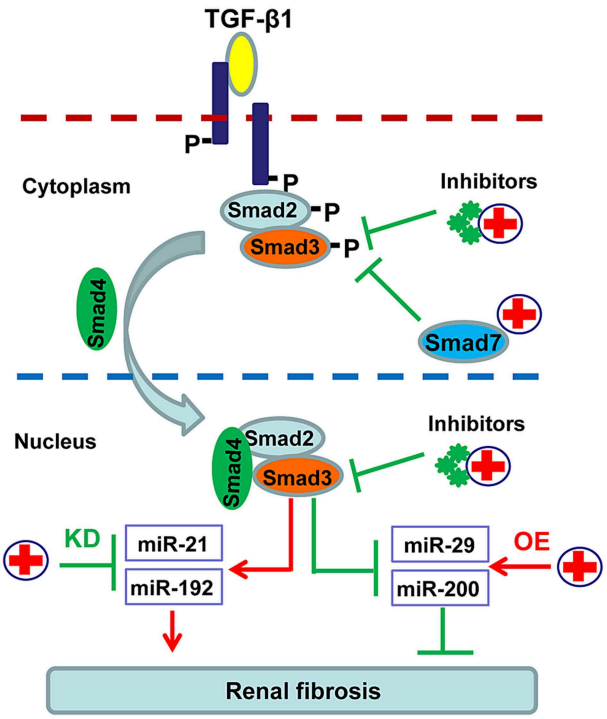

FIGURE 3 | Potential therapeutic strategies for renal fibrosis by specifically targeting downstream TGF-β/Smad signaling. Since renal fibrosis is mediated positively by Smad3 but negatively by Smad7, treatment for renal fibrosis can target Smad3 with specific inhibitors or Smad3-dependent microRNAs that regulate fibrosis, and/or by promoting Smad7 with gene therapy or specific agonists.

Specific Inhibition of Downstream Smads or Smad-regulated miRNAs

In order to avoid the side effects caused by complete blockade of TGF-β1 signaling, more focus has been paid on inhibiting the downstream targets of this signaling pathway including Smad3, Smad7, and Smad-dependent miRNAs (Ng et al., 2009). As shown in **Figure 3**, SIS3, a specific inhibitor of Smad3 phosphorylation, can attenuate renal fibrosis in diabetic nephropathy (Li et al., 2010). Accumulated evidence shows that targeting Smad3 by overexpressing renal Smad7 produces inhibitory effects on both renal inflammation and fibrosis in a variety of kidney disease models (Hou et al., 2005; Ka et al., 2007, 2012; Chen et al., 2011; Liu et al., 2014). Moreover, recent studies also revealed that overexpression of miR-29, miR-200 or inhibition of miR-21 and miR-192 can effectively decelerate the progression of renal fibrosis (Oba et al., 2010; Chung et al., 2010a; Qin et al., 2011; Zhong et al., 2011, 2013; Chen et al., 2014) (**Figure 3**).

BMP-7 is a natural antagonist for TGF-β through inhibiting the TGF-β/Smad3, which has been demonstrated on various renal disease models (Hruska et al., 2000; Zeisberg et al., 2003; Wang et al., 2006a; Sugimoto et al., 2007; Luo et al., 2010; Meng et al., 2013). Klotho, a single-pass transmembrane protein predominantly expressed in renal tubular epithelial cells, is capable of suppressing renal fibrosis by directly binding to type II TGF-β receptor to block the TGF-β-initiated signaling (Doi et al., 2011). Most recently, a study showed that an adaptor protein, Kindlin-2, recruits Smad3 to TGF-β type I receptor, therefore contributing to TGF-β/Smad3-mediating renal interstitial fibrosis (Wei et al., 2013). In addition, two well-known Smad transcriptional co-repressors Ski (Sloan-Kettering Institute proto-oncogene) and SnoN (Ski-related novel gene, non Alu-containing), elicit their anti-fibrotic effects on TGF-β by antagonizing Smad-mediated gene transcription (Yang et al., 2003a). Moreover, GQ5, a small molecular phenolic compound extracted from dried resin of *Toxicodendron vernicifluum*, has been shown to inhibit the interaction between TGF-β type I receptor and Smad3 through interfering the binding of Smad3 to SARA, thereby reducing the phosphorylation of Smad3 and downregulating the transcription of downstream fibrotic indexes including α-SMA, collagen I and fibronectin *in vivo* and *in vitro* (Ai et al., 2014). Furthermore, a number of miRNAs, such as let-7b and miR-29, are capable of regulating TGF-β signaling and altering the progression of renal fibrosis (Kato et al., 2011; Xiao et al., 2012; Wang et al., 2014).

Conclusion

An equilibrium shift of TGF-β/Smad signaling due to the hyperactivation of Smad3 but reduction of Smad7 may be a key pathological mechanism leading to renal fibrogenesis. Thus, rebalancing the TGF-β/Smad signaling by targeting Smad3 activity, up-regulating Smad7, as well as specifically modulating Smad3-dependent miRNAs related to fibrosis may represent an effective therapy for CKD associated with progressive real fibrosis.

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REVIEW
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Role of bone morphogenetic protein-7 in renal fibrosis

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Renal fibrosis is final common pathway of end stage renal disease. Irrespective of the primary cause, renal fibrogenesis is a dynamic process which involves a large network of cellular and molecular interaction, including pro-inflammatory cell infiltration and activation, matrix-producing cell accumulation and activation, and secretion of profibrogenic factors that modulate extracellular matrix (ECM) formation and cell-cell interaction. Bone morphogenetic protein-7 is a protein of the TGF-β super family and increasingly regarded as a counteracting molecule against TGF-β. A large variety of evidence shows an anti-fibrotic role of BMP-7 in chronic kidney disease, and this effect is largely mediated via counterbalancing the profibrotic effect of TGF-β. Besides, BMP-7 reduced ECM formation by inactivating matrix-producing cells and promoting mesenchymal-to-epithelial transition (MET). BMP-7 also increased ECM degradation. Despite these observations, the anti-fibrotic effect of BMP-7 is still controversial such that fine regulation of BMP-7 expression *in vivo* might be a great challenge for its ultimate clinical application.

Keywords: BMP-7, chronic kidney disease, renal fibrosis, cytokines, inflammation

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Introduction

Renal fibrosis, which characterized as glomerulosclerosis and tubulointerstitial fibrosis, is considered the hallmark of progressive renal injury and the final common pathway of multiple chronic renal diseases. Regardless of the primary causes, appearance of most "end stage kidney" manifested as extensive scar formation (fibrotic tubules and sclerotic glomeruli), thickened arteries, and infiltrated chronic inflammatory cells. Process of renal fibrosis involved activation of intrinsic kidney cells, infiltrated cells, and led to excessive accumulation and deposition of extracellular matrix and finally the loss of kidney function. TGF-β has been known as the key modulator of kidney (and that of other organs) fibrosis, and the role of TGF-β in renal fibrosis has been extensively studied. In recent years, another protein, BMP-7, that belongs to the TGF-β superfamily, has drawn great attention for its function in counteracting pro-fibrotic effects of TGF-β. BMP-7 is a homodimeric protein that with cysteine-knot. It plays an crucial role in renal development and only selectively expressed in several adult organs including the kidney. It is a natural negative regulator of nephrotic TGF-β/Smad signaling pathway. The fact that renal BMP-7 disappears during renal fibrogenesis (Wang et al., 2001; Morrissey et al., 2002; Yang et al., 2007) and, supply of BMP-7 either exogenously or endogenously results in prevention or even reversal of functional and structural changes of various nephropathies in animal models (Vukicevic et al., 1998; Hruska et al., 2000; Wang et al., 2003, 2006; Zeisberg et al., 2003a,b; Chan et al., 2008), strongly promises a renal protective function of BMP-7 in human kidney disorders. And its anti-fibrotic role in kidney disease has been extensively studied. A number of review articles have summarized the possible mechanisms and potential

therapeutic targets of renal fibrosis (Liu, 2006; Boor et al., 2010; Eddy, 2014; Kawakami et al., 2014; Lee et al., 2014; Munoz-Felix et al., 2014). This review focuses on role of BMP-7 in fibrotic kidney disease, and the possible mechanisms involved.

Individual Sections

Mechanisms of Renal Fibrosis

Fibrosis can be considered as ineffective wound healing process, in which excessive progression of scarring rather than resolution occurs. Fibroblasts play a pivotal role in the process, which is modulated by multiple cell types and molecules. In this respect, the cellular and molecular response of the damaged kidney attempted to prevent renal damage and preserve renal function, and as a matter of fact, almost all cell types of the kidney and a series of infiltrating cells are involved in the development of renal fibrosis, indicating the complexity of this process.

In early phase of renal injury, damage cells release cytokines and chemokines, which attract inflammatory cells infiltrated into injured sites (You and Kruse, 2002). Although inflammatory response is an important portion of the body defense mechanism, the non-resolving inflammation always become major driving force of fibrogenic process (Nathan and Ding, 2010; Schroder and Tschopp, 2010). The process of renal fibrosis is almost always accompanied with infiltration of inflammatory cells including macrophages, mast cells, lymphocytes, neutrophil as well as dendritic cells. After injury, the infiltrated inflammatory cells activated and release more chemotactic cytokines, vasoactive factors and increase production of profibrotic factors (Ferenbach et al., 2007; Ricardo et al., 2008; Vernon et al., 2010; Chung and Lan, 2011), thus sustained a profibrotic microenvironment in injured sites, and interacted with kidney intrinsic cells, which finally caused phenotypic and functional changes of kidney cells, increase the number of myofibroblast and ECM formation (Duffield, 2014; Mack and Yanagita, 2015). Therefore, persistent inflammation which was caused by injury trigger the onset of profibrotic stage and serve to sustain the profibrotic pressure in the tissue (Meng et al., 2014).

The sustained increase of profibrotic cytokines in microenvironment after renal injury inevitably leads to activation of matrix-producing cells, which plays a central role in renal fibrosis. Fibroblasts are considered as the major source of ECM in the kindney, besides, other cell types in renal tubulointerstitium such as tubular epithelial cells, vascular smooth muscle cells and macrophages are also capable of producing ECM. Myofibroblasts are terminally differentiated cells that rarely found in normal renal tissue. It functions for interstitial ECM synthesis and accumulation including collagen I, collagen III, fibronectin during wound healing or scar forming at injured sites. There are at least five source of myofibroblasts have been proposed in experimental kidney models or disease kidneys, including activation of endogenous fibroblasts (which was regarded as classical source of myofibroblasts), differentiation of pericytes, tubular epithelial to mesenchymal transition, bone marrow fibroblast infiltration or endothelial phenotypic transition (Duffield, 2014). Regardless of their origin, the presence of myofibroblasts are predictor of fibrotic progression, once detectable, they are prognostic indicators of fibrotic expansion. Intervening the activity of myofibroblasts and preventing their accumulation in the kidney has become a therapeutic target of antifibrotic treatment.

Activated fibroblasts from all sources produce large amounts of ECM components, leading to excessive accumulation and deposition of interstitial matrix, which is predominantly composed of collagen I, III, and fibronectin. In the physiological condition, the production and degradation of ECM is intensely regulated under a dynamic balance. Under the profibrotic state, this delicate balance is interrupted. Activation of profibrotic factors and inactivation of antifibrotic factors tilt the balance, increasing ECM formation while reducing their degradation, finally leading to excessive accumulation of ECM. Key fibrogenic factors include TGF-β1, connective tissue growth factor (CTGF), angiotensin II, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)2, among which, TGF-β1 has been regarded as key modulator of renal fibrosis (Bottinger, 2007), and the role of TGF-β in fibrosis has been extensively studied and well summarized (Branton and Kopp, 1999; Bottinger, 2007; Lan, 2011).

Transforming growth factor-β (TGF-β) is a multifunctional cytokine, functioned through its cognate receptors (type II and I) to activate a variety of canonical and non-canonical signaling pathways with large amount of profibrotic target genes (Bottinger, 2007). Although most intrinsic kidney cells can secret and respond to TGF-β, fibroblasts and myofibroblasts are particularly responsive to TGF-β stimulation. TGF-β1 mediates renal fibrogenesis by increasing ECM production and reducing degradation, besides, TGF-β1 is strong stimulant to induce phenotypic transformation of epithelial cells into mesenchymal cells (EMT) (Lan, 2003; Bi et al., 2012; Moustakas and Heldin, 2012). Blockade of TGF-β1 by neutralizing antibodies or gene silencing, decorin have demonstrated to reduce ECM production and ameliorate renal fibrosis in both *in vitro* and *in vivo* studies (Border and Noble, 1998), whereas overexpression of TGF-β1 in mouse liver develop progressive liver and renal fibrosis (Sanderson et al., 1995; Kopp et al., 1996). Studies targeted on TGF-β signaling have been done by many research groups, mainly focusing on reducing TGF-β production or blockade of TGF-β signal transduction. Clinical trials have also been done to evaluate efficacy of anti-TGF-β antibodies in fibrotic diseases (Mead et al., 2003; Denton et al., 2007), while results were less promising as anticipated. Anti-TGF-β treatment with pirfenidone, an orally active small molecule that inhibit TGF-β through reducing promoter activity and protein secretion, showed beneficial effect in diabetic mouse model (RamachandraRao et al., 2009) and in human focal segmental glomerulosclerosis (FSGS) (Cho et al., 2007), a recently completed placebo-controlled randomized clinical trial also demonstrated the efficacy of pirfenidone on improving GFR in overt DN (Sharma et al., 2011). However, due to the multiple pathophysiological functions of TGF-β, systemic administration of anti- TGF-β antibodies may have significant side effect given that mice knockout of TGF-β1 developed chronic inflammation in almost all organs (Boivin et al., 1995), and knockout of TGF-β2 die soon after their birth (Sanford et al., 1997). Therefore, strategies also target on TGF-β signaling regulation

including TGF-β receptor blockade, receptor Smad regulation or other downstream protein control.

CTGF is a cytokine with a molecular weight around 36–38 kD, it is thought to be an prominent profibrotic downstream modulator of TGF-β (Leask and Abraham, 2006). Under pathological circumstances like fibrotic diseases or skin scarring, overexpression of CTGF was observed (Leask and Abraham, 2004). Elevated of constitutive CTGF is a hallmark of tissue firbosis, it acts as co-factor of ECM, growthfactor and cytokines which created an permissive enviroment for other stimuli to induce profibrotic response (Leask and Abraham, 2006). Plasma level of CTGF was independent predictor of ESRD extend and the overall mortality (Nguyen et al., 2008b). Blockade of CTGF by using antisense oligonucleotides or specific down-regulation with interfering RNA (siRNA) ameliorates renal tubulointerstitial fibrosis and the progression of nephropathy (Yokoi et al., 2004; Guha et al., 2007). Phase I clinical trial of anti-CTGF monoclonal antibody FG-3019 in type 1 and 2 diabetic patients was well tolerated and associated with a decrease in albuminuria (Adler et al., 2010).

Efficacy of BMP-7 as a Therapeutic Drug in Renal Fibrosis

BMP-7, formerly called osteogenic protein-1/OP-1, is a member of the BMP-subfamily within the transforming growth factor β (TGF-β) superfamily. BMP-7 expression in normal kidney has the highest expression level in adult organs (Dudley et al., 1995; Luo et al., 1995). Under several disease states like ischemia-reperfusion injury (Simon et al., 1999), diabetic nephropathy (Wang et al., 2001), and hypertensive nephrosclerosis (Bramlage et al., 2010), BMP-7 has been reported to be down-regulated in the kidney, and increased again during the regenerative phase (Vukicevic et al., 1998; Simon et al., 1999). Hypothesis that BMP-7 possesses anti-fibrotic activity was first verified in a rat model of unilateral ureteral obstruction (UUO) (Hruska et al., 2000), based on previous observations that BMP-7 was a renal morphogen (Dudley et al., 1995), such that its loss and re-expression pattern mirrors preservation of renal function in acute renal injury (Vukicevic et al., 1998). The simplified model of renal interstitial fibrosis was generated by UUO, in which renal injury was mediated partly through activation of the angiotensin II -TGF-β cascade (El-Dahr et al., 1993; Diamond et al., 1994; Yoo et al., 1997; Fern et al., 1999). Exogenous administration of recombinant human BMP-7 blocked tubular epithelial cell apoptosis, reduced tubular cells dropped out and the accumulation of ECM. This observation suggested a convincing anti-fibrotic effect of BMP-7 in renal interstitial fibrosis.

Shortly after this observation, researchers sought to explore the role of BMP-7 in a rat model of STZ-induced diabetes, which resembles human type 1 diabetes. BMP-7 was given exogenously through i.v. injection. In this model, it was found that BMP-7 administration delayed the onset of diabetic nephropathy and prevented glomerulosclerosis; and even partially reversed diabetic kidney hypertrophy and restored GFR in the progression stage of DN (Wang et al., 2003). Subsequent studies also demonstrated a protective role of BMP-7 in STZ-induced diabetic kidney injury, manifesting as reduced urinary protein excretion, preserved podocyte nephrin expression (Xiao et al., 2009). In another STZ-induced diabetic model, the CD1 mouse, which tends to develop more severe and accelerated diabetic kidney injury, BMP-7 inhibited glomerular lesion and tubulointerstitial fibrosis (Sugimoto et al., 2007). Besides exogenously supplemented BMP-7, transgenic overexpression of BMP-7 in FVB/N mice induced with STZ led to reduced podocyte dropout, glomerulosclerosis and interstitial collagen accumulation (Wang et al., 2006).

The beneficial effects of BMP-7 are not just restricted to DN. In MRL/MpJ background and autoimmune disease-prone MRL/MpJ$^{lpr/lpr}$ mice, which developed chronic renal injury resembling lupus nephritis, BMP-7 dose dependently reduced interstitial ECM protein accumulation, tubular atrophy, serum creatinine and improved prognosis (Zeisberg et al., 2003a). In addition, BMP-7 improved kidney morphology and renal function in Col4A3 knockout mice that recapitulate Alport Syndrome, a genetic kidney disease. CD1 mice treated with nephrotoxic serum to induce acute glomerulonephritis had decreased ECM secretion and even prompted mesenchymal to epithelial transition when BMP-7 was administered (Sugimoto et al., 2007).

Among these studies, a prominent anti-fibrotic effect of BMP-7 in both glomerulus and tubulointerstitium was observed. Nevertheless, considering that BMP-7 was first discovered as growth factor that facilitated bone formation, concerns about ectopic bone formation and vascular calcification of using BMP-7 systemically seem reasonable. To date, no reports suggested these potential complications in their rodent models. Furthermore, there was evidence that BMP-7 even prevented vascular calcification (Davies et al., 2005; Hruska et al., 2005). Transgenic mice that over-expressed BMP-7 for 1 year had no observable ectopic bone formation either (Wang et al., 2006).

However, BMP-7 was not always effective and promising. For example, BMP-7 conferred no benefits in a rat protein-overload model (Ikeda et al., 2004). In this study, BMP-7 showed no effect in reducing ECM accumulation and proteinuria; on the contrary, BMP-7 tended to increase ECM gene expression in the kidney. Another study investigated on LDLR-deficient mice with kidney partial ablation also showed no effect of BMP-7 in preserving renal function, though BMP-7 corrected hyperphosphatemia, osteodystrophy and prevented vascular calcification (Davies et al., 2005). Most published studies of BMP-7 as a therapeutic intervention in animal models of kidney disease were listed in **Table 1**. Furthermore, clinical trials of BMP-7 analog THR-V-123 has been launched by Thrasos to study its efficacy on diabetic nephropathy, which results worth anticipated.

Mechanism of Anti-Fibrotic Effect of BMP-7

BMP-7 and TGF-β

TGF-β has been known as the key modulator of kidney (and that of other organs) fibrosis. TGF-β acts on multiple cell types in the kidney, including podocytes, mesangial cells, renal proximal tubular epithelial cells and interstitial fibroblasts. TGF-β induces pro-fibrotic gene transcription, and finally leads to the overexpression of extracellular matrix proteins, reduction of cell proliferation and differentiation, and elevation of resident epithelial cell apoptosis. Moreover, although evidence of

TABLE 1 | Published studies of BMP-7 as a therapeutic tool in animal models of kidney disease.

Animal model	Species	Disease model	Effect of BMP-7	References
IRI	Rat	Acute tubular necrosis	Preserved kidney function, increase survival rate	Vukicevic et al., 1998
UUO	Rat	Obstructive nephropathy	Preserved renal blood flow, prevent tubular atrophy, reduced tubulointerstitial and fibrosis	Hruska et al., 2000
MRL/MpJ$^{lpr/lpr}$	Mouse	Lupus nephritis	Inhibited tubular atrophy and interstitial fibrosis	Zeisberg et al., 2003a
COL4A3−/−	Mouse	Alport syndrome	Inhibited tubular atrophy and interstitial fibrosis	Zeisberg et al., 2003a
Nephrotoxic serum nephritis	CD1 Mouse	Acute glomerulonephritis	Induced MET, decreased ECM secretion	Zeisberg et al., 2003b, 2005
STZ induced diabetes	Rat	Type 1 diabetic nephropathy	Reversed kidney hypertrophy, restored GFR, reduced albumin excretion	Wang et al., 2003
Kidney Cx or 5/6 Nx (RRKM)	SD rat	CKD	Increased tubular regeneration in early stage of repair process	Dube et al., 2004
STZ induced diabetes	CD1 Mouse	Type 1 diabetic nephropathy	Inhibit glomerular lesion, and tubulointerstitial fibrosis	Sugimoto et al., 2007
STZ induced diabetes	FVB/N Mouse	Type 1 diabetic nephropathy	Reduced podocytes dropout, glomerular fibrosis and interstitial collagen accumulation	Wang et al., 2006
LDLR−/−	Mouse	CKD/VC/Atherosclerosis/	Down regulated osteocalcin expression	Davies et al., 2003
Unx + kidney partial ablation LDLR−/−	Mouse	Metabolic syndrome/Insulin resistance	Corrected hyperphosphatemia, osteodystrophy, and prevent VC, but no improvement of renal function	Davies et al., 2005
STZ induced diabetes	Rat	Type 1 diabetic nephropathy	Reduced urine protein excretion, preserved podocyte number and nephrin expression	Xiao et al., 2009
Protein overload	Rat	Kidney disease	Failed to reduced proteinuria and even showed increase of ECM gene expression	Ikeda et al., 2004

IRI, Ischemia-reperfusion injury; UUO, unilateral ureteral obstruction; STZ, streptozotocin; Unx, uninephrectomy; CKD, chronic kidney disease; VC, vascular calcification; Kidney Cx, decapsulation; 5/6 Nx, 5/6 nephrectomy; RRKM, rat remnant kidney model.

epithelial-to-mesenchymal transition (EMT) in human kidney remains insufficient, TGF-β have been shown to super-induce fibrotic progression in dozens of *in vitro* studies of tubular epithelial cells, by causing epithelial phenotypic lost and gain of fibroblast phenotypes. As a member of the TGF-β superfamily, BMP-7 was first speculated to promote fibrosis progression in the kidney, but interestingly, an abundance of researches concluded an opposite view.

Besides the anti-fibrotic effect of BMP-7 in all the animal models mentioned above, a variety of *in vitro* studies have suggested the anti-fibrotic effect of BMP-7 to be heavily (although may not exclusively) TGF-β-dependent. This raised the hypothesis that the anti-fibrotic effect of BMP-7 was mediated via counterbalancing the TGF-β signaling pathways. A mass of *in vitro* findings showed evidence to support this notion, and the interaction between BMP-7 and TGF-β in the kidney has also been discussed previously (Meng et al., 2013).

TGF-β induced extracellular matrix and other pro-fibrotic gene overexpression in mesangial cells. Although mesangial cells did not express BMP-7, BMP receptors were detectable on mesangial cells with active signals. Co-incubating murine mesangial cells with TGF-β and BMP-7 reduced TGF-β-stimulated collagen IV, fibronectin and CTGF overexpression. It is worth mentioning that BMP-7 reduces ECM accumulation without concomitant changes of their mRNA levels, suggesting that BMP-7 might act through affecting ECM protein degradation. Besides, TGF-β stimulation decreased matrix metalloprotease (MMP)-2 expression and this action could be abolished by BMP-7, possibly via reducing the activation of the plasminogen activator inhibitor (PAI-1) (Wang and Hirschberg, 2003). Furthermore, BMP-7 reduced nuclear accumulation of Smad3 and blocked the transcriptional up-regulation of CAGA-lux. Knock-down of Smad5 impaired the ability of BMP-7 to interfere with CAGA-lux activation (Wang and Hirschberg, 2004).

The effects of BMP-7 on tubular epithelial cells have also been extensively studied. The emerging importance of tubular epithelial cells (the most abundant cell type in the kidney) in kidney diseases cannot be overemphasized. Most studies in the animal models stated above mainly focused on tubulointerstitial damage, fueling attention of how BMP-7 functions on TECs.

Like mesangial cells, proximal tubular epithelial cells showed no expression of BMP-7, but constitutively express BMP receptors as described in the previously section. On the other hand, distal tubular cells express both BMP-7 and BMP receptors.

In a setting that study of BMP-7 on NP-1 cell (mouse distal tubule cell line) which stimulated with TGF-β (Zeisberg et al., 2003b), BMP-7 preserved epithelial cell phenotype and helped re-express E-cadherin and ZO-1 in NP-1 cells. They constructed a ligand-independent system with construct expressing ALK5 and a plasmid expressing Smad3 to mimic TGF-β intracellular signal, and a plasmid expressing Smad5 to mimic BMP-7. Mimicry of BMP-7 increased activity of E-cadherin promotor activity, while mimicry of TGF-β showed the opposite, suggesting Smad-dependent counteractions of TGF-β and BMP-7.

Researches have also been done in human proximal tubular epithelial cell line (HK2). BMP-7 dose dependently reduced

TGF-β-induced overexpression of a-SMA, fibronectin, collagen I and CTGF in HK2 cells (Xu et al., 2009). Similar effects reproduced elsewhere by other group (Luo et al., 2010) with further research. BMP-7 was found to reduce Smad3 DNA binding to a consensus Smad binding element probe without alteration of Smad3 phosphorylation or degradation. Co-incubation with TGF-β revealed that BMP-7 reduced Smad3 binding to the PAI-1 promotor in HK2 cells. Moreover, BMP-7 prevented TGF-β-induced SnoN lost. siRNA interference suggested that the effect of BMP-7 on Smad3 was SnoN-dependent.

Cyclosporine A (CsA) has been reported to induce EMT in HK2 cells with involvement of TGF-β and CTGF (McMorrow et al., 2005). BMP-7 inhibited CsA-induced TGF-β and CTGF overexpression in a dose dependent manner (Xu et al., 2010). BMP-7 also reduced aristolochic acid (AA)-induced TGF-β and collagen 3 secretion, preserved cell phenotype and cell viability in HK-2 cells (Wang et al., 2010). These findings suggested a strong TGF-β-dependent anti-fibrotic role of BMP-7.

However, not all the studies with BMP-7 in TGF-β-induced fibrosis showed a positive result. Dudas et al., used TGF-β as a stimulant to study the role of BMP-7 in both human and murine tubular epithelial cells. Contrary to the other results listed above, they failed to demonstrate an anti-fibrotic role of BMP-7 in human tubular cells (both primary cell and immortalized cell line). BMP-7 *per se* decreased E-cadherin expression (Veerasamy et al., 2009) and increased vimentin, CTGF and TGF-β1 expression. However, in TCMK-1 cell (mouse renal tubular epithelial cell), BMP-7 preserved E-cadherin expression in TCMK-1 cells (Dudas et al., 2009), suggesting different modulatory role of BMP-7 in different species.

BMP-7 and CTGF

CTGF is considered as inhibitor of BMP-7 signaling. In diabetic CTGF+/+ mice, phosphorylation of smad1/5, expression of BMP-7 target gene Id1 and MMP activity was significantly lower than in CTGF+/− mice (Nguyen et al., 2008a). The secreted CTGF can directly binds to BMP and TGF-β through its CR domain, which prevent BMP binds to its receptors and contrarily enhance TGF-β-receptor binding (Abreu et al., 2002), by which, CTGF act as fibrogenic switch that shifting balance from anti-fibrosis to fibrosis (Gressner and Gressner, 2008). However, there are also evidences suggested BMP-7 alone increase gene expression of CTGF in tubular cells (Dudas et al., 2009; Li et al., 2015), which make interactions between BMP-7 and CTGF more complicated and pending for further investigation.

BMP-7 and ECM

Another possible hypothesis is that BMP-7 exerts anti-fibrotic effect by affecting ECM formation and degradation. BMP-7 reduced TGF-β induced overexpression of ECM proteins in mesangial cells without affecting their gene levels, besides, BMP-7 preserves MMP2 activity and blocks PAI-1 promoter activation in TGF-β-stimulated mesangial cells (Wang and Hirschberg, 2003), however, there were also studies suggested that MMP2 and -9 may play minor roles in ECM degradation (Holmbeck et al., 1999; Visse and Nagase, 2003; Lee et al., 2006). Study of BMP-7 in protein-overloaded rats showed a slight decrease of FN and collagen protein levels (not reaching statistical significance) but an increase of TGF-β gene expression, while in this study, endogenous BMP-7 expression was increased (Ikeda et al., 2004). Studies done by our group also observed increase of FN and collagen gene expression in PTECs that incubated with BMP-7. On the other hand, applied BMP-7 to human renal adult fibroblasts cell line reduced collagen I expression and promote cell mesenchymal-to-epithelial phenotypic transition (Zeisberg et al., 2005). Considering BMP-7 as a morphogen in bone formation, and the promotion of ECM composition was also reported in other study (Tacke et al., 2007) that increase of BMP-7 concentration was correlated with liver fibrosis. Thus, whether BMP-7 reduce or increase ECM remains controversial, there might be fine regulation of BMP-7 level *in vivo*, which may affected its role shifting between anti-fibrotic or pro-fibrotic.

BMP-7 and Inflammation

Inflammatory response can be considered as the onset of wound healing, while non-resolution inflammation is a relentless driver of fibrogenesis that forms a vicious cycle to finally lead to irreversible fibrosis. Anti-inflammatory treatment on the onset and progression stage of renal fibrosis also attracted highly attention. Current studies of BMP-7 in the kidney mainly focused on its anti-fibrotic role, though many studies have suggested the possible anti-inflammatory effect of BMP-7. In the IRI model, BMP-7 reduced ICAM-1 expression and accumulation and activity of neutrophils (Vukicevic et al., 1998). Latter study with UUO model suggested BMP-7 reduced monocyte/macrophage infiltration in the tubulointerstitium (Hruska et al., 2000). Study that done by Stephen E Gould et al showed that co-incubated of TNF-α-stimulated human tubular cells with BMP-7 reduced overexpression of pro-inflammatory cytokines, including IL-6, IL-8, IL-1β, and MCP-1 (Gould et al., 2002). *In vitro* study with human mesangial cells suggested BMP-7 inhibited IL-1β-induced overexpression of MCP-1 expression in mesangial cells, which might exert by suppressing the JNK signaling pathways (Lee et al., 2003). BMP-7 also reduced overexpression of TNF-α, IL-6 that induced by polymeric IgA in mesangial cells (Chan et al., 2008). Moreover, there was study of BMP-7 on monocyte polarization, which found BMP-7 enhanced THP-1 polarized to M2 macrophage, and the increase of anti-inflammatory cytokines IL-10 and IL-1ra (Rocher et al., 2012). Our data also demonstrated BMP-7 reduced overexpression of proinflammatory cytokines in advanced glycation end products (AGEs)-stimulated PTECs (Li et al., 2015) and IgA-induced mesangial cells (Chan et al., 2008). This anti-inflammatory effect of BMP-7 might exert through suppressing activation of multiple signaling pathways including p38 and p44/42 MAPK, as well as reduction of ROS formation. **Table 2** listed most published studies of BMP-7 as a therapeutic intervention in different cell types of the kidney.

BMP-7 and Oxidative Stress

Role of oxidative stress in tissue fibrogenesis has been discussed extensively (Poli, 2000; Aragno et al., 2008; Zhao et al., 2008b; Nie and Hou, 2012). In the kidney, oxidative stress contributed to the onset and progression of renal fibrogenesis in different disease models (Zhang et al., 2004; Zhao et al., 2008a; Kim et al.,

TABLE 2 | Published studies of BMP-7 as a therapeutic tool in different cell types of kidney disease.

Cell type	Stimulants	Effect of BMP-7	References
Murine podocyte	HG 25 mM TGF-β 100 pM	Reduced capase-3 activity, reduced apoptosis	Mitu et al., 2007
Murine podocyte	HG	Restored synaptopodin and podocin	De Petris et al., 2007
Rat mesangial cell	HG 30 nM	Decreased ROS and TGF-β	Yeh et al., 2009
Mesangial cell	polymeric IgA	Reduced expression of TNF-α, IL-6, TGF-β, fibronectin	Chan et al., 2008
Mesangial cell	TGF-β 50–200 pM	Reduced ECM accumulation, maintained activity of MMP2	Wang and Hirschberg, 2003
Mesangial cell	TGF-β 12.5–200 pM	BMP-7-induced opposition to TGF-β required Smad5	Wang and Hirschberg, 2004
Human PTECs	HSA 5 mg/ml	Repressed NIK dependent chemokine synthesis	Lim et al., 2014
HK2 cell	AA 30, 60, 120 umol/L	Preserved cell phenotype and cell viability, reduced TGF-β and collagen 3 secretion	Wang et al., 2010
HK2 cell	TGF-β 1 ng/ml	Suppressed PAI-1, CTGF and TGF-β expression, preserved SnoN expression	Luo et al., 2010
HK2 cell	CsA 4.2 μM	Preserved epithelial cell phenotype, restored E-cadherin and decreased ECM secretion	Xu et al., 2010
HK2 cell	TGF-β 3 ng/ml	Reversed EMT decreased ECM expression	Xu et al., 2009
HK2 cell and RPTEC and TCMK-1 cells	TGF-β 10 ng/ml (RPTEC); 3 ng/ml (HK2)	Failed to attenuate EMT in RPTEC or HK-2 cells, BMP-7 alone decreased E-cadherin expression and increased vimentin, CTGF and TGF-β1 expression; Preserved E-cadherin in TCMK-1 cells	Dudas et al., 2009
HK2 cell	MCP-1 0.1, 1, 10, 50 ng/ml	Inhibited MCP-1 induced EMT through TGF-β-Smad 3 pathways	Tan et al., 2005
TK173	N/A	Increased fibroblast E-cadherin, Pax2, Wnt4 expression and E-cadherin promoter activity (MET)	Zeisberg et al., 2005
HK2 cell	U937 cell condition media TNF-a 10 ng/ml	Reduced U937-dependent TGF-β promoter activity and TGF-β synthesis through interaction with cell surface hyaluronan;	Zhang et al., 2005
NP-1 cell	TGF-β 3 ng/ml	Preserved E-cadherin and ZO-1 expression	Zeisberg et al., 2003b

TK173, human renal fibroblast cell line; NP-1, mouse distal tubular epithelial cells; HK2, human tubular epithelial cells; RPTEC, human primary tubular epithelial cells; TCMK-1, mouse renal tubular epithelial cells; HAS, human serum albumin; HG, high glucose; AA, Aristolochic.

2009; Singh et al., 2011). On the other hand, previous studies have demonstrated that BMP-7 protected cultured neurons from oxidative stress, and BMP-7 reduced H_2O_2 toxicity to the neuron and lipopolysaccharide (LPS) stimulation (Tsai et al., 2007; Sun et al., 2011), which indicated an anti-oxidative role of BMP-7. Subsequently, Yeh et al. (2009) showed that BMP-7 decreased HG-induced ROS in mesangial cells, and that neutralizing BMP-7 with anti-BMP-7 or knockdown BMP-7 expression would increase superoxide generation in the cells. This antioxidative activity of BMP-7 might be exerted through suppressing JNK and c-jun phosphorylation and the reduction of PKCζ.

Summary and Perspectives

Increasing evidence from different independent experiments has approved the anti-fibrotic effect of BMP-7 in renal fibrotic disease regardless of its primary causes. BMP-7 exerts anti-inflammatory, anti-fibrotic and anti-oxidative responses caused by various stimuli, reduces ECM production by suppressing matrix-producing cell activity and inducing MET, enhances ECM degradation by increasing the activity of MMP and reducing activation of PAI-1. Clinical trials have also been launched to investigate the efficacy of BMP-7 (analog) in both acute and chronic kidney disease. However, despite the increasing understanding of BMP-7 signaling transduction and regulation, there are also controversial results in its efficacy. For instance, there was no effect of BMP-7 in skin, lung or renal fibrosis, and some studies even showed that BMP-7 is a promoter of fibrosis. Based on the overall results from all *in vitro* and *in vivo* studies to date, the anti-fibrotic effect of BMP-7 seems promising, whereas the diverse regulatory effect of BMP-7 in ECM protein and gene expression, and its interaction with profibrotic mediators like CTGF indicates that the precise role of BMP-7 could shift between anti-fibrosis or profibrosis and the fine regulation of its level *in vivo* might be a major challenge in the systemic use of BMP-7 in clinical application.

Author Contributions

Rui Xi Li, Wai Han Yiu and Sydney C. W. Tang designed the study. Rui Xi Li and Wai Han Yiu carried out the experiments. Rui Xi Li analyzed the data and wrote the manuscript. Wai Han Yiu provided technical support. Sydney C. W. Tang edited the manuscript.

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REVIEW
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HIPK2 is a new drug target for anti-fibrosis therapy in kidney disease

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In vitro and animal studies continue to elucidate the mechanisms of fibrosis and have led to advancements in treatment for idiopathic pulmonary fibrosis and cirrhosis, but the search for treatments for renal fibrosis has been more disappointing. Here, we will discuss homeodomain-interacting-protein kinase 2 (HIPK2), a novel regulator of fibrosis that acts upstream of major fibrosis signaling pathways. Its key role in renal fibrosis has been validated *in vitro* and in several murine models of chronic kidney diseases (CKD).

Keywords: kidney, fibrosis, HIPK2, inflammation, signaling pathways

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Background: Fibrosis and ESRD

Fibrosis is the final common pathway for chronic kidney diseases (CKD). Renal fibrosis occurs as an overwhelming response to tissue injury. The attempt to repair damage begins with the recruitment of inflammatory cells, but ends with an unchecked inflammatory response that activates matrix-producing cells leading to tubular cell apoptosis, irreversible scarring, loss of renal function, and ultimately end stage renal disease (ESRD) (Chuang et al., 2012). CKD and ESRD are major public health problems with increasing prevalence worldwide and are associated with early mortality, poor quality of life, and high healthcare costs. Unfortunately, at present, there are no effective therapies to prevent or slow the progression of renal fibrosis, and the treatment options for patients with ESRD are limited to dialysis and renal transplant. The blockade of the renin–angiotensin–aldosterone system (RAAS) that nephrologists currently rely on for renal protection decreases the risk of progression by only 20% (Drawz and Rosenberg, 2013). To develop effective molecular targets for prevention and treatment of renal fibrosis, it is important to gain a better understanding of the mechanisms involved.

Here, we will review major signaling pathways that contribute to aberrant wound healing and fibrosis with a focus on homeodomain-interacting-protein kinase 2 (HIPK2) which acts upstream of several pro-fibrosis pathways.

Signaling Pathways in Renal Fibrosis and Targeted Therapies

Novel drugs targeting the signaling pathways of renal fibrosis have generally given mixed results and occasionally concerning side effects. The shortcomings of these experimental therapies are

Abbreviations: HIPK2, homeodomain-interacting-protein kinase 2; RAAS, renin–angiotensin–aldosterone system; TGF-β, transforming growth factor-β; BMP-7, bone-morphogenetic protein-7; CTGF, connective tissue growth factor; RTEC, renal tubular epithelial cells; SIAH-1, seven in absentia homolog-1.

likely due to the fact that the pathogenesis of fibrosis is complex; blockade of one pathway may still leave open many others for promoting inflammation and fibrosis.

A central mediator in renal fibrosis is transforming growth factor-β (TGF-β). TGF-β superfamily is highly conserved and plays a major role in a variety of cellular responses including cell proliferation, differentiation and apoptosis. TGF-β1, the predominant isoform, stimulates extracellular matrix production via Smad-dependent and Smad-independent mechanisms to inhibit extracellular matrix degradation and promote expression of pro-fibrosis genes in various cell types (Blobe et al., 2000; Massagué and Wotton, 2000). TGF-β has also been shown to exert pleiotropic effects in various cell types. In focal segmental glomerulosclerosis, hyperplastic podocytes exhibit increased TGF-β/Smad signaling which results in mesangial cell matrix overproduction (Kim et al., 2003). In animal models of diabetic nephropathy, increased TGF-β1 signaling leads to podocyte apoptosis and glomerulosclerosis (Kim et al., 2003).

Recently, Eli Lilly sponsored a phase 2 trial for a humanized neutralizing monoclonal antibody against TGF-β1 for the treatment of advanced diabetic nephropathy, but the study had to be prematurely terminated due to futility in efficacy. These data were presented at the ASN 2014 and several issues of the study design were raised by experts in the field during the meeting. There are also concerns regarding anti-TGF-β therapies, since in addition to its pro-fibrotic effects, it also exerts anti-inflammatory effects; indiscriminate complete blockade of TGF-β functions can promote inflammation.

Despite some disappointments in the field of renal fibrosis, the FDA has recently approved two promising anti-fibrosis drugs, pirfenidone and nintedanib, for the treatment of idiopathic pulmonary fibrosis (IPF). These drugs are appealing because they block chronic inflammation in addition to decreasing aberrant wound healing. Pirfenidone has well-established anti-fibrotic and anti-inflammatory properties in various *in-vitro* systems and animal models of fibrosis (Schaefer et al., 2011). A number of cell-based studies have shown that pirfenidone reduces fibroblast proliferation, inhibits TGF-β stimulated collagen production and reduces the production of fibrogenic mediators such as TGF-β (Lee et al., 1998; Nakayama et al., 2008; Lin et al., 2009). Pirfenidone has also been shown to reduce production of inflammatory mediators such as TNF-α and IL-1β (Phillips et al., 2005). These activities are consistent with the broader anti-fibrotic and anti-inflammatory activities observed in animal models of fibrosis. Pirfenidone is a drug developed by several companies worldwide for the treatment of IPF. In 2008, it was first approved in Japan for the treatment of IPF. While pirfenidone has established safety and efficacy in treating human pulmonary fibrosis and cirrhosis and is able to prevent kidney fibrosis in rodents, these benefits have not yet been confirmed in renal patients. In a study with 77 patients with kidney disease, pirfenidone improved renal function but failed to significantly reduce proteinuria (Ramachandrarao et al., 2009), suggesting that pirfenidone improves renal fibrosis but does not improve podocyte injury. Nintedanib is a small molecule tyrosine-kinase inhibitor targeting vascular endothelial growth factor, fibroblast growth factor receptor and platelet derived growth factor receptor as an anti-angiogenesis and anti-cancer agent (Roth et al., 2015). The role of Nintedanib has never been determined in kidney disease.

Tranilast is another agent that is an anti-allergic drug and has been used for the treatment of allergic disorders such as asthma, allergic rhinitis and atopic dermatitis. However, *in-vitro* studies show that it reduces collagen synthesis in fibroblasts, and inhibits the production of interleukin-6 in endothelial cells (Spiecker et al., 2002). Tranilast has been shown to improve renal function in the subtotal nephrectomy CKD model in rodents and to reduce albuminuria and slow progression of kidney disease in a small cohort of renal patients (Soma et al., 2002; Spiecker et al., 2002). However, when tranilast was used in patients with cardiovascular disease to prevent major cardiovascular events and restenosis after percutaneous coronary intervention, there was a rise in creatinine afterwards suggesting that tranilast has some nephrotoxicity (Holmes et al., 2002).

Bone-morphogenetic protein-7 (BMP-7) is part of the TGF-β family and actually opposes the pro-fibrogenic effects of TGF-β1 by blocking transcriptional upregulation of plasminogen activator inhibitor-1 and antagonizing TGF-β-induced epithelial-mesenchymal transition (Zeisberg et al., 2003). BMP-7 expression is reduced in both animal models of diabetic nephropathy (DN) and in DN patients, and is also downregulated in the rodent model of ischemic acute tubular necrosis (Vukicevic et al., 1998; Simon et al., 1999; De Petris et al., 2007). Consistent with its TGF-β antagonizing role, exogenous recombinant BMP-7 administration *in vivo* led to significant reduction of renal fibrosis in pre-clinical models of diabetic nephropathy and CKD (Wang, 2006). There are currently several phase 2 clinical trials of agonists of BMP-7 signaling underway.

Connective tissue growth factor (CTGF) modulates TGF-β and BMP-7 activities. CTGF is not expressed in normal kidney but is upregulated in human kidney disease where it enhances TGFβ-1 signaling resulting in myofibroblast activation, fibronectin accumulation and tissue fibrosis (Yokoi et al., 2001; Guha et al., 2007; Wang et al., 2011). In mouse models of kidney disease, the reduction of CTGF prevents renal fibrosis. In a small clinical trial, human anti-CTGF monoclonal antibody significantly reduced albuminuria in patients with diabetic nephropathy without significant adverse events (Adler et al., 2010). FibroGen, Inc. started a phase 2 clinical trial designed to evaluate the efficacy and safety of FG-3019, a fully human monoclonal antibody against CTGF in patients with type 2 diabetes and advanced kidney disease in 2009. Unfortunately, this clinical trial was terminated because of some regulation issues.

Despite some promising findings in animal studies and small clinical trials, currently, anti-fibrosis drugs have yet to be effectively translated into clinical practice in the field of nephrology. The current strongest tool for renal protection remains to be RAAS blockade which decreases the pro-fibrotic effects of angiotensin II, thereby decreasing TGF-β and TGF-β receptor levels in the kidney and decreasing expression of CTGF. However, RAAS blockade is insufficient against renal fibrosis as it only partially decreases the risk of progression, which highlights the urgency for the development of more effective treatments for renal fibrosis.

HIPK2 in Renal Fibrosis

In order to determine which signaling networks are critical in renal fibrosis Jin et al. developed a combined systems biology and experimental approach to identify major signaling pathways involved in gene expression changes in renal fibrosis (Jin et al., 2012; Chen et al., 2013). TG-26 mice were used because they are the murine model of HIV-associated nephropathy and are a model of CKD. Their pathology is characterized by diffuse segmental and global glomerulosclerosis with podocyte foot process effacement, as well as significant tubulointerstitial injury accompanied by heavy proteinuria, elevated blood urea nitrogen, edema and hypoalbuminemia (Rosenstiel et al., 2009). Jin et al. found differentially regulated transcription factors in the kidneys of Tg-26 mice compared to wild-type control mice and then identified upstream protein kinases that are likely to phosphorylate those transcription factors. HIPK2 was identified as one of the protein kinases with the highest ranks using this approach. Subsequent experiments using gene ablation of HIPK2 showed that it is a master regulator of kidney fibrosis in experimental models of CKD.

HIPK2 Structure and Function

HIPK2 is part of the family of conserved serine/threonine homeodomain-interacting kinases that localizes to nuclear bodies. Structurally, its N-terminal domain consists of a sumoylation site at lysine 25 and a 330 amino acid serine/threonine kinase domain with a key catalytic site at lysine residue, K221 (**Figure 1**) (Gresko et al., 2005; Hofmann et al., 2005); the homeobox-interacting domain (HID), a protein–protein interaction region, is followed by the speckle-retention signal (SRS) which contains two PEST motifs and is required for the subcellular localization of HIPK2 to nuclear bodies (Kim et al., 1998); and its C-terminus contains the auto-inhibitory domain (AID) with a ubiquitination site (K1182), which, when cleaved by caspases leads to full activation of HIPK2. HIPK2's pleotropic functions include serving as co-regulator of transcription factors, modulator of growth, development, morphogenesis and cell death, tumor suppressor, and regulator of response to DNA damage (Hofmann et al., 2001; D'Orazi et al., 2002; Link et al., 2007; Bitomsky and Hofmann, 2009; Calzado et al., 2014). A defective HIPK2-activated P53 system has been implicated in the pathogenesis of idiopathic pulmonary fibrosis (Ricci et al., 2013).

In renal tubular epithelial cells (RTEC), HIPK2 induced expression of pro-fibrosis markers and apoptosis in HIV-infected RTEC, likely due to its role in activating p53, TGF-β, and Wnt/Notch pathways, which are each known to increase expression of pro-fibrosis markers (Puca et al., 2010). Conversely, suppression of HIPK2 activity via kinase dead (KD)-HIPK2 expression prevented TGF-β-induced expression of fibrosis markers and attenuated HIV-induced apoptosis. In addition, overexpression of WT-HIPK2 in HIV-infected RTECs enhanced the expression of NF-κB targeted genes, while overexpression of KD-HIPK2 suppressed NF-κB activation and inhibited the expression of its downstream target genes. These data suggest that HIPK2 mediates not only the fibrosis pathway but also the inflammatory pathway. These results were further validated *in vivo* using three different murine models of CKD (unilateral ureteral obstruction or UUO, folic acid nephropathy and HIVAN) by genetic ablation of HIPK2. In all three separate models of CKD, loss of HIPK2 resulted in attenuation of renal fibrosis, consistent with the role of HIPK2 as a regulator of gene expressions involved in tubular injury and fibrosis.

HIPK2 Regulation is at Post-Translational Level

HIPK2 is upregulated in fibrosis in murine kidney disease and in human HIVAN, focal segmental glomerulosclerosis, diabetic nephropathy and IgA nephropathy. However, this upregulation appeared to be mediated through a post-translational regulation, as the mRNA levels did not differ in Tg26 kidneys, while there was a significant increase in protein detected by immunohistochemistry. Indeed, the HIPK2 level was found to be regulated through seven in absentia homolog-1 (SIAH-1) mediated proteasomal degradation. However, oxidative stress, such as from HIV infection, decreased SIAH-1 mRNA and protein expression *in vitro*, resulting in an accumulation of HIPK2. This inverse relationship between SIAH-1 and HIPK2 expression was confirmed *in vivo* in Tg26 mice, which had decreased SIAH-1 expression in the renal cortex. While these findings are intriguing, further reduction of HIPK2 staining in late HIVAN glomeruli did not correlate with SIAH-1 expression suggesting that there are other mechanisms besides SIAH-1 that

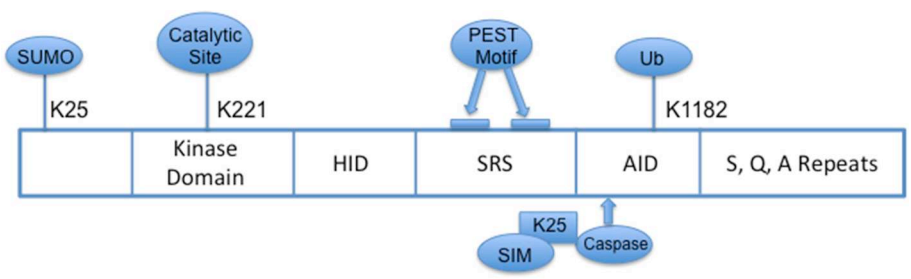

FIGURE 1 | Schema of HIPK2 Structure. HID, Homeoprotein-interacting domain; SRS, Speckle-retention signal; AID, auto-inhibitory domain; PEST, peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T); SIM, SUMO-interacting motif; Ub, ubiquitylation.

regulate HIPK2. In fact, studies suggest that HIPK2 expression is also downregulated by miR-141 interaction with HIPK2′s 3′ untranslated region which leads to upregulated e-cadherin, downregulated vimentin, and ultimately decreased epithelial to mesenchymal transition (Huang et al., 2015). The role of reactive oxidative species in HIPK2 expression was further confirmed when treatment with n-acetylcysteine, a reactive oxidative species scavenger, attenuated the HIV-induced increase in caspase 3 activity and epithelial-mesenchymal transition marker expression in RTECs *in vitro* (Jin et al., 2012) and resulted in increased SIAH1 expression and reduced HIPK2 expression in Tg26 mice, ultimately resulting in a partial reduction in proteinuria, and attenuation of azotemia and kidney fibrosis.

Pros and Cons of HIPK2 as Potential Anti-Fibrosis Target

The above data suggest that inhibition of HIPK2 would mitigate renal fibrosis progression in kidney disease. Importantly, genetic ablation of HIPK2 does not result in a significant phenotype, as opposed to the detrimental effects seen with partial NF-κB inhibition, making HIPK2 an attractive target for therapy (Doi et al., 1997). Although recent studies suggest that it is possible to develop specific HIPK2 inhibitors (Cozza et al., 2014), at present, there are no specific HIPK2 inhibitors available for clinical testing. Development of specific HIPK2 inhibitors is necessary, since unlike the HIPK2 KO mice that show no overt phenotype, HIPK1 and HIPK2 double KO mice are lethal.

One concern of using HIPK2 inhibitors as anti-fibrosis therapy is its tumor suppressive effects. HIPK2 suppresses tumor growth by activating p53 and inducing tumor cell apoptosis (Li et al., 2007; Wei et al., 2007; Stanga et al., 2010; D'Orazi et al., 2012; Mao et al., 2012). In addition to having tumor-suppressive effects, HIPK2 is also needed for TGF-β-mediated survival of midbrain dopaminergic neurons (Zhang et al., 2007; Lanni et al., 2010; Stanga et al., 2010; Chalazonitis et al., 2011). The potential tumor suppressive and neurologic effects of HIPK2 suggest that inhibitors of HIPK2 could have potential adverse effects which need to be carefully watched for in future clinical studies. However, it is likely that the effects of HIPK2 are quite different between tumor cells and non-tumor cells and HIPK2 inhibitors could be used in CKD patients without history of cancers using the same criteria that we currently use for immunosuppressive medication in patients with kidney disease.

Conclusion

Several key signaling pathways that mediate renal fibrosis have been identified. This has been shown both *in vitro* in cultured kidney cells and *in vivo* in animal models of kidney disease. However, currently, none of the drugs targeting these pathways have been proven to be effective anti-fibrosis therapy for kidney disease in large clinical trials. One potential reason is that renal fibrosis is mediated by a complex signaling network of several different pathways. Therefore, targeting one pathway is not enough to be effective. HIPK2 has been identified as a master regulator of kidney fibrosis and acts upstream of several major fibrosis-signaling pathways. Controlling HIPK2's activities might be an effective approach to reduce the progression of kidney fibrosis. However, it is important to note that HIPK2 also has some onco-suppressor functions and that loss of HIPK2 may also lead to neurodegenerative disease (Lanni et al., 2010). Therefore, we need to carefully monitor for potential side effects during future clinical studies. Further structural and functional studies of HIPK2 may aid in the development of more specific HIPK2 inhibitors as anti-fibrosis therapy without causing these side effects.

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REVIEW
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Role of non-classical renin-angiotensin system axis in renal fibrosis

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The renin–angiotensin system (RAS) is a major regulator of renal fibrosis. Besides the classical renin/Angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/AT1 and AT2 axis, multiple new axes have been recently described. The new members have added new dimensions to RAS, including the ACE2/Ang(1–7)/Mas receptor axis, the prorenin/(pro)renin receptor(PRR)/intracelluar pathway axis, and the Angiotensin A (Ang A), alamandine-Mas-related G protein coupled receptor D(MrgD) axis. This review summarized recent studies regarding role of the non-classical RAS axis in renal fibrosis, and its possible implications to the intervention of progression of chronic kidney disease.

Keywords: renin-angiotensin system, renal fibrosis, angiotensin-converting enzyme 2, (pro)renin, angiotensin(1–7)

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Introduction

Glomerulosclerosis and tubular interstitial fibrosis are the final common manifestation of a wide variety of chronic kidney diseases (Liu, 2006). One common feature of progressive renal disease is the proliferation of resident renal cells, excessive accumulation and deposition of extracellular matrix (ECM), and tissue retraction (Eddy, 2014). The renin–angiotensin system (RAS) is a major regulator of renal fibrosis (da Silveira et al., 2010). Both tissue and circulatory RAS are believed to be overactivated in the development of renal fibrosis (Navar, 2014). Tremendous studies have demonstrated that angiotensin II (AngII) play a powerful role in renal fibrosis by mediating the release of transforming growth factor-β (TGF-β) and activating inflammatory process (Mezzano et al., 2001). Moreover, our previous studies have suggested connective tissue growth factor (CTGF)/integrin-linked kinase (ILK) participated in the renal fibrosis development mediated by AngII (Liu et al., 2007). We also proposed the interaction of AngII and inflammation might be the critical node in pathogenic glomerulotubular feedback loop (Zhang and Liu, 2011). In addition to angiotensin-converting enzyme (ACE) dependent AngII generating system, chymase as an alternative AngII generating enzyme, was involved in the development of diabetic/hypertensive nephropathy (Huang et al., 2003). However, in recent years, RAS is far more complex than originally described (Chappell, 2012). New members have been added to the RAS family, which played critical roles in the process of renal diseases (Chappell, 2012; Simoes e Silva et al., 2013). This review will focus on the role of new RAS members and the mechanism in promoting glomerulosclerosis and tubularinterstitial fibrosis.

ACE2/Ang(1–7)/Mas Receptor Axis

Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase. ACE2 converts angiotensin II (Ang II) to angiotensin 1–7 [Ang(1–7)], which binds to Mas (Vickers et al., 2002). ACE2 shares

40–42% homology with ACE but shows different biochemical activities (Donoghue et al., 2000). ACE and ACE2 co-localize in the brush border of mouse proximal tubules (Ye et al., 2006). However, they localize to different cell types in glomeruli. Within the glomerulus, ACE2 is present in glomerular epithelial cells and glomerular mesangial cells. Glomerular ACE is mainly expressed in endothelial cells. In contrast to the fibrogenic and proliferative actions of the ACE/Ang II/AT1 axis, it has been suggested that the ACE2/Ang(1–7)/Mas axis exerts anti-fibrogenic and anti-proliferative actions (Ferrario, 2011).

ACE2 and Renal Fibrosis

Many studies have suggested a protective role for ACE2 in different models of renal damage or diseases, including subtotal nephrectomy (Dilauro et al., 2010), ischaemia/reperfusion kidney injury (da Silveira et al., 2010), unilateralureteral obstruction (UUO) (Liu et al., 2012). Acquired genetic ACE2 deficiency accelerates renal injury in those experimental models. Dilauro et al. reported that kidney ACE2 is downregulated in the early period after 5/6 nephrectomy (5/6 NX). Inhibition of ACE2 with MLN-4760 (MLN) in 5/6 NX mice increases albuminuria via an AT1 receptor dependent mechanism, independent of blood pressure (Dilauro et al., 2010). In studies from Zhong et al., wild-type mice were infused with Ang II and treated daily with recombinant human ACE2. It showed that ACE2 could attenuate Ang II-induced pressor response and normalize renal Ang II levels and oxidative stress. Importantly, ACE2 could prevent Ang II-induced tubulointerstitial fibrosis (Zhong et al., 2011). Regarding glomerularsclerosis, it has been shown that glomerular staining for fibronectin was increased in both db/db and db/m mice that were treated with ACE2 inhibitor (MLN-4760) compared with vehicle-treated group (Ye et al., 2006).

ACE and ACE2 activity play an important role to balance the expression of angiotensin II and angiotensin(1–7) (Chappel and Ferrario, 2006). The integral relationship of ACE/ACE2 is critically involved in process of renal fibrosis. Our previous study has suggested that treatment of HK-2 cells to bovine serum albumin (BSA) led to a significant dysregulation of ACE/ACE2 expression in time and concentration dependent manner (Liu et al., 2009). However, in a recent study with cultured podocytes, insulin increases ACE2 expression and favors an "anti-angiotensin II" regarding ACE/ACE2 gene expression balance and decreases fibronectin expression as a marker of fibrosis. In the presence of albumin, the effect of insulin on ACE2 disappeared. The authors suggest that albuminuria might block the beneficial effect of modulating RAS balance for preventing diabetic nephropathy (Marquez et al., 2014). Moreover, urinary albumin excretion (UAE) increased significantly in ACE2 inhibitor-treated group as compared with vehicle-treated db/db mice (Ye et al., 2006). These studies suggested the close correlation between albumin and ACE2. ACE2 reduction could accelerate albuminuria, while albumin in turn could disrupt the balance of ACE/ACE2. The interplay between ACE and ACE2 may govern the formation and metabolism of Ang effector peptides, the regulators that determines their balance need more studies (Brosnihan et al., 2005). Moreover, it is still unclear whether the protective effects of ACE2 is due to the reduction of AngII, the formation of Ang(1–7) or the increased ratio of Ang(1–7) to AngII.

Transforming growth factor-β1 (TGF-β1) is a key mediator in the process of renal fibrosis (Lan and Chung, 2012). The interaction between TGF-β and ACE2-Ang(1–7)-Mas has been observed. In NRK-52E cells, TGF-β1 decreases ACE2 and Mas and Ang(1–7) conversion from Ang II. The combination of Ang-(1–7) and Mas could attenuate TGF-β1 (but not high glucose)-induced fibronectin (Chou et al., 2013). Liu et al. showed that deletion of ACE2 resulted in increasing ratio of intrarenalAng II/Ang(1–7) and enhanced renal fibrosis in the UUO nephropathy. These changes were attributed to the increase in the intrarenalAng II signaling (AT1-ERK1/2 mitogen-activated protein kinase), TGF-β/Smad2/3, and NF-κB signaling pathways. The authors concluded that enhanced AngII-mediated TGF-β/Smad and NF-κB signaling might be one of the mechanisms by which loss of ACE2 enhances renal fibrosis and inflammation (Liu et al., 2012).

Ang(1–7) and Renal Fibrosis

Multiple small peptides can be derived from degradation of AngII and have been demonstrated to have local physiological effects in the kidney. Those peptides include Ang(1–7) (Alzayadneh and Chappell, 2014), Ang(2–8) (Kemp et al., 2012), Ang(3–8) (Chai et al., 2004), Ang(3–4) with Ang(1-5), and Ang(1–4) as intermediate peptides (Axelband et al., 2009). Among those peptides, Ang(1–7) is a biologically active heptapeptide from the degradation of angiotensin II by ACE2, the role of which in kidney disease have been studied most extensively (Ferrao et al., 2014). Santos et al. firstly found a G protein-coupled receptor for Ang(1–7), the Mas receptor. It provides the molecular basis for the physiological actions of this peptide (Santos et al., 2003). Although there is now much evidence that Ang(1–7) may exert a protective role in experimental models of renal diseases, a few studies have revealed controversial results. Consequently, the role of Ang(1–7) in the progression of CKD requires further clarification, especially its therapeutic role in different types of kidney diseases.

Ang(1–7) has been demonstrated to be able to ameliorate streptozotocin induced diabetic renal injury. In mouse model of streptozotocin induced diabetic nephropathy, large dose of Ang(1–7) improved renal function, attenuated glomeruli sclerosis, oxidative stress, and cell proliferation (Zhang et al., 2015). Moreover, the effects of large-dose Ang(1–7) alone and in combination with valsartan were superior to valsartan alone, but the combination had no significant synergistic effect compared with Ang(1–7) alone (Zhang et al., 2015). Mori et al. administered Ang(1–7) or saline to 5-mo-old db/db mice. The treatment reduced kidney weight and ameliorated mesangial expansion and increased urinary albumin excretion. The protective effect on renal fibrosis was correlated with dephosphorylation of the signal transducer and activator of transcription 3 (STAT3) pathway (Mori et al., 2014).

In contrast, another study using a moderate dose of Ang(1–7) to treat diabetic nephropathy showed no benefitial effect in reducing the levels of serum creatinine and creatinine clearance

(Singh et al., 2010). Shao et al. reported that treatment of STZ induced diabetic rat by constant Ang(1–7) vein injection for 6 weeks, renal function was found to be even worse than diabetic rats (Shao et al., 2008). In the 5/6 NX mouse model of chronic kidney disease (CKD), treatment of 5/6 NX mice with Ang(1–7) increased kidney and plasma levels of Ang(1–7) but did not change levels of blood pressure, urinary albumin excretion (Dilauro et al., 2010).

The underlying mechanism for Ang(1–7)'s role in renal fibrosis needs to be further clarified before the therapeutic role to be determined. A few studies have explored the molecular mechanisms of the renoprotection induced by Ang(1–7). Alzayadneh et al. found that Ang(1–7) could abolish Advanced glycation end products (AGEs)-induced activation of the MAP kinase ERK1/2 to a similar extent as the TGF-β receptor kinase inhibitor (Alzayadneh and Chappell, 2014). Ang(1–7) could also decrease the expression of collagen IV, TGF-β1, VEGF, NOX4, p47phox, PKCα, and PKCβ1, and the phosphorylation of Smad3 (Zhang et al., 2015). AGEs exposure in NRK-52E renal epithelial cells for 48 h significantly reduced the intracellular levels of Ang(1–7) approximately 50%. Treatment with Ang(1–7) could reverse AGEs-induced cellular hypertrophy and myofibroblast transition (Alzayadneh and Chappell, 2014). Hyperlipidemia is an independent risk factor for renal disease, and lipid deposition is closely correlated with glomerulosclerosis. Huang et al. found that Ang(1–7) could inhibit low-density lipoprotein accumulation and decreases cholesterol levels via modulating the low-density lipoprotein receptor (LDLr) -sterol regulatory element-binding protein (SREBP)-cleavage activating protein (SCAP) negative feedback system through the Mas receptor (Shao et al., 2008). Interestingly, Ang(1–7) antagonize the effect of Ang II on lipid accumulation. Our previous studies showed that Ang II increased lipid droplet accumulation in human renal mesangial cells (HMCs) via enhanced translocation of the SCAP/SREBP-2 complex from the endoplasmic reticulum (ER) to the Golgi in HMCs (Ma et al., 2013). Since the close correlation between renal lipid accumulation and glomerulosclerosis, tubulointerstitial fibrosis, especially in diabetic kidney disease has been shown, we thought that Ang(1–7) might prevent the progression of renal fibrosis through its inhibition on lipid accumulation (Wang et al., 2005). This could be a interesting aspect in exploring the therapeutic role of Ang(1–7) in treating renal fibrosis.

Consequently, to address the theraputic potential of Ang(1–7), the related issues that need to be clarified include: (1) the effect of different doses of Ang(1–7); (2) the effect of its combination with an angiotensin receptor blocker; (3) the mechanism by which it exerts the protective effect.

Prorenin/(pro)renin Receptor (PRR)/intracellular Signaling Axis and Renal Fibrosis

A new axis in the RAS research field provoking much interest recent years is the prorenin/PRR/intracelluar pathway axis. Renin is synthesized in juxtaglomerular cells of the afferent arterioles of the kidney as preprorenin. Prorenin was generated by cleavage of a 23 amino acid peptide at carboxyl terminus of preprorenin. Prorenin is activated to renin by cleavage of 43-amino acid N-terminal prosegment by proteases (Song and Yosypiv, 2011). The receptor binding renin and prorenin, named the PRR, was cloned in 2002 (Nguyen et al., 2002). The binding induces prorenin enzymatical activation without cleavage of the prosegment, and triggers intracellular signaling pathways independent of the RAS. Through activating multiple intracellular pathways, prorenin/PRR participate in the development of renal fibrosis independent of ANG II. Recent findings have found additional role of PRR, participating in the functions of vacuolar proton ATPase and constituting the Wnt receptor complex (Oshima et al., 2014). The novel biological effect of PRR is that it regulated several cellular homeostatic processes including autophagy which was described in a recent review (Binger and Muller, 2013).

Prorenin/PRR in Resident Kidney Cells

In multiple types of resident kidney cells, it has been shown that prorenin/PRR could activate several intercellular signaling pathways leading to renal fibrosis. In the human kidney, confocal microscopy has identified that the PRR localized in mesangium of glomeruli and in the subendothelium of coronary and kidney artery (Nguyen et al., 2002). However, a number of studies showed predominant expression of PRR in the collecting duct, particularly on the apical membrane of intercalated cells (Advani et al., 2009; Gonzalez et al., 2013). Thus, the cellular localization of PRR in addition to its molecular form is intriguing. The protein is shown to accumulate intracellular or in plasma membrane or in the trans-Golgi. Indeed, Prieto et al. found that hyperglycemia induces PRR trafficking alterations and increase PRR abundance in plasma membrane (PM) in the collecting duct. Recent evidence demonstrated that PRR is predominantly activated by AngII, which is mediated by (cycloxygenase-2) COX-2/E-prostanoid4 pathway (Wang et al., 2014a,b).

In renal mesangial cells, Zhang et al. showed that elevated prorenin could be activated through receptor binding without being proteolytically converted to renin. This activation leads to increased expression of PAI-1 and transforming growth factor-β1 via AngII-independent and AngII-dependent mechanisms (Zhang et al., 2012). Melnyk et al. also showed that two distinct pathways were activated by renin and prorenin, a TGF-dependent pathway and a TGF-independent pathway (Melnyk et al., 2009). Additionally, high glucose exposure enhanced TGFβ1-CTGF signaling cascade through PRR (Huang et al., 2011). In renal epithelial cell, Saito et al. demonstrated that indoxyl sulfate, which is a uremic toxin, could induce expression of PRR and prorenin. PRR knock-down inhibited indoxyl sulfate-induced expression of TGF-β1 and α-smooth muscle actin in HK-2 cells. The up-regulation of prorenin expression and activation of PRR was related with reactive oxygen species and activation of Stat3 and nuclear factor-κB (Saito et al., 2014). In human embryonic kidney cells (HEK), both renin and prorenin stimulate TGF-β, fibronectin and PAI-1 expression via a Nox4 dependent mechanism. The study indicates a PRR/Nox4 pathway in the development of kidney fibrosis

through the generation of superoxide anions (Clavreul et al., 2011).

Thus, prorenin and PRR participate to an overall switch toward a pro-fibrotic state of the kidney cells, including renal mesangial cells, proximal tubular cells and embryonic kidney cells. Prorenin/PRR could induce multiple pathways of intercellular signaling via AngII-independent mechanism, leading to the development of renal fibrosis (Ichihara et al., 2006a).

Prorenin/PRR in Animal Models

Activation of PRR by prorenin have been implicated in the development and progression of renal fibrosis in animal models. Kaneshiro et al. showed that in (pro)renin receptor-transgenic rat, proteinuria and significant glomerulosclerosis was developed. In kidney, mitogen-activated protein kinases (MAPK) were activated and expression of TGF-β1 was enhanced, and these changes were AngII-independent (Kaneshiro et al., 2007). Ichihara et al. showed that in AT1a receptor-deficient (ATKO) mice, prorenin/PRR/MAP kinases ERK1/2 axis plays a pivotal role in the development of diabetic nephropathy. Treatment of the diabetic WT or ATKO mice with ACE inhibitor failed to prevent the diabetic nephropathy completely, while chronic infusion of mouse HR decoy peptide (HRP) (HRP inhibits prorenin binding to PRR and non-proteolytic activation) (Ichihara et al., 2004) completely prevented the increase in urinary protein excretion and the development of glomerulosclerosis (Ichihara et al., 2006b). In spontaneously hypertensive rats (SHR), inhibition of non-proteolytic activation of prorenin by HRP decreased renal AngII levels and attenuated the development and progression of proteinuria and glomerulosclerosis, the effect is independent of circulating RAS or arterial pressure (Ichihara et al., 2006a). These findings suggest that prorenin/PRR may induce renal fibrosis through multiple intracellular signaling, either alone or in concert with activation of renal tissue RAS.

However, a recent study indicated that increased expression of PRR is not sufficient to induce renal injury. Rosendahl et al. generated a mouse that constitutively overexpressed PRR, they found no difference in systolic blood pressure or albuminuria between wild type and PRR overexpressing mice. And no renal or cardiac fibrosis was detected with histological examination in mutant mice. The authors suggest that PRR might not play central role in organ damage *per se* (Rosendahl et al., 2014). Interestingly, transgenic overexpression of the prorenin in rats does not cause renal fibrosis, whereas leads to myocardial hypertrophy, proteinuria, and hypertension (Peters et al., 2008). Nguyen et al. proposed in a well summarized review on PRR, that prorenin may be an amplifier of fibrosis, in addition to high glucose, inflammation, or immunological cytokines (Nguyen and Muller, 2010).

Moreover, recent studies suggest that PRR is not always devil to kidney. Oshima et al. reported that PRR might be necessary for the maintenance of normal podocyte structure and function. Deficiency of the PRR in murine podocytes resulted in disruption of the filtration barrier and accumulation of intracellular vesicles (Oshima et al., 2011). More studies are needed to determine the contribution of prorenin/PRR in renal fibrosis.

Other New Members of RAS:Angiotensin A (Ang A), Alamandine-Mas-Related G Protein Coupled Receptor D(Mrgd)

Jankowski et al. first discovered a novel human Ang-derived peptide, Ang A (Ala-Arg-Val-Tyr-Ile-His-Pro-Phe) with increased level in end-stage renal failure patients compared with healthy humans (Jankowski et al., 2007). However, Yang et al. demonstrated that Ang A displays similar *in vitro* and *in vivo* properties as AngII, the effect of this peptide is mediated via AT1a receptors. Ang A might be not a naturally occurring peptide that modulates the pressor and renal hemodynamic effects of AngII (Jankowski et al., 2007). Additional studies are needed to characterize the role of this peptide in the kidney function and renal fibrosis.

By using mass spectrometry, Lautner et al. found a new member of RAS, Alamandine. Alamandine is formed from catalytic hydrolysis of the angiotensin A by human ACE2. The peptide binds to Mas-related G protein coupled receptor D(MrgD), the effects include vasorelaxation, blunting of isoproterenol-induced heart fibrosis, and anti-hypertensive action in SHR (Lautner et al., 2013). Alamandine is an endogenous peptide of heart tissue and also presents in human blood (Lautner et al., 2013). Lautner et al. revealed that nephropathic patients have increased plasmatic concentration of alamandine. Administration of alamandine produced a long-term antihypertensive effect and decrease of collagen I, III, and fibronectin accumulation in the heart (Lautner et al., 2013). However, the role of these novel RAS components in renal fibrosis is still lacking investigation.

Can We Block/Activate New Member of RAS to Ameliorate Renal Fibrosis?

XNT(1-[(2-dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one) is a recently described synthetic activator of ACE2 (Hernandez Prada et al., 2008). It has been reported to exert various organ-protective effects, which are attributed to the activation of ACE2. XNT could attenuate thrombus formation, control pulmonary hypertension, reverse cardiac fibrosis (Ferreira et al., 2009). Administration of XNT to SHR resulted in the decrease in blood pressure, which was also associated with improvements in renal fibrosis (Hernandez Prada et al., 2008). However, a recent study reported by Haber et al. showed that neither plasma nor kidney ACE2 activity in Ang II infusion animal model was affected by XNT. *In vitro* and *in vivo* experiments confirmed a lack of enhancement of ACE2 enzymatic activity by XNT. They concluded that the biological effects of this compound are ACE2-independent (Haber et al., 2014). Studies on the therapeutic effect of XNT in renal fibrosis are still very limited. More observations are needed to prove its potential in treating renal fibrosis.

Current anti-RAS therapy only has limited efficiency, partly because of compensatory upregulation of renin expression. Handle region peptide (HRP) mimics part of the prosegment of prorenin and inhibits prorenin binding to PRR and

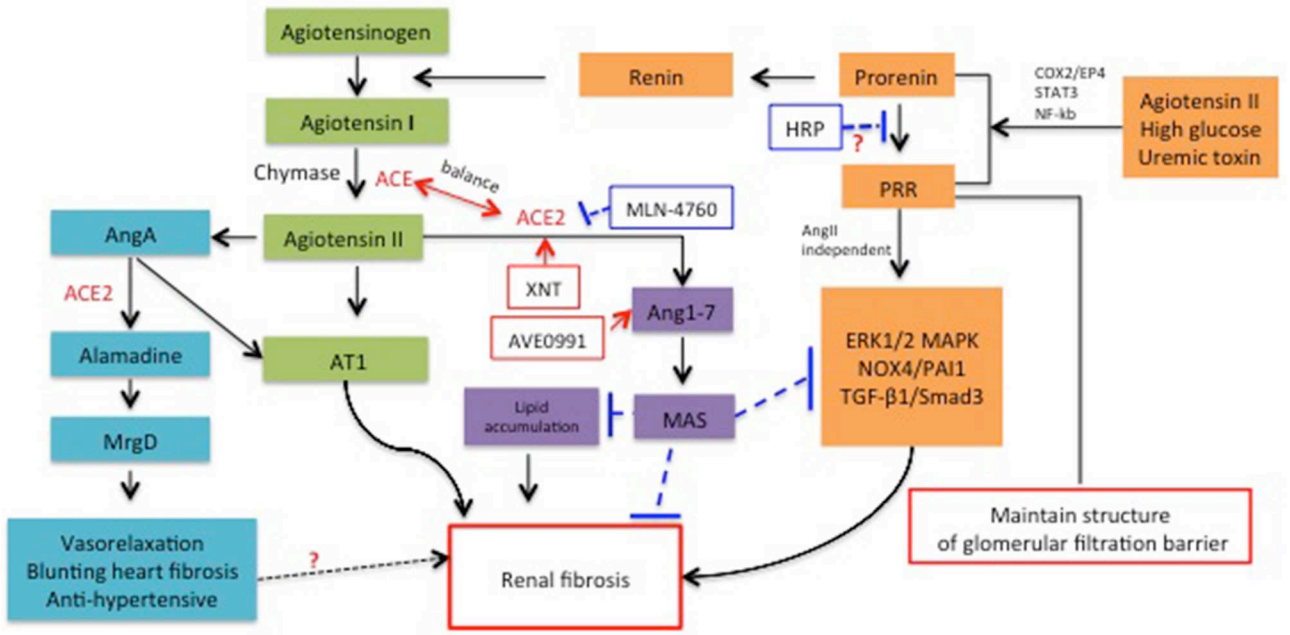

FIGURE 1 | Schematic representation for the role of new members of RAS system in renal fibrosis and the potential molecules targeted RAS for therapeutic application.

non-proteolytic activation (Suzuki et al., 2003). It was originally considered as a PRR antagonist, which evokes much interest. However, its therapeutic effect *in vivo* was still in controversial. HRP has been shown to prevent glomerulosclerosis in diabetic nephropathy (Ichihara et al., 2004). Afterwards, other *in vivo* studies have shown no therapeutic effects, which arise skepticism (Muller et al., 2008). Importantly, it is still unclear to what degree PRR blockade adds benefits in combination with classic RAS blockade. A recent study showed that in diabetic TGR (mREN2)27 rats, HRP given on top of aliskiren (a direct inhibitor of human renin), did not alter the effects of aliskiren on blood pressure, RAS activity, or aldosterone. However, it counteracted the beneficial effects of aliskiren in the kidney (te Riet et al., 2014). In SHR, it also showed that HRP counteracted the beneficial effects of aliskiren on blood pressure, coronary function, and cardiac hypertrophy in an angiotensin-independent manner (van Esch et al., 2011). Thus, the therapeutic effect of HRP for renal fibrosis is still in controversial which need more observation.

Ang(1–7)/Mas receptor axis plays a countermeasure role to that of angiotensin II/AT1 receptors. Ang(1–7)/Mas receptor might be a potential target for developing oral active agonist treating renal fibrosis. Wiemer et al. firstly described a non-peptide mimic of Ang(1–7), compound AVE 0991 which efficiently mimics the effects of Ang(1–7) on the endothelium (Wiemer et al., 2002). Suski et al. showed that Ang(1–7) peptidomimetic AVE0991 partially reversed atherosclerosis-related changes in apoE(-/-) mice (Suski et al., 2013). In adriamycin (ADR)-induced nephropathy, treatment with AVE 0991 improved renal function and attenuated histological changes (da Silveira et al., 2010). Ang(1–7) mimic peptide might hold a promise in future, but it need more investigations in multiple models of kidney injury.

Since the unsatisfied effect of RAS blockade on controlling renal fibrosis, recently, Zhou et al. aimed to find a treatment strategy to simultaneously target multiple RAS genes. They found that overexpression of either β-catenin or Wnt ligands induced the expression of all RAS genes. Interestingly, a small-molecule-weight β-catenin inhibitor ICG-001 abolished RAS activation. Meanwhile, ICG-001 therapy restored expression of nephrin, podocin, and Wilms' tumor 1, attenuated interstitial myofibroblast activation, repressed matrix expression, and inhibited renal inflammation and fibrosis. The results indicated that blockade of Wnt/β-catenin signaling can simultaneously repress multiple RAS genes, thereby leading to the reversal of established proteinuria and kidney injury (Zhou et al., 2015).

Further research on the contribution of those novel axes to renal fibrosis is in warranted. The clarification of the molecular mechanism mediating renal fibrosis may lead to the development of new approaches in the design of agonist or antagonists of RAS axis.

Summary

In addition to classical renin/ACE/Ang II/AT1 and AT2 axis, recent studies have shown that multiple new members of RAS system play critical role in both glomerularsclerosis and tubularsclerosis, with synergic or antagonistic effect to classical RAS (summarized in **Figure 1**). However, the exact mechanisms through which the new players contribute to renal fibrosis are still

unclear and the therapeutic potential targeting those members are still in controversial. Thus, based on the review mentioned above, the issues that need further observations include: How did the microenvironment (lipid accumulation, albumin, inflammation etc.) change the ACE/ACE2 axis balance and how to regulate it properly? How did the PRR been switch to pro-fibrotic factor and what is the exact role in development of renal fibrosis? What is the role of Ang A, alamandine-MrgD in renal fibrosis? Shall we develop more reliable approaches to prevent or reverse renal fibrosis with agonist or antagonists of new RAS members? Obviously, understanding these issues will provide more strategies for the intervention of renal fibrosis.

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REVIEW
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The JNK Signaling Pathway in Renal Fibrosis

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Fibrosis of the glomerular and tubulointerstitial compartments is a common feature of chronic kidney disease leading to end-stage renal failure. This fibrotic process involves a number of pathologic mechanisms, including cell death and inflammation. This review focuses on the role of the c-Jun amino terminal kinase (JNK) signaling pathway in the development of renal fibrosis. The JNK pathway is activated in response to various cellular stresses and plays an important role in cell death and inflammation. Activation of JNK signaling is a common feature in most forms of human kidney injury, evident in both intrinsic glomerular and tubular cells as well as in infiltrating leukocytes. Similar patterns of JNK activation are evident in animal models of acute and chronic renal injury. Administration of JNK inhibitors can protect against acute kidney injury and suppress the development of glomerulosclerosis and tubulointerstitial fibrosis. In particular, JNK activation in tubular epithelial cells may be a pivotal mechanism in determining the outcome of both acute kidney injury and progression of chronic kidney disease. JNK signaling promotes tubular epithelial cell production of pro-inflammatory and pro-fibrotic molecules as well as tubular cell de-differentiation toward a mesenchymal phenotype. However, the role of JNK within renal fibroblasts is less well-characterized. The JNK pathway interacts with other pro-fibrotic pathways, most notable with the TGF-β/SMAD pathway. JNK activation can augment TGF-β gene transcription, induce expression of enzymes that activate the latent form of TGF-β, and JNK directly phosphorylates SMAD3 to enhance transcription of pro-fibrotic molecules. In conclusion, JNK signaling plays an integral role in several key mechanisms operating in renal fibrosis. Targeting of JNK enzymes has therapeutic potential for the treatment of fibrotic kidney diseases.

Keywords: apoptosis, ASK1, kidney disease, kidney inflammation, p38 MAPK, SMAD3

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INTRODUCTION

Kidney disease can be classified as acute or chronic and can affect the glomerular, tubular, and interstitial compartments of the kidney. Acute kidney injury can be directed at the glomerulus (glomerulonephritis), tubules (acute tubular necrosis), or interstitium (acute interstitial nephritis). Precipitants of acute kidney injury are varied but include immunological, hypoxic, and toxin mediated causes. A wide range of insults including immunity, hypertension, diabetes, and prior acute injury can induce progressive chronic disease which is hallmarked by inflammation and fibrosis leading to end-stage kidney disease.

Renal fibrosis involves a loss of renal parenchyma and an excessive deposition of extra-cellular matrix. This is evident in all progressive forms of chronic kidney disease irrespective of the

initial injury. Glomerulosclerosis and, in particular, interstitial fibrosis correlates with loss of renal function and is considered a common pathway leading to end stage renal disease (Mackensen-Haen et al., 1981; Nath, 1992). A wide range of factors can promote renal fibrosis of which pathologic cell death and chronic inflammation are considered to be major mechanisms underpinning this destructive process (Meng et al., 2014). Tubular epithelial cells can become active drivers of the inflammatory and fibrotic processes following either direct tubular damage or tubular damage secondary to glomerular injury (Meng et al., 2014). A number of signaling pathways have been implicated in the fibrotic process (e.g., TGF-β/SMAD, Wnt/β-catenin, Jagged/Notch, EGF-R, and JAK/STAT). This review focuses on the role of the c-Jun amino terminal kinase (JNK) in renal fibrosis.

OVERVIEW OF THE JNK PATHWAY

JNK is one of three well-characterized mitogen-activated protein kinase (MAPK) pathways which transduce extra-cellular signals to control processes such as cell proliferation, differentiation, migration, and apoptosis (Bode and Dong, 2007). Each of these MAPK pathways are activated via a cascade of phosphorylation reactions. In the case of JNK, upstream MAP3K family members phosphorylate and activate MAP2K enzymes (MKK4 and MKK7), which, in turn, phosphorylate and activate JNK (**Figure 1**). Rapid activation of the pathway is facilitated by scaffold proteins, such as JNK interacting protein 1 (JIP1) (Avruch, 2007). On the other hand, inactivation of the JNK pathway occurs by dephosphorylation performed by a group of dual specificity phosphatases (Patterson et al., 2009).

Upon activation, JNK can phosphorylate serine and threonine residues in specific protein substrates. JNK derives its name from its ability to phosphorylate serine residues at positions 63 and 73 in the amino-terminal domain of the c-Jun proto-oncogene (Bode and Dong, 2007; Wagner and Nebreda, 2009). There are three genes in the JNK family (*Jnk1/Mapk8*, *Jnk2/Mapk9,* and *Jnk3/Mapk10*). JNK1 and JNK2 enzymes are expressed in most cells of the body, including the kidney, whereas expression of JNK3 is limited to the brain, heart, and testis (Gupta et al., 1996; Kumar et al., 2015). Deletion of both *Jnk1* and *Jnk2* genes causes fetal lethality, whereas deletion of either *Jnk1* or *Jnk2* results in viable and healthy mice (Wagner and Nebreda, 2009). Alternative splicing of mRNA from these three genes gives rise to at least 10 different JNK enzyme isoforms ranging between 46 and 55 kDa (Gupta et al., 1996). The JNK pathway is broadly expressed but context specific, indicating that specific stimuli can activate distinct cellular pathways and responses (Davis, 2000).

An important aspect of the JNK pathway is that it can be activated in response to a range of stimuli that have been implicated in acute and chronic kidney injury, including: pro-inflammatory cytokines, danger-associated molecules pattern ligands (alarmins), oxidative stress, pro-fibrotic factors, and nephrotoxins (**Figure 1**). Thus, JNK signaling may contribute to renal fibrosis through induction of apoptosis and inflammation as well as a direct contribution to fibrosis itself.

JNK in Apoptosis

JNK is best known for its role in the induction of cell apoptosis (Davis, 2000). JNK is directly involved in the mitochondrial (or intrinsic) pathway of apoptosis (**Figure 1**). Activation of JNK by oxidative stress causes mitochondrial release of cytochrome c into the cytoplasm leading to caspase activation and apoptosis (Tournier et al., 2000). This is thought to operate via B cell lymphoma (Bcl-2) and Bcl-2 like 1 gene (Bcl-xs) oncoproteins on the surface of mitochondria (Maundrell et al., 1997). However, JNK signaling is not always pro-apoptotic and can be context specific. For example, in fibroblasts JNK can act to suppress TNF-stimulated apoptosis, but JNK can also potentiate TNF-stimulated cell death via increased production of reactive oxygen species (Ventura et al., 2004). Of note, JNK activation can also promote cell survival through the induction of autophagy. Under conditions of cell starvation, JNK1 but not JNK2, can phosphorylate Bcl-2 causing disruption of the Bcl-2/beclin-1 complex and activation of the autophagy response (Wei et al., 2008).

JNK in Inflammation

The JNK pathway can promote an inflammatory response in both leukocytes and non-leukocytes. Although the nature of the stimuli inducing JNK activation may vary depending upon the cell type and nature of tissue injury, a primary mechanism through which the JNK pathway promotes inflammation is the transcription factor, AP-1 (**Figure 1**). JNK can phosphorylate c-Jun which enables dimerization with c-Fos to make AP-1 which transcribes a wide range of genes that orchestrate the inflammatory response, including cytokines (e.g., TNF-α), chemokines (e.g., CCL2), and leukocyte adhesion molecules (e.g., VCAM-1/CD106) (Ip and Davis, 1998). Thus, activation of JNK in endothelial cells can facilitate adhesion and transmigration of leukocytes via up-regulation of chemokines and adhesion molecules, while JNK activation in epithelial cells (e.g., in the injured kidney, lung, or liver) can recruit and activate leukocyte populations via chemokine and cytokine production. A second phosphorylation target of JNK is activating transcription factor 2 (ATF-2) which also transcribes genes that contribute to the inflammatory response (Yu et al., 2014). In addition, JNK signaling also plays a role in activation of Th1 and Th2 subsets of T cells (Davis, 2000).

Nuclear factor-kappaB (NF-kB) is a major factor that promotes transcription of genes involved in the inflammatory and anti-apoptotic responses (Workman and Habelhah, 2013). Many of the same stimuli (e.g., TNF-α, IL-1, LPS, oxidative stress) can induce both JNK/AP-1 and NF-kB activation which drives inflammation (Workman and Habelhah, 2013). In addition, JNK can directly activate NF-kB by promoting IkBα degradation (Ruan et al., 2015). Thus, while JNK/AP1 and NF-kB can coordinate the inflammatory response, NF-kB acts to limit JNK-dependent cell death (Workman and Habelhah, 2013; Ruan et al., 2015).

Thus, activation of the JNK pathway has the potential to promote renal fibrosis through its pro-apoptotic and pro-inflammatory actions. In addition, as discussed below, there is

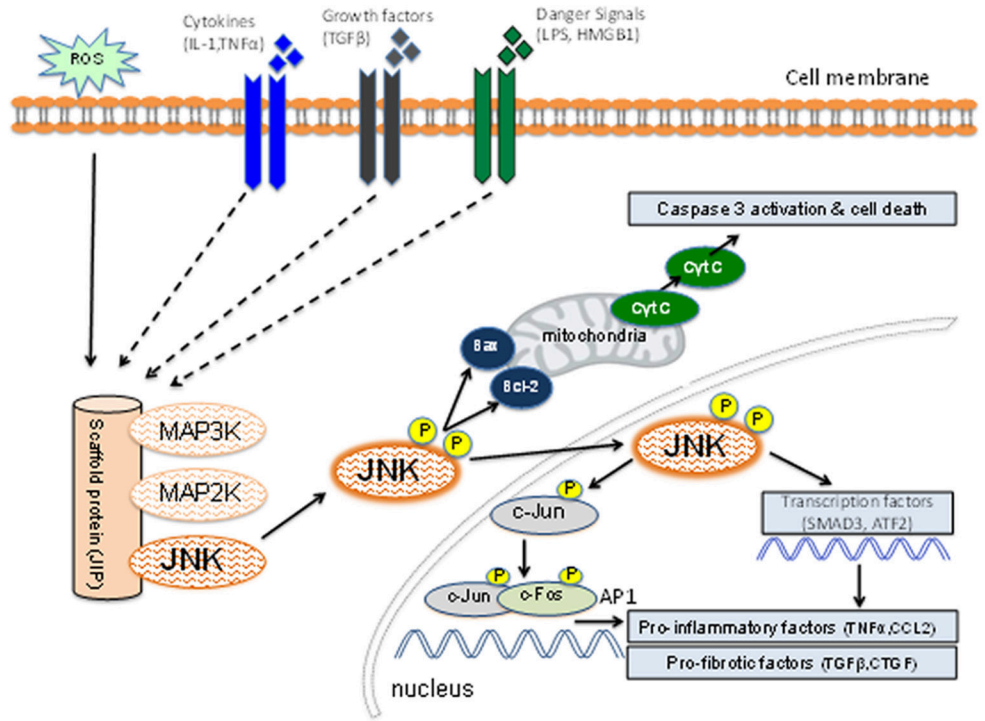

FIGURE 1 | Schematic diagram of the JNK signaling pathway. Inflammatory cytokines, danger-associated molecules pattern ligands (alarmins), and pro-fibrotic growth factors can induce activation of members of the mitogen-activated protein kinase kinase kinase (MAP3K) family. Other factors such as reactive oxygen species (ROS) and osmotic stress can also lead to MAP3K activation. Individual members of the MAP3K and MAP2K families plus JNK are held in close proximity by the scaffold protein (JNK-interacting protein-1) which facilitates a rapid series of phosphorylation reactions leading to phosphorylation of the activation site of JNK. The activated JNK then dissociates from this complex and can promote mitochondria-dependent apoptosis through B cell lymphoma (Bcl-2) and Bcl-2 associated x-protein (Bax). Active JNK can promote transcription of genes involved in the inflammatory and fibrotic responses through phosphorylation of c-Jun [to promote activator protein-1 (AP-1) function], SMAD3 and ATF2. JNK activation can promote cell death, inflammation and fibrosis.

now evidence that JNK signaling can act directly to potentiate the fibrotic response.

Inhibition of the JNK Pathway

A standard approach to defining the function of a signaling pathway is through genetic and/or pharmacologic inhibition. In the case of JNK signaling, deletion of one or more of *Jnk1-3* genes has been highly informative both in terms of individual gene function and for identifying redundancy in the signaling pathway (Wagner and Nebreda, 2009). Two main approaches have been used for pharmacologic inhibition of JNK activity. A series of small molecule inhibitors such as SP600125 and CC-401 have been developed which inhibit JNK1-3 with IC_{50} values of 25–90 mM (Bennett et al., 2001; Ma et al., 2007a), while CC-930 inhibits JNK2/3 (6–7 nM) more potently than JNK1 (61 nM) (Plantevin Krenitsky et al., 2012). A second approach has been to block the JNK-interacting protein 1 (JIP1) scaffold protein from binding JNK enzymes. This utilizes a 20 amino acid peptide from the JIP1 protein to compete for JNK binding. Conjugated of the JIP1 peptide with a 10 amino acid HIV–TAT transporter sequence provides a cell-permeable molecule that can inhibit all JNK enzymes (Vivès et al., 1997). These strategies have been instrumental in determining the role of JNK in kidney injury and fibrosis.

JNK SIGNALING IN GLOMERULAR DISEASE

Two main approaches have been used to detect activation of the JNK pathway in tissues. First, tissue lysates can be examined for phosphorylation of the activate site of the JNK enzyme ($JNK^{Tyr183/185}$) by Western blotting or JNK activity can be directly measured in a kinase assay. Second, immunohistochemistry staining for phospho-$JNK^{Tyr183/185}$ can identify and localize cell types in which JNK signaling occurs. An indirect method to assess JNK signaling in tissue lysates or sections is to measure c-Jun^{Ser63} phosphorylation (referred to as p-c-Jun) since JNK is the only kinase known to phosphorylate c-Jun in this position.

Immunohistochemistry staining has identified JNK activation in collecting duct and parietal epithelial cells in normal mouse, rat and human kidney (Flanc et al., 2007; Ma et al., 2007a; Kanellis et al., 2010). While p-c-Jun staining can be seen in a small number of parietal epithelial cells in normal kidney, this is not apparent in collecting ducts which lack c-Jun expression under normal

conditions (De Borst et al., 2007; Ma et al., 2007a; Kanellis et al., 2010).

JNK Activation in Glomerular Disease

Immunohistochemistry staining for p-JNK and p-c-Jun in renal biopsies have identified marked JNK activation in most types of glomerulonephritis as well as in diabetic nephropathy (De Borst et al., 2007; de Borst et al., 2009). Numerous glomerular cells exhibit JNK activation across all diseases and this correlates with the degree of macrophage infiltration and glomerulosclerosis, but not with proteinuria or renal function (De Borst et al., 2007; de Borst et al., 2009).

JNK activation has also been identified in a diverse range of animal models of glomerular disease, including; crescentic glomerulonephritis, diabetic nephropathy, minimal change disease, Alport's syndrome, and salt-sensitive hypertension (Nishiyama et al., 2004; Park and Jeong, 2004; Flanc et al., 2007; Lim et al., 2011; Nakagawa et al., 2016). In addition, increased JNK activation has been identified in the aging kidney (Kim et al., 2002).

JNK Blockade in Experimental Glomerular Disease

Acute anti-glomerular basement membrane (GBM) glomerulonephritis features JNK activation in glomerular podocytes and parietal epithelial cells as well as infiltrating macrophages (**Figures 2A,B**). As disease progresses, JNK activation becomes evident in cells within glomerular crescents, including multi-nucleated macrophage giant cells, together with extensive JNK activation in tubular epithelial cells

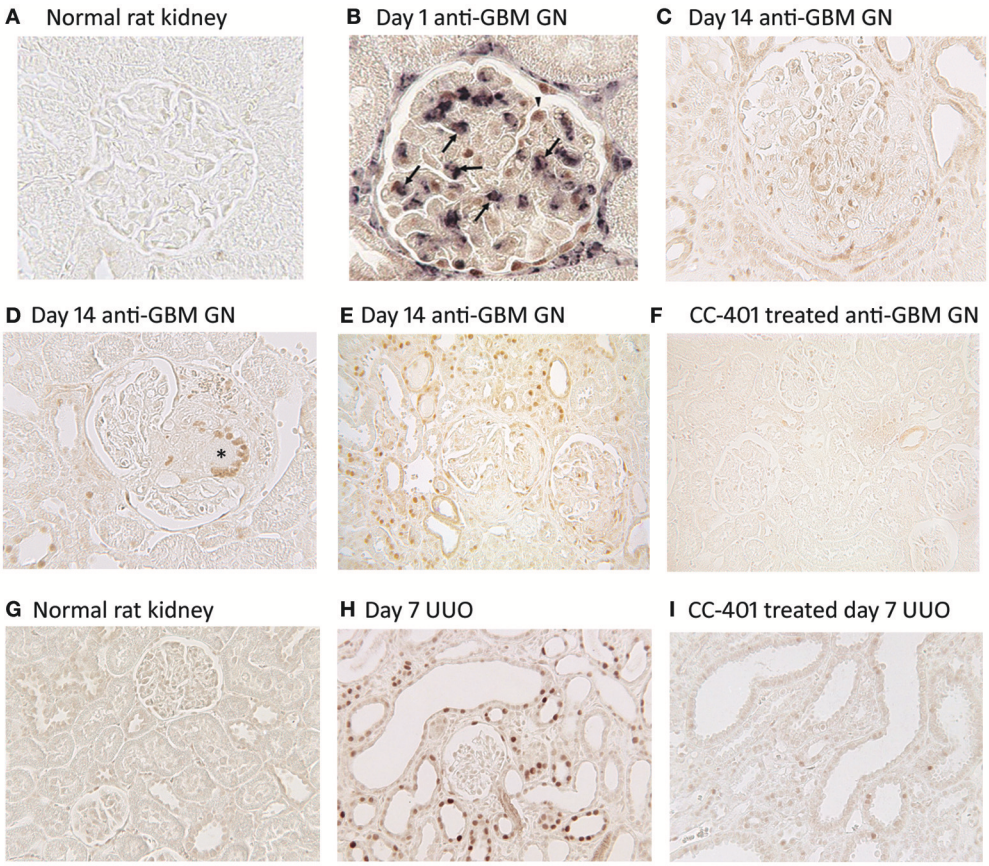

FIGURE 2 | JNK activation in kidney injury. Detection of JNK activation by immunohistochemistry staining for c-Jun phosphorylated at Ser63 (p-c-Jun). **(A–F)** JNK activation in rat anti-glomerular basement membrane (GBM) glomerulonephritis (GN). **(A)** Normal rat glomeruli lack p-c-Jun staining. **(B)** Two color immunostaining for p-c-Jun (brown nuclei) and ED1+ macrophages (purple/blue) in a glomerulus on day 1 of anti-GBM disease. Arrows indicate cells stained for both ED1 and p-c-Jun, demonstrating JNK signaling in macrophages. Arrowhead shows p-c-Jun immunostaining in a podocyte-like cell. Parietal epithelial cells staining for p-c-Jun is also evident. **(C)** Glomerulus on day 24 of anti-GBM disease shows nuclear p-c-Jun staining of cells in the capillary tuft and in the crescent. **(D)** Day 24 of anti-GBM disease showing p-c-Jun staining in a multinucleated giant cell in a glomerular crescent (*). **(E)** Lower power view of day 24 anti-GBM disease showing p-c-Jun+ cells in the glomerular tuft, in crescents, in many tubular epithelial cells and in some interstitial cells. **(F)** Treatment with the JNK inhibitor CC-401 over days 7–24 of anti-GBM disease blocks c-Jun phosphorylation. **(G–I)** JNK activation in the rat unilateral ureteric obstruction (UUO) model. **(G)** Normal rat kidney shows a lack of p-c-Jun staining in glomeruli and tubules. **(H)** Day 7 after UUO shows extensive JNK activation in both atrophic tubules and in tubules with normal morphology. Glomeruli do not show inflammation in this model and they lack p-c-Jun staining. **(I)** Treatment with the JNK inhibitor CC-401 over days 0–7 of the UUO model blocks c-Jun phosphorylation. Reproduced with permission from Flanc et al. (2007) **(A,B)**; Ma et al. (2009) **(C–F)**, and; Ma et al. (2007a) **(G–I)**.

during secondary tubulointerstitial damage (**Figures 2C–E**). Administration of a JNK inhibitor (CC-401) abrogated phosphorylation of c-Jun in diseased kidneys demonstrating effective JNK blockade (**Figure 2F**). This resulted in significant protection from acute and progressive proteinuria. JNK blockade also protected against glomerular damage, including crescent formation and sclerosis (Flanc et al., 2007). However, this protection was independent of glomerular neutrophil and T cell infiltration. In a subsequent study, intervention with CC-401 during ongoing renal injury prevented crescent formation and halted the development of renal failure and renal fibrosis (Ma et al., 2009). While JNK blockade did not prevent macrophage recruitment into the kidney, it did suppress the M1 pro-inflammatory macrophage response (Flanc et al., 2007; Ma et al., 2009). This is consistent with the established role for macrophages in causing renal injury in this model (Han et al., 2011), and with adoptive transfer studies in which selective JNK blockade in macrophages prevented their activation within the glomerulus and thereby prevented acute glomerular injury in this model (Ikezumi et al., 2004).

In contrast to the anti-GBM model, JNK signaling does not play a clear pathologic role in diabetic nephropathy. Activation of JNK has been described in human diabetic nephropathy (De Borst et al., 2007), streptozotocin-induced diabetic nephropathy in the spontaneous hypertensive rat and in the *db/db* model of spontaneous type 2 diabetic nephropathy (Ijaz et al., 2009; Lim et al., 2011). However, blockade of JNK signaling using either a selective kinase inhibitor (CC-930) or a JIP1 peptide-based inhibitor failed to protect the kidney from mesangial expansion and caused a modest increase in the severity of podocyte damage and albuminuria (Ijaz et al., 2009; Lim et al., 2011). Why JNK blockade exacerbated podocyte damage in diabetic nephropathy is unclear since JNK blockade protects cultured podocytes against injury caused by TNF-α or by antibody plus complement (Peng et al., 2002; Ikezumi et al., 2008).

Of note, podocytes express JunD, an atypical component of the AP-1 family, and JunD confers protection against glomerlosclerosis in anti-GBM disease and subtotal nephrectomy models (Pillebout et al., 2003; Hernandez et al., 2008; Cook et al., 2011). While JNK can phosphorylate and activate JunD, it is not the only kinase which can perform this function, and this redundancy probably explains why JNK blockade is protective in anti-GBM disease whereas *JunD* gene deletion is detrimental (Flanc et al., 2007; Ma et al., 2009; Cook et al., 2011). Activation of the JNK pathway may also facilitate glomerulosclerosis through PDGF and angiotensin II induced proliferation of mesangial cells (Kawano et al., 2003; Zhang et al., 2005).

JNK activation has also been described in glomerular endothelial cells (De Borst et al., 2007). Previous studies have shown that cytokine induced *Ccl2* gene transcription in cultured human endothelial cells depends on the cooperative action of AP1 and NF-kB (Martin et al., 1997), and that uremic toxins can activate glomerular endothelial cells via JNK signaling (Shen et al., 2016). However, a direct role for endothelial cell JNK activation in regulating glomerular inflammation remains to be demonstrated.

In summary, JNK signaling can promote glomerular necrosis and fibrosis via effects upon macrophages, endothelial cells, and mesangial cells. JNK signaling can induce podocyte damage and albuminuria, while JNK activation promotes glomerular crescent formation via effects upon macrophages and possibly on parietal epithelial cells.

JNK SIGNALING IN TUBULOINTERSTITIAL INJURY AND FIBROSIS

JNK signaling in tubular epithelial cells can be seen as a key determinant in the development of tubulointerstitial injury and fibrosis based upon the common finding of tubular JNK activation in human and experimental kidney disease. This can result in cell death via apoptosis or necrosis, activation of pro-inflammatory and pro-fibrotic responses, and tubular cell de-differentiation leading to activation of a mesenchymal gene signature (**Figure 3**).

Prominent JNK activation in many tubular epithelial cells is a striking aspect of many forms of human glomerular disease

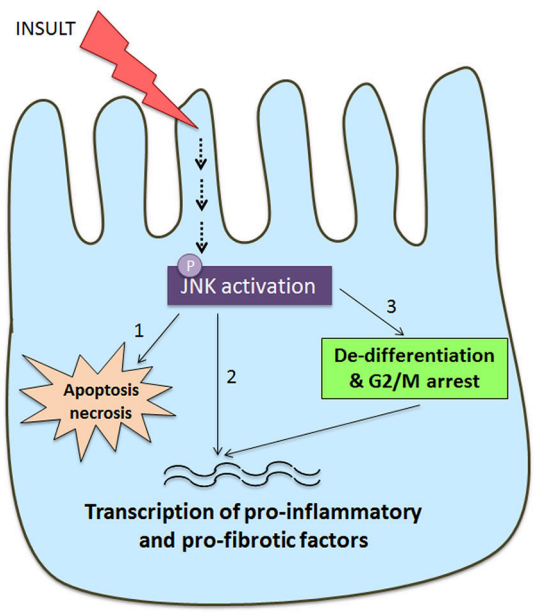

FIGURE 3 | Schematic diagram of JNK signaling in the tubular epithelial cell response to damage**.** Many insults to the tubular epithelial cell, such as oxidative stress and nephrotoxins, as well as cytokines and growth factors can result in activation of the JNK pathway. JNK signaling in tubular epithelial cells can result in tubular cell damage and consequent tubulointerstitial damage via three pathways. (1) JNK activation can directly induce apoptosis and necrosis via mitochondrial dependent mechanisms. (2) JNK phosphorylates a number of transcription factors (e.g., c-Jun, SMAD3, ATF2) resulting in tubular production of pro-inflammatory and pro-fibrotic molecules that promote recruitment and activation of macrophages and myofibroblasts. (3) JNK activation is implicated TGF-β induced de-differentiation of tubular cells toward a partial mesenchymal phenotype with production of pro-fibrotic factors. This switch toward a pro-fibrotic mesenchymal phenotype can also be induced by JNK-dependent arrest at the G2/M phase of the cell cycle when tubular cells are undergoing a repair response.

(De Borst et al., 2007; de Borst et al., 2009). Indeed, JNK activation in tubulointerstitial cells gave a significant correlation with declining renal function, macrophage infiltration, tubular damage, and interstitial fibrosis (De Borst et al., 2007; de Borst et al., 2009). Two color immunohistochemistry identified JNK activation in damaged tubules and in areas of macrophage infiltration and fibrosis (De Borst et al., 2007), suggesting an active role for tubular JNK signaling in this process. Support for this proposal comes from studies of animal models of anti-GBM glomerulonephritis and Alport's syndrome in which prominent tubular JNK activation develops secondary to glomerular injury (Ma et al., 2009; Nakagawa et al., 2016). Indeed, commencing JNK inhibitor treatment (CC-401) in established anti-GBM disease resulted in marked protection from interstitial macrophage infiltration, tubular damage and interstitial fibrosis (Ma et al., 2009); however, this could also be, in part, due to inhibition of the primary glomerular injury.

JNK in Acute Kidney Injury

It is now well-recognized that episodes of acute kidney injury (AKI) are linked to subsequent development of chronic kidney disease (Zuk and Bonventre, 2016), emphasizing the importance of limiting the severity of AKI. A number of factors can induce AKI including sepsis, drug toxicity, and ischaemia. Indeed, AKI is a predictable outcome in a number of settings, including cardiac bypass surgery, kidney transplantation, and treatment with chemotherapeutic drugs such as cisplatin.

Tubular epithelial cells exhibit dramatic activation of the JNK pathway within minutes of reperfusion of the ischaemic kidney. This has been identified in post perfusion human renal transplant biopsies (Kanellis et al., 2010). The highest levels of JNK activation were seen in kidneys from deceased donors when compared with kidneys from living donors, with the degree of JNK activation correlating with ischaemic time in deceased donor allografts (Kanellis et al., 2010). Animal models recapitulate this rapid JNK activation in tubular epithelial cells following ischaemia/reperfusion (I/R) injury (Wang et al., 2007; de Borst et al., 2009; Kanellis et al., 2010). Systemic treatment with a JNK inhibitor (CC-401 or SP600125) prior to bilateral renal I/R injury protects against tubular damage and acute renal failure (Wang et al., 2007; Kanellis et al., 2010). Notably, delaying JNK inhibitor treatment until 1 h after reperfusion conferred no benefit, confirming that the early peak of JNK activation is the main pathologic event to be targeted (Kanellis et al., 2010). The postulated mechanisms for the protection seen with systemic JNK blockade are reduced tubular necrosis and apoptosis, reduced expression of pro-inflammatory molecules (e.g., TNF-α and CCL2) by tubular epithelial cells and reduced interstitial accumulation and activation of macrophages and T-cells (Wang et al., 2007; de Borst et al., 2009; Kanellis et al., 2010).

Endothelial cell activation is an important mechanism in renal I/R injury with blockade of the adhesion molecule CD54 (ICAM-1) suppressing leukocyte recruitment and renal injury (Kelly et al., 1996). Systemic delivery of a JNK inhibitor (CC-401) reduced endothelial and tubular CD54 expression and infiltration by T cells and macrophages (Kanellis et al., 2010). A direct role for endothelial JNK activation in inflammation is suggested by studies in which JNK blockade in cultured microvascular endothelial cells prevented diapedesis of CD4+ T cells across the endothelial layer (Dragoni et al., 2017). Furthermore, the direct delivery of a JIP-1 peptide-based JNK inhibitor to the renal endothelium via renal artery infusion reduced the severity of renal dysfunction in subsequent renal I/R injury, although leukocyte recruitment was not investigated (Doi et al., 2013).

Acute kidney injury induced by the nephrotoxin cisplatin is also associated with prominent tubular JNK activation, particularly in older mice which are more susceptible to developing AKI (Guan et al., 2017). Administration of a JNK inhibitor (SP600125) substantially protected against cisplatin-induced tubular damage and acute renal failure (Francescato et al., 2007). Indeed, numerous *in vitro* studies have shown that JNK signaling directly contributes to tubular epithelial cell death in response to noxious stimuli such as ATP depletion (Ma and Devarajan, 2008), reactive oxygen species (Pat et al., 2003; Arany et al., 2004), and nephrotoxic molecules (de Graauw et al., 2005). Thus, there is good evidence to conclude that JNK activation in tubular epithelial cells is a key determinant in the outcome of acute kidney injury.

JNK in Primary Tubulointerstitial Fibrosis

JNK activation in tubular epithelial cells is a common response to various stressors. The role of JNK signaling in renal interstitial fibrosis was first examined using unilateral ureteric obstruction (UUO). The UUO model features a rapid development of interstitial fibrosis due to a primary stretch-induced injury to tubular epithelial cells. There is dramatic and persistent JNK signaling in dilated and atrophic tubules as well as in tubules with normal morphology following ureteric obstruction (**Figures 2G,H**). Gene deletion of either *Jnk1* or *Jnk2* did not affect the prominent tubular JNK activation in the obstructed kidney or the development of renal fibrosis, indicating redundancy between JNK1 and JNK2 in this model (Ma et al., 2007a). However, treatment with a JNK inhibitor (CC-401) efficiently blocked JNK signaling (**Figure 2I**), and provided substantial protection against interstitial fibrosis in terms of myofibroblast accumulation and collagen deposition, as well as reducing mRNA levels of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (Ma et al., 2007a). Tubular apoptosis is also evident on day 7 of the UUO model and this was significantly reduced in *Jnk1* (but not *Jnk2*) deficient mice as well as with CC-401 treatment, providing the first demonstration that JNK1 has a selective role in apoptosis of kidney cells (Ma et al., 2007a).

JNK and Tubular De-Differentiation

The contribution of tubular epithelial cells to the development of renal interstitial fibrosis is a topic of great interest. While tubular epithelial cells can undergo complete morphologic and functional transition into myofibroblasts in a TGF-β/JNK dependent mechanism *in vitro* (Fan et al., 1999; Mariasegaram et al., 2010), this transition is a rare event *in vivo* in human and experimental kidney disease (Ng et al., 1998; Jinde et al., 2001). Recent lineage tracing studies in mouse models of kidney disease have either

found no evidence to support this mechanism (Grgic et al., 2012), or suggest that tubular epithelial cells account for less than 5% of interstitial fibroblasts (LeBleu et al., 2013). Nevertheless, studies have shown that even partial de-differentiation of tubular epithelial cells—as a result of incomplete repair following tubular cell injury—can result in expression of a mesenchymal and pro-fibrotic gene expression program (Yang et al., 2010; Lan et al., 2012; Zhu et al., 2016). Using several models of tubular injury (severe I/R injury, UUO, and aristolochic acid nephropathy), a subset of tubular epithelial cells were shown to be arrested in the G2/M phase of the cell cycle (Yang et al., 2010). *In vitro* studies identified a role for JNK in aristolochic acid-induced G2/M arrest in cultured tubular epithelial cells, and administration of a JNK inhibitor (SP600125) in a model of severe I/R injury suppressed both cell cycle arrest and development of interstitial fibrosis (Yang et al., 2010). Consistent with this finding, JNK activation is seen in tubular epithelial cells with *de novo* vimentin expression (a marker of de-differentiation) on day 14 after severe I/R injury and in human glomerular disease featuring severe tubulointerstitial damage (Lan et al., 2012). In addition, the differentiation factor Numb has been identified as promoting tubular cell G2/M arrest during interstitial fibrosis in association with activation of JNK signaling (Zhu et al., 2016). However, a study of cultured tubular epithelial cells found that JNK inhibition could suppress TGF-β1 induced PDGF-B production, but did not affect G2/M cell cycle arrest (Wu et al., 2013), questioning whether these two responses are directly linked.

Direct evidence that tubular de-differentiation drives interstitial fibrosis comes from studies using conditional gene deletion in tubular epithelial cells. Deletion of key mesenchymal proteins, Snail1 or Snail1/TWIST, in tubular epithelial cells resulted in reduced levels of interstitial fibrosis across three different disease models (UUO, folic acid nephropathy, and nephrotoxic serum nephritis) (Grande et al., 2015; Lovisa et al., 2015). A subset of tubular cells were shown to lose their epithelial markers and acquired mesenchymal markers, such as vimentin, without leaving the tubules, thereby undergoing a process of de-differentiation, rather than a complete transition into fibroblasts (Grande et al., 2015; Lovisa et al., 2015). Of note, inducible over-expression of c-Jun, and thereby over-activation of AP-1, in the tubular compartment is sufficient to induce tubular atrophy, severe tubulointerstitial fibrosis and renal failure (Wernig et al., 2017).

JNK in Tubular Cell and Fibroblast Activation

Tubular epithelial cells are a major site of JNK activation in kidney disease and systemic JNK blockade demonstrates a pathologic role for this pathway in models of tubulointerstitial damage and fibrosis. However, since JNK signaling can be activated in many cell types, including endothelial cells and infiltrating macrophages (Flanc et al., 2007; de Borst et al., 2009), systemic blockade does not provide clear delineation of JNK function in tubular epithelial cells in disease. In the absence of studies using conditional gene deletion, this issue has been addressed using cultured tubular epithelial cells.

Tubular epithelial cells are a major source of chemokines (e.g., CCL2) and pro-fibrotic factors (e.g., TGF-β1, PDGF-B) in tubulointerstitial fibrosis (Chow et al., 2006; Ma et al., 2007b; de Borst et al., 2009), and this is suppressed by systemic JNK blockade (Ma et al., 2007a; de Borst et al., 2009). Consistent with these findings, studies in cultured tubular epithelial cells have shown that IL-1β and TGF-β1 induced MCP-1 expression (de Borst et al., 2009), and IL-1 and TNF-α induced IL-6 production (de Haij et al., 2005), operate in a JNK-dependent fashion. Similarly, tubular cell secretion and activation of TGF-β1 in response angiotensin II and aristolochic acid stimulation operates via JNK signaling (Naito et al., 2004; Ma et al., 2007a; Rui et al., 2012). Finally, the transition of tubular epithelial cells toward a mesenchymal phenotype in response to TGF-β1, alpha 2-antiplasmin, or aristolochic acid operates via JNK signaling (Mariasegaram et al., 2010; Zhou et al., 2010; Kanno et al., 2014).

While JNK activation in tubular cells and infiltrating macrophages can promote fibroblast activation and collagen production, it is less clear whether JNK signaling is important in the fibroblast itself. Studies of cultured fibroblasts have identified a role for JNK signaling in proliferation induced by PDGF, TGF-β1, and aldosterone (Khalil et al., 2005; Huang et al., 2012). However, the role of JNK in cell proliferation is highly context dependent. For example, PDGF-B induced proliferation is JNK dependent in pulmonary artery fibroblasts isolated from animals exposed to hypoxia but not in fibroblasts from control animals (Panzhinskiy et al., 2012), and PDGF-induced proliferation of pericytes was not affected by JNK inhibition (Ren et al., 2013).

In summary, JNK activation in tubular epithelial cells can promote cell death following severe injury or promote progressive tubulointerstitial inflammation and fibrosis in response to a less severe chronic insult. This chronic pathologic process is also promoted by JNK activation in interstitial macrophages, fibroblasts, and endothelial cells.

JNK AND TGF-β1/SMAD SIGNALING

JNK interacts with a number of pathways in the fibrotic process of which the best characterized interaction is with the TGF-β/SMAD pathway. The TGF-β/SMAD signaling pathway plays a central role in the development of renal fibrosis (Meng et al., 2016). This is regulated at many levels including; TGF-β production and secretion, activation of latent TGF-β, and the activity of the SMAD2/3/4 complex to promote transcription of pro-fibrotic molecules (Meng et al., 2016). JNK signaling can enhance pro-fibrotic TGF-β/SMAD signaling through at least three distinct mechanisms. First, JNK activation induced by factors such as angiotensin II, IL-1, TNF-α, and aristolochic acid can increase TGF-β production through activation of AP-1 which binds to distinct sites in the *Tgfb* gene promoter to increase transcription (Kim et al., 1990; Naito et al., 2004; Lee et al., 2006; Rui et al., 2012). In addition, TGF-β induces a positive feedback loop to continue MAPK stimulation via TAK1, another upstream p38/JNK activator (Yamaguchi et al.,

1995). Second, JNK signaling can enhance tubular production of thrombospondin-1 which, in turn, induces activation of the latent form of TGF-β1 (Naito et al., 2004). Third, JNK can directly phosphorylate residues in the linker regions of SMAD3 which enhances SMAD3 transcriptional activity (Velden et al., 2011). In addition, a direct interaction between JNK and SMAD3 has been shown in the UUO model of renal fibrosis (Sun et al., 2013). Immunoprecipitation studies showed increased levels of JNK activation as well as increased phosphorylation of SMAD3 at its linker region are very early events following UUO surgery. This interaction is also important in stimulating fibroblast proliferation and collagen production (Sun et al., 2013). However, the JNK pathway can also act to limit TGF-β/SMAD signaling. In contrast to the other SMAD proteins, SMAD7 is a negative feedback inhibitor. It competes with the SMAD2/3 complex for binding to the TGF receptor, thereby decreasing TGF-β signaling (Yan and Chen, 2011). Mice deficient in SMAD7 have increased susceptibility to renal fibrosis, whereas those with overexpression of SMAD7 have attenuated fibrosis (Chen et al., 2011). TGF–β induced up-regulation of inhibitory SMAD7 in enhanced by JNK signaling (Uchida et al., 2001).

TARGETTING KINASES UPSTREAM OF JNK IN RENAL FIBROSIS

Targeting JNK directly using small molecule compounds has identified a pro-inflammatory and pro-fibrotic role for JNK in most, but not all, types of experimental kidney disease. However, given that JNK has a clear function in normal physiology, as shown by fetal lethality of combined *Jnk1*/*Jnk2* gene deletion (Yang et al., 1997), it may be desirable to target only JNK signaling induced by specific stimuli. In theory, this can be done by blocking individual enzymes in the upstream MAP2K or MAP3K families, although this may also result in inhibiting other signaling pathways. One example is transforming growth factor β-activated kinase 1 (TAK1/MAP3K7), a member of the MAP3K family (Wang et al., 2001). As the name implies, TAK1 can be activated by the pro-fibrotic factor TGF-β1, although the LPS receptor (TLR4) and the IL-1 and TNF-α receptors are more potent activators of this enzyme. TAK1 signaling leads to activation of JNK, p38 MAPK, and NF-kB pathways (Wang et al., 2001; Dai et al., 2012). As *Map3k7* gene deletion is embryonic lethal, conditional global gene deletion was used to investigate this pathway in the UUO model (Ma et al., 2011). Short term *Map3k7* deletion significantly reduced renal fibrosis and inflammation in the obstructed kidney. This was associated with inhibition of JNK, p38 and NF-kB activation and so a specific role for TAK1/JNK signaling could not be distinguished (Ma et al., 2011). While the TAK1 inhibitor 5Z-7-oxozeaenol has been shown to inhibit the development of diabetic nephropathy (Xu et al., 2016), this finding is difficult to interpret as this compound inhibits a wide range of kinases including PDGFR-α, VEGR-R3, Flt3, VEGF-R1, and MEK1 (Jogireddy et al., 2009).

A MAP3K family member, apoptosis signal-regulating kinase 1 (ASK1/MAP3K5) activates only JNK and p38 pathways (Tobiume et al., 2001). In addition, *Map3k5* gene deficient mice are viable and healthy (Gerits et al., 2007) indicating that ASK1 is not essential in physiologic JNK and p38 signaling. *Map3k5* deficient mice exhibit substantial protection from both renal fibrosis and tubular cell apoptosis in the UUO model (Ma et al., 2014). This protective effect was associated with a partial inhibition of JNK activation and a complete inhibition of p38 MAPK activation. Since selective p38 MAPK inhibitors can also suppress interstitial fibrosis in the UUO model (Stambe et al., 2004), this finding does not distinguish between the individual contributions of ASK1/JNK vs. ASK1/p38 signaling. Similarly, the ability of an ASK1 inhibitor to suppress diabetic glomerulosclerosis was associated with partial JNK inhibition and complete inhibition of p38 MAPK signaling (Tesch et al., 2015).

FUTURE STUDIES AND THERAPEUTIC POTENTIAL

Most studies in models of renal fibrosis have relied upon pan-JNK inhibitor drugs. However, many questions remain unanswered. In particular, we have little understanding of the relative contribution of JNK1 vs. JNK2 in different types of renal injury. Also, the use of systemic inhibitors does not provide detailed information regarding the function of JNK activation in different cells types following renal injury. Conditional gene deletion will be important to further our understanding of how JNK signaling promotes different aspects of kidney disease. This will also be important for determining whether we should try and deliver JNK inhibitors to specific cell types, such as tubular epithelial cells, rather than systemic administration. In addition, we need to understand the upstream components in JNK activation in different cell types as this could provide alternative therapeutic targets.

Mixed progress has been made in clinical trials of JNK inhibitors. Following the demonstration that JNK blockade could suppress renal fibrosis (Ma et al., 2007a), JNK inhibitors have been shown to alleviate fibrosis in lung disease (van der Velden et al., 2016) and liver disease (Gautheron et al., 2014). However, a phase 2 trial of the JNK inhibitor CC-930 in idiopathic pulmonary fibrosis was halted due to acute liver toxicity, although reductions in markers of lung fibrosis were noted (van der Velden et al., 2016). This has resulted in development of another JNK inhibitor, CC90001, which is in a phase I trial in patients with idiopathic pulmonary fibrosis (NCT02510937). The JIP1 inhibitor Brimapitide (also known as AM-111 or XG-102) (Vivès et al., 1997) is currently in clinical trials to reduce intraocular inflammation post-cataract surgery (Chiquet et al., 2017), and to prevent acute sensorineural hearing loss (Suckfuell et al., 2014). However, this peptide-based JNK inhibitor has yet to be evaluated in models of renal fibrosis. One alternative strategy to target JNK/p38 MAPK signaling is blockade of the upstream kinase,

ASK1, which is currently in phase 2 clinical trials in patients with stage 3/4 diabetic kidney disease (NCT02177786) and in patients with non-alcoholic steatohepatitis associated liver fibrosis (NCT02466516).

In conclusion, pharmacological blockade of JNK signaling has clear potential for the treatment of acute and chronic kidney diseases. However, whether this is best achieved by systemic JNK inhibition, cell-type specific delivery of JNK inhibitors, use of isoform-specific JNK inhibition, or via targeting an upstream component of the JNK pathway has yet to be determined.

AUTHOR CONTRIBUTIONS

All authors determined the scope and structure of the review. KG led the drafting of the manuscript. All authors critically revised the manuscript and approved the final version.

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REVIEW
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Treatment of chronic kidney diseases with histone deacetylase inhibitors

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Histone deacetylases (HDACs) induce deacetylation of both histone and non-histone proteins and play a critical role in the modulation of physiological and pathological gene expression. Pharmacological inhibition of HDAC has been reported to attenuate progression of renal fibrogenesis in obstructed kidney and reduce cyst formation in polycystic kidney disease. HDAC inhibitors (HDACis) are also able to ameliorate renal lesions in diabetes nephropathy, lupus nephritis, aristolochic acid nephropathy, and transplant nephropathy. The beneficial effects of HDACis are associated with their anti-fibrosis, anti-inflammation, and immunosuppressant effects. In this review, we summarize recent advances on the treatment of various chronic kidney diseases with HDACis in pre-clinical models.

Keywords: histone deacetylases, chronic kidney diseases, renal fibrosis, renal fibroblasts

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Introduction

Histone and non-histone protein acetylation has been widely studied in the field of cancer research (Kwon et al., 2009; Mahalingam et al., 2010; Selinger et al., 2011). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) can mediate the acetylated/deacetylated states of histones (Bush and McKinsey, 2010). HATs induce acetylation of histones H3 and H4 on lysine amino groups (Spencer and Davie, 1999) whereas HDACs remove acetyl groups from the acetylated proteins. Acetylation of the ε-amino groups of lysine residues in nucleosomal histone tails by HATs is considered necessary for the chromatin structure to relax, allowing activation of transcriptional activators and initiation of gene induction. Inhibition of HDACs with HDAC inhibitors (HDACis) also enhances the deposition of acetylated histones H3 and H4, thereby modifying chromatin structure and regulating gene transcription (Turner, 1993; Van Lint et al., 1996). In addition, HDACs are able to catalyze deacetylation of many non-histone proteins, thus, they are also called lysine deacetylases to describe their functions more precisely (Glozak et al., 2005). HDACis have been reported to regulate gene transcription positively or negatively in a gene-specific manner (Marks et al., 2000).

HDACs are divided into four groups, mainly according to the homology of yeast HDACs. Class I HDACs (HDAC1, 2, 3, and 8) are critically connected with yeast RPD3 gene. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) are related to yeast Hda1 gene. Class III HDACs (SIRT1-7) are homologous to silent information regulator 2 (Sir2) and have no sequence similarity to class I and II HDACs; these Sir2 proteins, also called sirtuins are unaffected by known class I/II HDACis. Class IV (HDAC11) has conserved residues in its catalytic regions that are shared by both class I and II HDACs. Class I and II HDACs need Zn^{2+} for their enzymatic reaction. Class IV also has a Zn^{2+} based reaction mechanism. However, class III HDACs do not require Zn^{2+} for their catalysis, but strictly depend on the cofactor NAD^{+} (Pang and Zhuang, 2010).

Currently, the expression profiles and distribution of HDACs in the kidney have not been completely clarified. It has been documented that class I HDAC isoforms are expressed in the cortex of developmental kidney, renal fibroblasts, and renal tubular cells (Pang et al., 2011; Chen and El-Dahr, 2013; Tang et al., 2013). HDAC5 and 6 have been identified in the renal tubules (Marumo et al., 2008; Liu et al., 2012b). SIRT1 is expressed in both renal tubules (Zhou et al., 2013) and fibroblasts (Ponnusamy et al., 2014) and HDAC11 is expressed in renal tubules (Kim et al., 2013). HDACs have been shown to be involved in a variety of cellular functions such as proliferation, survival, differentiation, and immunological responses (Van Beneden et al., 2013).

Most functional roles of HDACs are revealed by application of HDACis, which are classified into four categories according to chemical structures: hydroxamates (e.g., vorinostat), cyclic peptides (e.g., romidepsin), aliphatic acids (e.g., phenylbutyrate), and benzamides (entinostat) (Miller et al., 2003; Marks et al., 2004; Dokmanovic and Marks, 2005; Ma et al., 2009). HDACis primarily target the zinc domains to exert the biological effects through cell cycle arrest, differentiation and apoptosis in a variety of tumor models (Acharya et al., 2005). Treatment of renal cell carcinoma with HDACis also resulted in tumor growth inhibition (Ramakrishnan and Pili, 2013). To date, two HDACis, vorinostat, and romideps, have been approved by the FDA to treat cutaneous and peripheral T cell lymphoma. Other 20 different HDACis have been tested in the clinic (West and Johnstone, 2014). The common side-effects of HDACis in humans include fatigue, nausea, and vomiting and resolve upon treatment withdrawal (Minucci and Pelicci, 2006).

Although the tumor has been the primary target for HDACis, HDAC inhibition has also shown beneficial effects in some non-neoplastic disorders. HDACis are effective in attenuating the pathogenesis of several forms of chronic kidney disease and improving renal function. In this article, we highlight the therapeutic application of HDACis in pre-clinical models of renal injury and discuss the mechanisms involved (**Figure 1**).

HDACs in Renal Interstitial Fibrosis

Progression and development of renal injuries ultimately leads to renal interstitial fibrosis (Neilson, 2006; Wynn, 2008). Kidney fibrosis is characterized with activation and proliferation of renal interstitial fibroblasts as well as accumulation of extracellular matrix (ECM) components. During development of renal fibrosis, multiple cytokine, and growth factor signaling pathways are activated and involved in this process. Emerging evidence indicates that HDACs are also implicated in renal fibrogenesis. The mechanisms by which HDACs mediates renal fibrogenesis remain elusive, but may be associated with regulating the expression of inflammatory and profibrotic genes and activation of cell signaling pathways that mediate renal fibrosis. In the earlier studies, Pang et al. (2009) demonstrated that treatment with trichostatin A (TSA), a pan HDACi that can block both class I and class II HDACs, attenuates renal fibrosis in a murine model of unilateral ureteral obstruction (UUO). TSA treatment also significantly inhibits expression of α-SMA and fibronectin, two hallmarks of activated fibroblasts (Pang et al., 2009). Moreover, silencing of HDAC1 or HDAC2 using specific siRNA blocked renal fibroblast proliferation and reduced phosphorylation of STAT3 (signal transducer and activator of transcription 3), a signaling molecule associated with proliferation of renal fibroblasts and development of renal fibrosis (Pang et al., 2011). Recently, Manson et al. (2014) demonstrated that TSA treatment

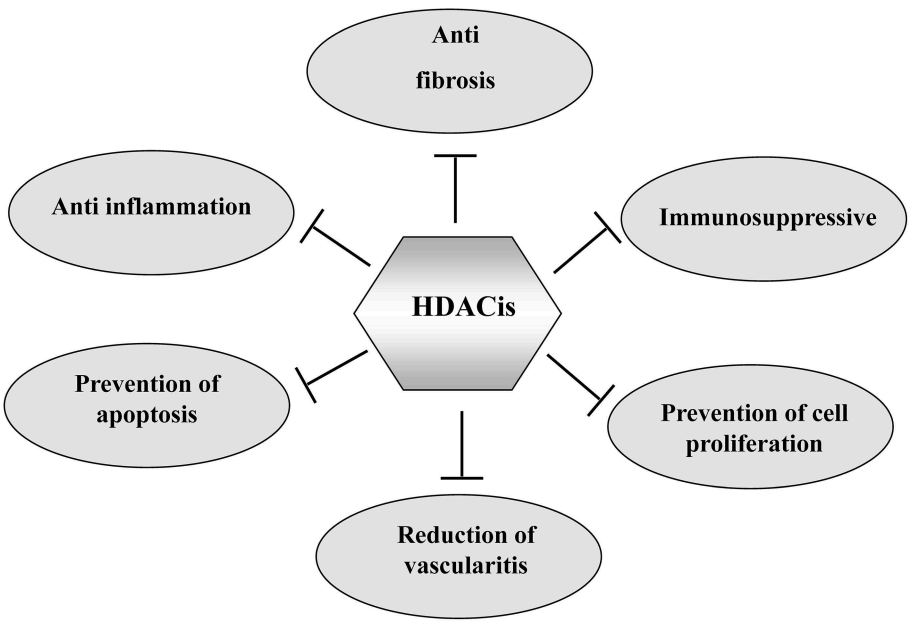

FIGURE 1 | The mechanisms by which HDACIs attenuate chronic kidney diseases. HDACIs can protect against chronic kidney diseases through multiple mechanisms as indicated.

preserves the expression of Bmp-7 transcription and attenuates the pathogenesis of renal injury in obstructive nephropathy. As BMP-7 is a potent anti-fibrotic molecule, restoration of BMP-7 expression by TSA represents another mechanism by which HDACis protect against chronic kidney injury. In addition, Marumo et al. (Liu et al., 2013) showed that HDAC inhibition also alleviates renal fibrosis through suppression of inflammatory responses in the injured kidney.

Since non-selective blockade of class I and class II HDACs does not allow elucidation of their individual roles in renal fibrogenesis, Liu et al. (2013) further examined the effect of MS-275, a selective class I inhibitor, on UUO injury and renal interstitial fibroblast activation and proliferation. Administration of MS-275 inhibited both renal fibroblast activation and proliferation and attenuated progression of kidney fibrosis. MS-275 treatment also inhibited UUO-induced production of TGF-β1 and phosphorylation of Smad3 and EGFR (epidermal growth factor receptor). These results suggest that specific blockade of only class I HDAC can inhibit renal fibrogenesis through a mechanism involved in the inactivation of the TGF-β1/Smad3 and EGFR signaling pathways. Although there is no study thus far to compare the pharmacological effect of TSA and MS-275 on renal fibroblast activation and development of renal fibrosis, it has been reported that pre-treatment with either valproic acid, another inhibitor of class I or TSA attenuates glomerulosclerosis and tubulointerstitial fibrosis to the similar degree. Delayed administration of these two inhibitors also showed comparable effects on the inhibition of renal fibrosis (Van Beneden et al., 2013). Thus, it appears that class I HDACs play a pre-dominant role in regulating renal fibrogenesis. Class III HDACs have been implicated in the regulation of renal fibrosis. It is evident that specific inhibition of SIRT1/2 alleviates progression of renal fibrogenesis and reduces renal fibroblast activation (Ponnusamy et al., 2014). Mechanistic studies showed that blocking SIRT1/2 inhibits activation of epidermal growth factor receptor (EGFR) and platelet derived growth factor receptors (PDGFR), two growth factor receptors associated with renal fibrosis (Chen et al., 2011; Liu et al., 2012a).

Collectively, these studies indicate that HDACs contribute to renal fibroblast activation and fibrogenesis. Additional studies are needed to clarify the role of individual HDAC isoforms in mediating these processes and elucidate the mechanisms involved in a great detail.

HDACs in Polycystic Kidney Diseases

Autosomal dominant polycystic kidney disease (ADPKD) is very common hereditary kidney diseases in humans, affecting 1/500 in the United States (Gabow, 1993). In ADPKD patients, a large number of bilateral kidney cysts displace normal kidney parenchyma, leading to end-stage renal disease (ESRD). ADPKD is mainly caused by gene mutations in one of two genes: PKD1, which accounts for approximately 85–95% of the cases and PKD2, which affects about 5–15% of the cases (Peters and Sandkuijl, 1992). ADPKD is characterized by development of multiple bilateral renal cysts, and increased renal epithelial cell proliferation and fluid secretion. As the gene product of *PKD2*, polycystin-2 (PC2), either alone or in complex with the gene product of *PKD1*, polycystin-1 (PC1), functions as a calcium-permeable cation channel and regulates intracellular Ca^{2+} levels, alteration of signaling pathways regulated by calcium such as cAMP-dependent B-Raf and ERK (extracellular signal-regulated kinase) activation resulted in abnormal proliferation of tubule epithelial cells (Yamaguchi et al., 2006). In addition, activation of many other signaling pathways and transcription factors such as EGFR and p53 is also involved in the development and growth of polycystic kidneys (Harris and Torres, 2014).

Emerging evidence has revealed the regulatory role of HDACs in the pathogenesis of polycystic kidneys. Xie et al., showed (Xia et al., 2010) that histone deacetylase 5 (HDAC5) is a target of polycystin-dependent fluid stress sensing in renal epithelial cells in mice. Stimulation of polarized epithelial monolayers with fluid flow induced phosphorylation and nuclear export of HDAC5 whereas dwonregulation of HDAC5 or treatment with TSA reduced cyst formation in $Pkd2^{-/-}$ mouse embryos. Cao et al. (2009) demonstrated that TSA treatment can affect both body curvature and laterality, two pathological changes associated with cyst formation in zebrafish and block cyst formation in pkd2 knockdown animals. Treatment with valproic acid (VPA), a class I specific HDACi, also delays the development of cyst production and improves renal function in a mouse ADPKD model. In addition, Fan et al. showed that administration of TSA in pregnant mice prevented cyst formation in *Pkd1* mutant embryonic kidneys (Fan et al., 2012). TSA treatment can ameliorate p53-induced repression of the PKD1 expression, Chang et al. (2006) and Thivierge et al. (2006). As EGFR activation and nuclear translocation of β-catenin are essential for ADPKD, the role of HDAC6 in regulating these biological responses was examined. HDAC6 inhibition blocks EGF-induced β-catenin nuclear localization, leading to inhibition of epithelial cell proliferation and promotion of EGFR degradation (Li et al., 2008). These studies suggest that class I/II HDAC activation is essential for PKD development and that HDACis may be possible drug treatments for PKD.

A recent study further reveals that SIRT is also involved in the pathogenesis of ADPKD (Zhou et al., 2013). SIRT1 upregulation was observed in embryonic and post-natal Pkd1-mutant mouse renal epithelial cells and tissues whereas double conditional knockouts of PKD1 and SIRT1 as well as inhibition of SIRT1 with a pan-sirtuin inhibitor (nicotinamide) or a SIRT1-specific inhibitor (EX-527) resulted in delayed renal cyst formation. Silence or inhibition of SIRT1 also reduced renal epithelial cell proliferation, but potentiated apoptosis. Further studies show that SIRT1 mediates cystic epithelial cell proliferation through altering retinoblastoma (RB) protein acetylation/phosphorylation and promotes their survival via p53 deacetylation. This study elucidates a functional role of SIRT1 in regulating ADPKD and provides a molecular basis for using SIRT1 inhibitors to interfere with cyst formation (Zhou et al., 2013).

HDACs in Diabetic Nephropathy

Diabetic nephropathy (DN) is characterized by ECM protein accumulation in glomerular mesangium and tubulointerstitium

with thickening of glomerular and tubular basement membranes, ultimately progressing to glomerulosclerosis and tubulo-interstitial fibrosis (Mauer et al., 1984). The earliest finding of renal involvement in DN is glomerular hypertrophy, which is caused by glomerular hyper-filtration. Although targeting diverse signaling pathways has been reported to attenuate the pathogenesis of DN, two animal studies have demonstrated the inhibitory effect of HDACis on DN. Gilbert et al. showed that vorinostat administration resulted in attenuation of renal hypertrophy in rats (Gilbert et al., 2011). Advani et al. demonstrated that vorinostat was effective in decreasing albuminuria and mesangial matrix accumulation in streptozotocin–wild-type mice (Advani et al., 2011). *In vitro*, treatment with VPA and SK-7041, two class I-selective HDACis, can also reduce expression of ECM components in renal epithelial cells (NRK52-E) (Noh et al., 2009). In addition, HDAC2 activity was upregulated in the kidneys of strotozotocin (STZ) induced diabetic rats and db/db mice. Treatment with N-acetylcysteine, an antioxidant, decreased TGF-β1 mediated activation of HDAC2 in NRK52-E cells (Noh et al., 2009). These data suggest that HDACs are required for the development of DN and that reactive oxygen species may play an essential role in mediating TGF-β1-induced activation of HDAC2.

EGFR activation has been shown to be implicated in the DN (Chen et al., 2014; Zhang et al., 2014). To understand whether EGFR expression is associated with the HDAC activity, Gilbert et al. (2011) further investigated the effect of vorinostat on the expression of EGFR in the early stage of diabetes. They found that daily treatment with vorinostat in diabetic rats for 4 weeks remarkably reduced EGFR expression and subsequently inhibited kidney growth and glomerular hypertrophy. In cultured rat proximal tubule cells, treatment with vorinostat also decreased EGFR expression, concomitant with cellular proliferation inhibition. Therefore, HDACs may regulate early DN through activation of the EGFR signaling pathway.

Podocyte damage accelerates the development of DN, characterized by loss of cytoskeleton protein integrity, such as nephrin. An early study showed that miR-29a is a potent regulator that inhibits fibrotic matrix expression in high glucose–stressed renal proximal tubule cells (Du et al., 2010). A recent study (Lin et al., 2014) indicated that miR-29a is protective against diabetes-induced podocyte damage, glomerular fibrosis and inflammation, and renal dysfunction. However, HDAC4-dependent H3K9 hypoacetylation counteracts miR-29a transcription in high glucose–stressed podocytes, suggesting that HDAC4 may be an important mediator in diabetic podocytopathy. Indeed, stimulation of podocytes with high glucose, advanced glycation end products, or transforming growth factor-β can increase HDAC4 expression and specific silencing of HDAC4 reduces podocyte injury in streptozotocin-induced diabetic rats and diabetic db/db mice (Wang et al., 2014). Further studies showed that the protective effect of HDAC4 inhibition is associated with prevention of autophagy defects and suppression of renal inflammation (Wang et al., 2014). Therefore, HDAC4 is a critical epigenetic mediator in the pathogenesis of DN, and specific inhibition of HDAC4 could serve as a therapeutic approach for DN and related renal diseases.

While application of HDACis is effective in the attenuation of DN, the combination of HDACis with other inhibitors might have additive or synergistic effects. Although such studies have not been performed in the DN, the combination of an ACE inhibitor with a HDACi has been reported to provide a better renal protection in a mouse model of HIV-associated nephropathy (Zhong et al., 2013). These two inhibitors can affect several important pathways involved in kidney inflammation and fibrosis, such as NF-κB, interleukin-1, TGF-β, mitogen-activated protein kinase, and apoptosis signaling (Zhong et al., 2013). Thus, examination of the therapeutic effect of HDACis in the treatment of DN in combination with other drugs is warranted.

HDACs in Lupus Nephritis

Systemic lupus erythematosus (SLE) is a very common autoimmune disease (Alderaan et al., 2015). Two studies have examined the role of HDACs in the pathogenesis of SLE in the MRL-lpr/lpr murine model of lupus. Mishra et al. (2003) demonstrated a remarkable reduction in proteinuria, glomerulonephritis, and spleen weight after treatment with TSA. TSA was also effective in the downregulation of IL-12, IFN-γ, IL-6, and IL-10 expression levels in splenocytes of this model. Regna et al. showed that HDAC inhibition with ITF2357, a specific inhibitor of class I and II HDAC, reduces sera and urinary markers of lupus, and suppresses expression of several inflammatory cytokines (IL-1β, TNF-α, IL-6, and IFN-γ) and improves kidney histopathology (Regna et al., 2014). These data suggest that class I and II HDACs contribute to the development of lupus and application of HDACis may have therapeutic benefits in the treatment of SLE.

Aristolochic Acid Nephropathy

Aristolochic acid nephropathy is a progressive renal interstitial fibrosis, frequently associated with urothelial malignancies (Debelle et al., 2008). Recently, Novitskaya et al. (2014) examined the effect of a HDACi, 4-(phenylthio)butanoic acid (PTBA) analog methyl-4-(phenylthio)butanoate (M4PTB), on the kidney injury induced by aristolochic acid. They found that treatment with M4PTB promotes renal recovery and reduces renal fibrosis after aristolochic acid injury in mice. These beneficial effects are associated with increased renal tubular cell proliferation and decreased G2/M arrest of regenerating renal tubular epithelial cells. Furthermore, M4PTB treatment decreased peritubular macrophage infiltration and expression of macrophage chemokines such as CX3Cl1 and CCL2. Since an increased number of renal epithelial cell arrested at G2/M phase of cell cycle represents a maladaptive repair process that leads to renal fibrosis (Yang et al., 2010), class I/II HDACs may contribute to the development of CKD after aristolochic acid injury.

HDACs in Transplant Kidney Injury

Calcineurin inhibitors (CNIs) decrease the rate of acute rejection in renal transplantation patients, but side effects such as

TABLE 1 | Effects of HDAC inhibitors on experimental kidney disorders.

Disease models	HDAC inhibitors	Selectivity	Effects of HDAC inhibitors	Mechanisms	References
Renal interstitial fibrosis	TSA	HDAC I/II	Attenuates renal fibroblast proliferation, α-SMA, fibronectin expression	Inhibits STAT3 activation induced by UUO	Pang et al., 2009, 2011
	Sodium valproate	HDACI	Attenuates macrophage infiltration and fibrotic changes	Reduces CSF-1 expression induced by TNF-α in renal tubular cells	Marumo et al., 2010
	MS-275	HDACI	Inhibits renal fibroblast activation	Inhibits TGF-β/Smad3 and EGFR signaling	Liu et al., 2013
	TSA, VPA	HDAC I/II	Hamperes glomerulosclerosis and tubulointerstitial fibrosis	N/A	Van Beneden et al., 2013
	Sirtinol	SIRT1/2	Inhibits renal fibroblast activation and proliferation as well as renal fibrogenesis	Inhibits EGFR and PDGFRβ signaling.	Ponnusamy et al., 2014
Polycystic kidney diseases	TSA	HDAC I/II	Attenuates p53 induced repression of the PKD1 promoter	Deacetylates p53 and binds with Sp1	Van Bodegom et al., 2006
	TSA	HDAC I/II	Reduces cyst formation	N/A	Xia et al., 2010
	TSA, VPA	HDACI/II	Suppress kidney cyst formation	N/A	Cao et al., 2009
	EX-527	SIRT1	Delays renal cyst formation	Inhibits cystic epithelial cell proliferation and induces cystic epithelial cell apoptosis	Zhou et al., 2013
Diabetic nephropathy	TSA, VPA,	HDAC I/II	Attenuate ECM accumulation and	Suppresses TGF-β1 induced HDAC2	Noh et al., 2009
	SK7041	HDAC I	EMT	activation	
	Vorinostat	HDAC I/II	Attenuates cellular proliferation, blunts renal growth, and glomerular hypertrophy	Downregulates EGFR expression	Gilbert et al., 2011
	SAHA	HDAC I/II	Decreases albuminuria, mesangial collagen IV deposition, and oxidative-nitrosative stress	Reduces eNOS expression in mouse kidneys and in cultured human umbilical vein endothelial cells	Advani et al., 2011
	Sodium butyrate	Pan HDAC inhibitor	Improves renal function	Inhibits apoptosis and DNA damage	Khan and Jena, 2014
Lupus nephritis	TSA, SAHA	HDAC I/II	Reduces proteinuria, glomerulonephritis and spleen weight	Downregulates IL-12, IFN-γ, IL-6, and IL-10 expression	Mishra et al., 2003
	ITF2357	HDAC I/II	Improves kidney histopathology	Suppresses expression of IL-1β, TNF-α, IL-6, and IFN-γ	Regna et al., 2014
Aristolochic acid nephropathy	PTBAs	Pan HDAC inhibitor	Accelerate recovery and reduce post-injury fibrosis	Decrease G2/M arrest and reduce macrophage infiltration	Novitskaya et al., 2014
Transplant kidney injury	FR276457	Pan HDAC inhibitor	Prolongs allograft survival	Suppresses mononuclear cell infiltration and vasculitis, and inhibits the proliferation of Jurkat cells by targeting activity of NF-κB.	Kinugasa et al., 2009

CSF-1, colony stimulating factor 1; EGFR, epidermal growth factor receptor; HDAC, histone deacetylase; PTBA, 4-(phenylthio)butanoic acid; SAHA, suberoylanilide hydroxamic acid; STAT3, signal transducer and activator of transcription 3; α-SMA, α-smooth muscle actin; TSA, Trichostatin A; VPA, valproic acid; TNF-α, tumor necrosis factor; TGF-β, transforming growth factor-β.

nephrotoxicity, neurotoxicity, and diabetogenicity (Shapiro et al., 1994), limit application of these drugs. Furthermore, CNIs are less effective in preventing chronic allograft rejection and the promotion of tolerance. Therefore, it is essential to search for novel and safer immunosuppressants with different mechanisms. Histone deacetylases (HDACs) are known to mediate transcription of genes that trigger immunological responses (Johnstone, 2002; Remiszewski, 2002). Studies examining the effect of HDACis on kidney injury after transplantation suggest that HDACis can initiate immunosuppression and prolong graft survival (Takahashi et al., 1996; Mishra et al., 2001). Edens et al. showed that HDACis were able to induce antigen-specific energy in lymphocytes (Edens et al., 2006). Tao et al. reported that HDAC inhibition improved the generation and function of regulatory T cells (Tao and Hancock, 2007). Moreover, Kinugasa et al. demonstrated that FR276457 had a remarkable immunosuppressive effect in a heterotopic cardiac transplant rat model (Kinugasa et al., 2008) and was able to prolong the median survival time (MST) in transplanted grafts in a canine renal transplant model. The combination of FR276457 with tacrolimus can also prevent allograft rejection. Histopathological analysis indicated that FR276457 suppressed mononuclear cell infiltration and vasculitis. Therefore, HDAC inhibition may prolong the MST in transplanted grafts when

administered alone or combined with other immunosuppressive agents.

Conclusion and Future Directions

Numerous studies have shown that treatment with HDACis is able to inhibit activation and proliferation of cultured renal interstitial fibroblasts and attenuate renal fibrosis in animal models. HDAC inhibition is also beneficial for other chronic kidney diseases caused by the diverse etiologies, as listed in **Table 1**. Given the large number of distinct HDACs, most studies in this field are currently conducted by using pan-HDACis or class-specific HDACis. Thus, there is a need to identify specific functions of each HDAC and to develop small molecule inhibitors that can selectively modulate the activities of individual HDAC isoforms. In addition, it is necessary to clarify the profile of HDAC-modulated proteins in the setting of renal fibrosis and other kidney diseases by using some novel techniques such as proteomics to globally analyze protein lysine acetylation in response to HDAC inhibition. Although numerous clinical trials for application of HDACIs in tumors have been reported, there is no clinical trial thus far to test the therapeutic effects of those inhibitors in patients with CKD. Therefore, further investigation of the mechanisms, efficacy and toxicity of HDACis in the pre-clinical model of CKD will be helpful for initiating clinical trials to assess the feasibility of HDACis in the treatment of this disease in the future.

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MicroRNAs in renal fibrosis

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MicroRNAs (miRNAs) are endogenous short non-coding RNAs that regulate most of important cellular processes by inhibiting gene expression through the post-transcriptional repression of their target mRNAs. In kidneys, miRNAs have been associated in renal development, homeostasis, and physiological functions. Results from clinical and experimental animal studies demonstrate that miRNAs play essential roles in the pathogenesis of various renal diseases. Chronic kidney diseases (CKD) is characterized by renal fibrosis. Transforming growth factor beta (TGF-β) is recognized as a major mediator of renal fibrosis because it is able to stimulate the accumulation of extracellular matrix (ECM) proteins to impair normal kidney function. Recently, emerging evidence demonstrate the relationship between TGF-β signaling and miRNAs expression during renal diseases. TGF-β regulates expression of several microRNAs, such as miR-21, miR-192, miR-200, miR-433, and miR-29. MiR-21, miR-192, and miR-433 which are positively induced by TGF-β signaling play a pathological role in kidney diseases. In contrast, members in both miR-29 and miR-200 families which are inhibited by TGF-β signaling protect kidneys from renal fibrosis by suppressing the deposition of ECM and preventing epithelial-to-mesenchymal transition, respectively. Clinically, the presence of miRNAs in blood and urine has been examined to be early biomarkers for detecting renal diseases. From experimental animal studies of CKD, targeting microRNAs also provides evidence about therapeutic potential of miRNAs during renal diseases. Now, it comes to the stage to examine the exact mechanisms of miRNAs during the initiation and progression of renal diseases. Therefore, determining the function of miRNAs in renal fibrosis may facilitate the development of both early diagnosis and treatment of renal diseases.

Keywords: microRNAs, kidney diseases, renal fibrosis, TGF-β signaling, biomarkers

INTRODUCTION

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs that regulate various cellular processes such as death, differentiation, proliferation, metabolism, and pathophysiology of many diseases via the regulation of target gene expression. During recent decades, the understandings of miRNAs in molecular mechanisms on various disease processes are expanding rapidly. In the kidney diseases, miRNAs also play a key role in renal fibrosis.

MiRNAs bind to their respective target mRNAs and recruit the RNA-induced silencing complex (RISC). There are multiple steps of the biogenesis of miRNAs. Firstly, after having transcribed by RNA polymerase II or RNA polymerase III as long stem-loop primary miRNA (Pri-miR) in the nucleus, the Pri-miR is then cleaved into a double-stranded shorter miRNA precursor (Pre-miR) by RNase III enzyme Drosha and its partner DGCR8 (DiGeorge syndrome critical region 8) (Lee et al., 2003; Gregory et al., 2004). These Pre-miR will next be exported into the cytoplasm by the Ran-GTP and Exportin-5 (Du and Zamore, 2005). Pre-miR is further cleaved into the mature form, a 20–22 base pairs (bp) double-stranded RNA, in the cytoplasm by another RNase III enzyme Dicer. This mature miRNA-miRNA duplex is unwound and the functional strand ("guide strand") is loaded onto the RISC (Filipowicz, 2005). The mature miRNA induces the RISC complex to bind to the 3′ untranslated region (3′ UTR) of a target messenger RNA (mRNA). This will result in post-transcriptional gene silencing by mRNA degradation or by translation inhibition. Therefore, miRNAs is able to suppress target gene expression by mRNA degradation, translation inhibition or transcriptional inhibition.

Early studies by microarray assays demonstrate that the abundance of miR-192, -194, -204, -215, and -216 are high in the kidney when compared with other organs (Sun et al., 2004; Tian et al., 2008). These studies suggest the potential role of miRNAs in kidney function. So far, more and more miRNAs have been described in the development, hemostasis and diseases in the kidney (Kato et al., 2009; Li et al., 2010; Bhatt et al., 2011; Lorenzen et al., 2011). As many comprehensive reviews about the biogenesis of miRNAs and the role of miRNAs in normal kidney have been published (Du and Zamore, 2005; Filipowicz, 2005; Saal and Harvey, 2009; Wessely et al., 2010; Bhatt et al., 2011; Lorenzen et al., 2011; Chung et al., 2013b; Li et al., 2014), this review will focus on recent novel findings into the implications for miRNAs in renal fibrosis.

ROLE OF TGF-β SIGNALING IN RENAL FIBROSIS

Renal fibrosis is the common feature of chronic kidney disease (CKD) progressing to end-stage renal failure. Renal fibrosis is generally characterized either by interstitial extracellular matrix (ECM), or myofibroblast accumulation, and destruction of renal tubules (Bottinger, 2007; Liu, 2011). Transforming growth factor-beta (TGF-β) is the well-known master cytokine/growth factor in fibrosis (Roberts, 1998; Wang et al., 2005; Meng et al., 2010, 2012a,b; Lan and Chung, 2011) (**Figure 1**). Smad2 and Smad3 are the important downstream mediators of TGF-β signaling (Massague and Chen, 2000; Miyazono, 2000; Chung et al., 2010b; Zhou et al., 2010a; Chen et al., 2011; Li et al., 2013b; Meng et al., 2013). During fibrosis, TGF-β is capable of inducing many fibrogenic genes, such as ECM proteins, via Smad2, Smad3, or mitogen-activated protein kinases (MAPKs) (Hoffman et al., 1998; Schnaper et al., 2003; Chung et al., 2009). Recent studies show that TGF-β also regulates several miRNAs during renal fibrosis. TGF-β1 induces miR-21, miR-192, miR-491-5p, miR-382, miR-377, miR-214, and miR-433, but suppresses the miR-29 and miR-200 families (Kriegel et al., 2010, 2012a; Kantharidis et al., 2011; Lan and Chung, 2012; Chung et al., 2013a) (**Table 1**). All these TGF-β-regulated miRNAs have been shown to participate in the events during renal fibrosis (**Figure 1**). Furthermore, expression of these miRNAs is altered when the kidneys are injured in the experimental mouse models of renal injury (Kantharidis et al., 2011; Lan and Chung, 2012; Chung et al., 2013a), suggesting that they play essential roles in TGF-β-induced fibrosis. In this review, we will discuss the recent findings of five groups of TGF-β-regulated miRNAs, including miR-21, miR-29, miR-192, miR-200, and miR-433 because they have been shown to modulate TGF-β-induced renal fibrosis. MiR-21, miR-192, and miR-433 promote fibrosis while miR-29 and miR-200 families inhibit fibrosis.

MICRORNAs IN TGF-β-INDUCED RENAL FIBROSIS

MiR-29

Three members of the miR-29 family are encoded from two distinct genomic loci in both human and rodent genomes (Kriegel et al., 2012b). All miR-29 members have the identical seed binding sequence and they all bind to the identical set of target genes (Kriegel et al., 2012b). Findings from clinical studies and experimental models suggest the anti-fibrotic effects of miR-29.

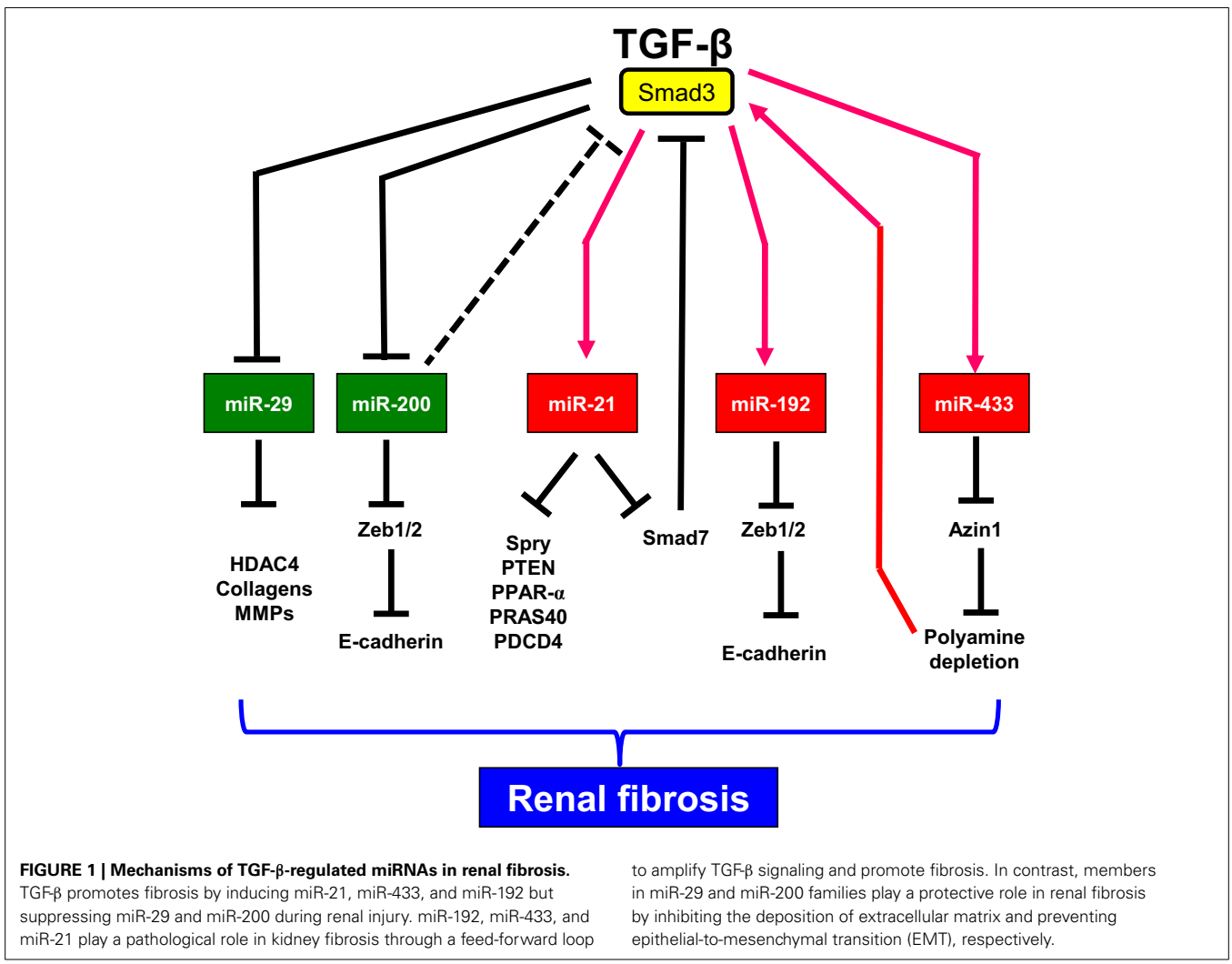

FIGURE 1 | Mechanisms of TGF-β-regulated miRNAs in renal fibrosis. TGF-β promotes fibrosis by inducing miR-21, miR-433, and miR-192 but suppressing miR-29 and miR-200 during renal injury. miR-192, miR-433, and miR-21 play a pathological role in kidney fibrosis through a feed-forward loop to amplify TGF-β signaling and promote fibrosis. In contrast, members in miR-29 and miR-200 families play a protective role in renal fibrosis by inhibiting the deposition of extracellular matrix and preventing epithelial-to-mesenchymal transition (EMT), respectively.

Table 1 | Roles of microRNAs in animal models of kidney diseases.

microRNA	Mouse model of kidney diseases	Pathological output	References
miRNAs WHICH ARE INDUCED BY TGF-β			
miR-21	UUO, ISI, DN in *db/db* mice	Fibrosis and inflammation	Zhong et al., 2011, 2013; Chau et al., 2012
miR-192	UUO, STZ induced DN, DN in *db/db* mice	Fibrosis and EMT	Kato et al., 2007; Chung et al., 2010a; Putta et al., 2012
miR-216a	STZ induced DN, DN in db/db mice	Increases col1a2 expression	Kato et al., 2010
miR-377	Spontaneous and STZ induced DN	Fibronectin expression	Wang et al., 2008
miR-491-5p	UUO (rat)	Induces Par-3 degradation	Zhou et al., 2010b
miR-382	UUO	Suppresses E-cadherin	Kriegel et al., 2012a
miR-433	UUO	Fibrosis, polyamine depletion	Li et al., 2013a
miR-29a,b,c	UUO, DN in *db/db* mice	Fibrosis	Du et al., 2010; Qin et al., 2011; Chen et al., 2014; Lin et al., 2014
miR-200a,b,c, miR-141, miR-429	UUO STZ induced DN, adenine-induced	EMT	Oba et al., 2010; Wang et al., 2011; Xiong et al., 2012

EMT, epithelial-to-mesenchymal transition; DN, diabetic nephropathy; ISIS, ischemia reperfusion injury; STZ, streptozotocin; UUO, Unilateral Ureteral Obstruction.

Abundance of miR-29s is always high in the kidney, lung, and heart (Kriegel et al., 2012b). However, their abundance is greatly reduced in animal models and human samples of fibrotic diseases in heart, lung, and kidney (van Rooij et al., 2008; Qin et al., 2011; Xiao et al., 2012; Zhang et al., 2014).

By employing microarrays and real-time PCR assays in the mouse model of unilateral ureteral obstruction (UUO), abundance of miR-29a, -29b, and -29c is substantially decreased in the fibrotic kidney of UUO wild-type mice but significantly induced in Smad3 knockout (KO) mice in which renal fibrosis was inhibited (Qin et al., 2011). This negative relationship of miR-29 in TGF-β-dependent fibrosis is further confirmed by the reduced expression of miR-29a and miR-29b in renal TECs, lung, and cardiac fibroblasts after treatment of TGF-β1 (van Rooij et al., 2008; Qin et al., 2011; Xiao et al., 2012).

Consistent with the results in heart that suppression of miR-29b increases the levels of fibrotic markers (van Rooij et al., 2008; Ye et al., 2010; Zhang et al., 2014), overexpression of miR-29 reduces but inhibition of miR-29 enhances abundance of fibrotic markers in mouse embryonic fibroblasts (MEF) and TECs under diabetic condition, salt-induced hypertensive conditions, or after TGF-β treatment, confirming the anti-fibrotic influence of miR-29 (Du et al., 2010; Liu et al., 2010b; Qin et al., 2011; Chen et al., 2014) (**Figure 1**). These reults are further confirmed by mouse models of unilateral ureteral obstruction and diabetic nephropathies (Qin et al., 2011; Chen et al., 2014). Delivery of *miR-29b* gene either before or after established obstructive and diabetic nephropathies effectively suppresses the progression of renal fibrosis. The ability to inhibit TGF-β-mediated deposition of ECM by miR-29 may be mechanism of how miR-29 protects kidney from fibrosis because more than 20 different ECM-related genes are predicted to be miR-29 targets and some of them are positively regulated by TGF-β signaling (van Rooij et al., 2008). A recent study demonstrates that increasing miR-29a action also protects against diabetic podocytopathy by suppressing HDAC4 signaling, nephrin ubiquitination, and urinary nephrin excretion associated with diabetes and restoring nephrin acetylation (Lin et al., 2014). In conclusion, miR-29 is a downstream inhibitor of TGF-β-mediated fibrosis and may have therapeutic potential for diseases involving fibrosis.

MiR-200

The miR-200 family includes miR-200a, -200b, -200c, -429, and -141. This family is known to maintain epithelial differentiation (Howe et al., 2012) because they were firstly discovered by their ability to restore an epithelial phenotype in breast cancer cell lines by inhibiting ZEB1 and ZEB2, the E-cadherin transcriptional repressors (Burk et al., 2008; Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008) (**Figure 1**). During obstructive and diabetic nephropathy, abundance of miR-200a and miR-141 are reduced in the fibrotic kidneys (Wang et al., 2011; Xiong et al., 2012). These findings are confirmed by *in vitro* studies that abundance of the miR-200 family in TECs is reduced in a TGF-β/Smad-dependent manner (Wang et al., 2011; Xiong et al., 2012). However, opposite results are shown in another study that renal expression of miR-200s is elevated in the mouse model of UUO (Oba et al., 2010). However, the differences of miR-200 expression in these mouse models of kidney diseases are possibly due to the differences in the origin of cell lines examined, the treatments performed, and the use of different animal models between studies. Side by side comparison of these mouse models and cell culture studies should be performed to understand the exact mechanism. In spite of the difference of miR-200 expression in fibrotic kidneys, the anti-fibrotic role of miR-200 family is confirmed by gene delivery of miR-200b in fibrotic kidney. A single injection of miR-200b precursor is sufficient to inhibit the up-regulation of collagens and fibronectin in obstructed kidneys (Oba et al., 2010).

MiR-21

MiR-21 is one of the first microRNAs to be described as an oncomir because it is associated in the genesis and progression of human cancers (Jazbutyte and Thum, 2010). MiR-21 expression is closely related to fibrosis and it is up-regulated by TGF-β 1 (Zavadil et al., 2007; Davis et al., 2008, 2010). The first report of miR-21 in fibrosis is firstly shown in heart failure (Thum et al.,

2008). Its expression is induced in cardiac fibroblasts of the failing hearts and delivery of miR-21 antagomir into a mouse model of cardiac hypertrophy inhibits interstitial fibrosis and restores the cardiac function (Thum et al., 2008). Similarly, elevation of miR-21 expression is found in the patients with idiopathic pulmonary fibrosis and in mice with bleomycin-induced lung fibrosis (Liu et al., 2010a). Suppressing miR-21 by antisense oligonucleotides inhibits lung fibrosis in mice (Liu et al., 2010a).

Although the abundance of miR-21 is low in normal kidneys, its abundance is greatly increased in both patient samples of kidney diseases and animal models of CKD and acute kidney injury (AKI) (Godwin et al., 2010; Zhong et al., 2011, 2013; Chau et al., 2012; Xu et al., 2012; Wang et al., 2013). From the studies in mouse models of obstructive and diabetic nephropathy, high abundance of miR-21 is observed in both tubulointerstitial and glomerular area where fibrosis happens (Zhong et al., 2011, 2013; Wang et al., 2013). In another study of ischemia-reperfusion injury, similar elevation of renal miR-21 is also observed (Godwin et al., 2010; Xu et al., 2012). These results suggest the pathological role of miR-21 in renal diseases.

Similarly, miR-21 positively regulates expression of ECM and α-SMA in tubular epithelial cell (TEC) and mesangial cells (MCs) after treatment of TGF-β1 or under diabetic condition (Zhong et al., 2011, 2013). In addition, knockdown of miR-21 inhibits but overexpression of miR-21 in kidney cells enhances renal fibrosis under diabetic condition or after treatment with TGF-β1 (Zarjou et al., 2011; Zhong et al., 2011, 2013).

Targeting miR-21 should possess a therapeutic potential to ameliorate the disease related to fibrosis because inhibition of miR-21 is effectively to decrease fibrosis in rodent models of heart, lung, and kidney diseases (Thum et al., 2008; Liu et al., 2010a; Zhong et al., 2011). In the mouse models of diabetic and obstructive nephropathy, inhibition of miR-21 also improves kidney function and halts the progression of renal injury (Zhong et al., 2011, 2013).

The results from miR-21 KO mice further confirm the pathological role of renal fibrosis (Chau et al., 2012). Consistence with the results of unilateral ureteral obstruction (UUO) and renal ischemia reperfusion injury (ISI) models, *miR-21* gene deficiency in mice reduces renal fibrosis, tubule atrophy, and P42/P44 MAP kinase pathway activation in diseased kidneys when compared with wild type mice (Chau et al., 2012). Furthermore, a negative relationship between the presence of miR-21 and genes which are involved in lipid metabolism, fatty acid oxidation, and redox regulation is found in miR-21 KO kidneys. More interestingly, this study also demonstrate that suppression of peroxisome proliferator-activated receptor-α (PRAR-α) by miR-21 may be one of mechanisms of how miR-21 promotes renal fibrosis by (**Figure 1**) (Chau et al., 2012).

The investigation of how miR-21 regulates fibrosis is still ongoing. Results from the studies in cardiac fibrosis show that *Phosphatase and tensin homolog* (*PTEN*) and *Sprouty* (*SPRY*) are two of potential targets of miR-21 (Thum et al., 2008; Roy et al., 2009) (**Figure 1**). It is found that miR-21 can suppress PTEN to activate phosphatidylinositide 3-kinases (PI3K) and Akt activity, and next increases MMP-2 abundance (Roy et al., 2009). As SPRY is an inhibitor of Ras/MEK/ERK, suppression of SPRY by miR-21 will activate ERK to promote TGF-β-induce fibrosis (Ding et al., 2008). In heart, suppression of miR-21 reduces ERK-MAPK activity and interstitial fibrosis (Thum et al., 2008). Recent study on diabetic nephropathy shows that *Smad7* and AKT1 substrate 1 (*PRAS40*), a negative regulator of Tor complex 1 (TORC1), are potential targets of miR-21 (Dey et al., 2011; Zhong et al., 2013). Negative correlation between miR-21 and *proinflammatory programmed cell death 4* (*PDCD4*) has also been reported in TEC with induction of ischemia (Godwin et al., 2010). As more and more miR-21 target genes which are related to fibrosis are found, further studies should be done to clarify how miR-21 exactly controls these target genes during renal fibrosis.

MiR-192

As mentioned above, abundance of miR-192 is high in the normal kidney, as compared with other organs (Sun et al., 2004; Tian et al., 2008). Pro-fibrotic role of miR-192 has been report in several studies from rodent models of kidney diseases, and in both MCs and TECs (Kato et al., 2007; Chung et al., 2010a; Putta et al., 2012). High abundance of miR-192 is detected in glomeruli isolated from diabetic mice (Kato et al., 2007). Treatment with TGF-β or high glucose in MCs and TEC up-regulates miR-192 expression (Kato et al., 2007; Chung et al., 2010a). Suppression of zinc finger E-box binding homeobox 1/2 (Zeb1/2) expression by miR-192 may be one of the mechanism of how miR-192 regulates TGF-β-induced collagen expression in MCs (Kato et al., 2007) (**Figure 1**). Similarly, inhibition of miR-192 reduces but overexpression of miR-192 enhances TGF-β1-induced collagen accumulation in TEC (Chung et al., 2010a). These results are further confirmed by a study in a mouse model of type I diabetes. An *in vivo* inhibition of renal miR-192 greatly up-regulates renal expression of Zeb1/2 and inhibits proteinuria, and expression of collagen and fibronectin (Putta et al., 2012). The pathological role of miR-192 in diabetic nephropathy is then supported by the findings in miR-192 KO mice (Deshpande et al., 2013). Deletion of *miR-192* gene in type I diabetic mice reduced albuminuria, proteinuria, renal fibrosis, and hypertrophy as compared to diabetic wild-type mice (Deshpande et al., 2013). Taken together, these studies demonstrate a pro-fibrotic role of miR-192 in TGF-β-dependent renal fibrosis observed in animal models of diabetic and obstructive nephropathy (Kato et al., 2007; Chung et al., 2010a; Putta et al., 2012).

However, the reverse is true in human nephropathy (Krupa et al., 2010; Wang et al., 2010). Remarkably, reduction of miR-192 expression is observed in human TECs after TGF-β1 treatment or in the human diseased kidneys (Krupa et al., 2010; Wang et al., 2010). This reduction of miR-192 expression correlates with tubulointerstitial fibrosis and a low GFR in diabetic patients. These significant differences in miR-192 expression in human and animal models of diabetic nephropathy requires further investigation to identify role and mechanism of miR-192's action during renal fibrosis in different species.

MiR-433

Early studies of miR-433 focus on its role in cancer (Jung et al., 2000; Luo et al., 2009). Recently, miR-433 has been found to be one of the important components of TGF-β/Smad3 driven renal

fibrosis (Li et al., 2013a). Similar to miR-21 and miR-192, renal miR-433 expression is induced after UUO. *In vitro*, TGF-β promotes fibrosis in TEC by inducing miR-433 expression (Li et al., 2013a). This induction requires the activation of TGF-β signaling. In addition, inhibition of miR-433 suppresses but overexpression of miR-433 enhances TGF-β1-induced collagen matrix accumulation in TEC (Li et al., 2013a). More importantly, suppression of miR-433 *in vivo* reduces renal fibrosis and halts the progression of renal fibrosis in established obstructive nephropathy. Antizyme inhibitor 1 (Azin1), a protective protein in kidney fibrosis, is found to be a target of miR-433 that overexpression of Azin1 is able to suppress expression of fibrotic proteins in TEC (Li et al., 2013a). As Azin1 promotes polyamine synthesis and polyamine depletion can activate TGF-β signaling by increasing the expression levels of TGF-β1, TβRI, and Smad3 (Patel et al., 1998; Liu et al., 2003), elevation of miR-433 during renal fibrosis forms a positive feedback loop to amplify TGF-β signaling by suppressing Azin1 expression.

REGULATION OF FIBROSIS-RELATED microRNAs BY TGF-β SIGNALING

Although the precise mechanism of how TGF-β signaling regulates miRNA expression during renal fibrosis is still continuing, recent evidence demonstrates that TGF-β signaling induces the synthesis of fibrosis-related microRNAs either by increasing transcription, or by enhancing posttranscriptional processing of primary miRNA transcript. Davis et al demonstrate that TGF-β signaling enhances the processing of primary transcripts of some microRNAs into its active form by the Drosha complex, such as miR-21 (Davis et al., 2008). Smad3, as one of the receptor-Smads, physically interacts with the Drosha complex to stimulate the production of mature miR-21 from the pri-miR-21 transcript (**Figure 1**). In addition, a consensus sequence (R-SBE) is found to be located within the stem region of the primary transcripts of TGF-β-regulated-miRs (pri-T-miRs) (Davis et al., 2010). The direct binding between Smads and the R-SBE will initialize the TGF-β-induced recruitment of Drosha, and DGCR8 to pri-T-miRs and enhance the processing of pri-T-miRs (Davis et al., 2010).

Our laboratory demonstrates that TGF-β/Smad3 signaling is able to regulate the transcription of miR-21, miR-192, miR-433, and the miR-29 family during renal diseases (Chung et al., 2010a; Qin et al., 2011; Zhong et al., 2011; Li et al., 2013a) (**Figure 1**). These results are further supported by the results from in rodent models of obstructive and remnant kidney diseases induced in mice lacking Smad3, Smad7, or having conditional knockout (KO) for Smad2 or overexpressing renal Smad7 (Chung et al., 2010a, 2013a; Qin et al., 2011; Zhong et al., 2011). This notion is firstly supported by the *in vitro* studies that TGF-β inhibits miR-29 expression but upregulates the expression of miR-21, -192, and -433 *via* the Smad3-dependent mechanism as revealed in MCs and TECs overexpressing Smad7, or knocking down for Smad2 or Smad3 and in Smad2 or Smad3 KO mouse embryonic fibroblasts (MEF) (Chung et al., 2010a, 2013a; Qin et al., 2011; Zhong et al., 2011). In addition, we also find that Smad3 physically interacts with Smad-binding site (SBE) located in their promoters to regulate the expression of these miRNAs (Chung et al., 2010a; Qin et al., 2011; Zhong et al., 2011; Li et al., 2013a). Binding of Smad3 on SBE in the promoters can either promote transcription and post-transcriptional processing of miRNAs, such as miR-21, -192, and -433, or suppress the transcription, such as miR-29b (Chung et al., 2010a; Qin et al., 2011; Zhong et al., 2011; Li et al., 2013a). Furthermore, Smad7, which is an inhibitory Smad, is able to defend kidneys from fibrosis because it can regulate TGF-β/Smad3-mediated miRNAs by maintaining renal miR-29b but inhibiting miR-21, -192, and -433 (Chung et al., 2010a, 2013a; Lan and Chung, 2012).

In addition, the feedback loop occurs as microRNAs can also regulate the TGF-β/Smad3 signaling (**Figure 1**). During renal injury, TGF-β induces the miR-21 expression and this elevation of miR-21 suppresses Smad7 expression and, in turn, enhances the TGF-β signaling (Liu et al., 2010a; Zhong et al., 2013). As a feed-forward loop, miR-21 may amplify TGF-β signal during renal injury. MiR-21 promotes renal fibrosis may employ the activation of ERK/MAP kinase signaling in fibroblasts (Thum et al., 2008).

In contrast, TGF-β expression can be negatively regulated by miRNAs. For instance, miR-200a is able to suppress TGF-β expression. Although both TGF-β1 and TGF-β2 suppress miR-200a expression in renal cells (Wang et al., 2011), TGF-β2 is also one of the target genes for miR-200a. Overexpression of miR-200a inhibits TGF-β2 expression, Smad3 activity, and TGF-β1-induced fibrosis (Wang et al., 2011). These results reveal a possible feedback between TGF-β2 and miR-200a. Similarly, miR-29 is shown to inhibit TGF-β1 and TGF-β2 (van Rooij et al., 2008; Zhang et al., 2014). Thus, miR-29 may exert its anti-fibrotic effects through inhibition of TGF-β signaling. In addition, recent studies also demonstate that TGF-β1 can be extensively post-transcriptionally regulated by miR-744 and miR-663 (Tili et al., 2010; Martin et al., 2011).

In addition, polyamine depletion activates TGF-β signaling (Patel et al., 1998; Rao et al., 2000; Liu et al., 2003) and our laboratory recently demonstrated that depletion of cellular polyamine levels by targeting Azin1 with a Smad3-dependent miR-433 exaggerates TGF-β-induced renal fibrosis (Li et al., 2013a). Both *in vitro* and *in vivo* studies show that elevated expression of TGF-β1, TβRI, miR-433, and phosphorylated Smad3 after TGF-β1 treatment is inhibited by overexpressing Azin1 or knocking down miR-433. This inhibition is accompanied by restoring cellular polyamine levels. These results support our hypothesis that TGF-β/Smad3-miR433 signaling mediates renal fibrosis. Other studies demonstrates that the miRNAs also regulate TGF-β signaling during renal fibrosis. For instance, TGF-β receptor 1 (TGF-βR1) is found to be identified as a target of miR-130b but during fibrosis, miR-130b is down-regulated by TGF-β1 (Castro et al., 2014). All these results demonstrate the close relationship and the complexity between miRNAs and TGF-β-induced renal fibrosis.

CLINICAL APPLICATION OF microRNAs IN KIDNEY DISEASES

BIOMARKERS

The presence of miRNAs in blood and urine suggests the potential of miRNAs to be biomarkers of kidney diseases. Recently, more and more investigations imply the circulating miRNAs

are potential biomarkers for cancer growth and organ injuries because miRNAs are stable and tissue specific as well as they can be identified and quantitated (Velu et al., 2012). The potential of miRNAs to be biomarkers for kidney diseases has been investigated recently. For example, remarkably high levels of circulating miR-21 is found in patients with severe interstitial fibrosis and tubular atrophy (Glowacki et al., 2013). An another study also shows that a total of 27 microRNAs at significantly different levels are found in urine from the patients at different stages of diabetic nephropathy (Argyropoulos et al., 2013). Furthermore, these 27 miRNAs have previously been found to participate into in signaling pathways of renal fibrosis during diabetic kidney disease. In addition, urinary levels of miR-29b and -29c are related to proteinuria and renal function in immunoglobulin A (IgA) nephropathy while miR-93 levels in urine are closely correlated with glomerular scarring (Wang et al., 2012). Now it should be time to search for patterns of these miRNAs that are released into the blood or urine from by diseased kidneys.

THERAPEUTIC POTENTIAL

MiRNAs should have therapeutic potential of miRNAs in kidney diseases because miRNAs play an essential roles in renal injury. Furthermore, sequence complementarity between mRNA and miRNA offers a feasible and specific approach to develop a miRNA drug that specifically targets gene(s) or miRNA(s) which have pathologic effect on certain disease. Recent improvements in chemical engineering enable us to develop chemical modified miRNAs that are stable in the circulation, can freely move into cells to target specific mRNA or miRNA and silence it (Lorenzen et al., 2011). Conventional construction of overexpression or shRNA plasmids provides an alternative to restore or suppress miRNA transcription, respectively (Qin et al., 2011; Zhong et al., 2011). In mouse models of kidney disease, restoration of miR-29 and -200 families, or inhibition of miR-21, -192, and -433 inhibits renal fibrosis (Oba et al., 2010; Qin et al., 2011; Zhong et al., 2011, 2013; Putta et al., 2012; Chung et al., 2013a). Thus, application of miRNAs or their inhibitors provides a novel and effective therapeutic approach to the treatment of kidney diseases.

The delivery method and safety are the main concern in the therapeutic application of miRNAs. So far, systemic delivery of chemical-engineered oligonucleotides is the most method to inhibit miRNA function (Lorenzen et al., 2011). One of the possible drawback it that this method may also suppress the function of miRNAs in organs other than the diseased one. To overcome it, specific gene delivery system to limit miRNA expression in specific organs is also developing (Lan et al., 2003; Xiao et al., 2012). For example, ultrasound-microbubble-mediated gene transfer developed has been shown to be able to deliver miRNA overexpression or knockdown plasmids specifically into the living kidneys (Lan et al., 2003; Qin et al., 2011; Zhong et al., 2011, 2013; Chung et al., 2013a). For the success gene therapy, it is also essential to control the transgene expression at the desired therapeutic levels to minimize the side-effect. To achieve this, an optimal dosage of miRNAs should be considered and investigated to avoid any undesirable side-effects caused by over doses of overexpression or inhibition of miRNA expression.

The risks of off-target effects and non-specific immune response are also the main concern in miRNA therapy. For instance, the one of main considerations to apply miR-21 as a therapeutic agent for fibrotic diseases is that the inhibition of miR-21 expression will result in an induction of apoptosis (Li et al., 2009; Godwin et al., 2010; Zhong et al., 2011). Similarly, strong pro-apoptotic effect of miR-29b may also impede the development of miR-29b gene therapy as overexpression of microRNA-29b upregulate cell death of multiple myeloma cells (Zhang et al., 2011). Therefore, miRNA therapeutics still await for further improvement on the controllable delivery system specific for cells and organs.

SUMMARY AND PERSPECTIVES

After the discovery of miRNAs and characterization of their functions in kidney diseases in last two decades, it is still plenty of room for improving our understanding about the specific role and mechanism of miRNAs in renal pathophysiology. How to accurately identify miRNA targets becomes one of the key issues which hinder our progress of miRNAs in renal research because the short seed sequence of a miRNA allow it to regulate multiple target genes. Although target prediction programs provide us a large number of potential miRNA targets, the overlap among various algorithms is so minimal that only a small portion of these targets can be validated experimentally. Even after the conservation of the 3′ UTR among species is included in the investigation, the number of targets predicted is far more than those for validation. In addition, the power of miRNAs also relies on their capability of targeting multiple genes that contribute to a pathway or phenotype. However, this also introduces the difficulty to search for real targets of miRNAs. We hope that the advances in the high-throughput validation and proteomic analysis will provide a solution to identify miRNA targets.

Another difficulty of miRNA research is to understanding the regulation of miRNA expression because possibly more than one mediators or pathways participate in regulating miRNA expression. For example, within a given miRNA cluster, miRNAs may show the same pattern of expression but some of cluster members may provide different expression patterns if they do not follow the pattern (Khella et al., 2012b). Furthermore, it is common that some miRNAs may be encoded from more than one genomic loci, such as miR-29b, with very different promoter contexts (Kriegel et al., 2012b). How to control the miR-29b expression from 2 genomic loci still awaits for further investigation. In addition, intronic miRNAs provides an interesting question as they do not always have the same expression pattern as their host gene (Baskerville and Bartel, 2005). Furthermore, it is found that both strands of the miRNA are sometimes coexpressed and they usually target different sets of genes (Khella et al., 2012a). All these unsolved questions are required to have further studies to understand the mechanism about how to regulate miRNA expression. Deep sequencing is becoming a valuable tool to provide us a comprehensive view of gene expression patterns and quantification of transcript levels. This information may assist us to correlate the expression of miRNAs with the target transcripts.

Finally, microRNAs are vital downstream effectors of TGF-β-induced renal fibrosis. The further understanding of the role

and mechanism of miRNAs during TGF-β-induced renal fibrosis should provide us a novel and effective strategy to halt disease progression.

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Renal erythropoietin-producing cells in health and disease

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Erythropoietin (Epo) is an indispensable erythropoietic hormone primarily produced from renal Epo-producing cells (REPs). Epo production in REPs is tightly regulated in a hypoxia-inducible manner to maintain tissue oxygen homeostasis. Insufficient Epo production by REPs causes renal anemia and anemia associated with chronic disorders. Recent studies have broadened our understanding of REPs from prototypic hypoxia-responsive cells to dynamic fibrogenic cells. In chronic kidney disease, REPs are the major source of scar-forming myofibroblasts and actively produce fibrogenic molecules, including inflammatory cytokines. Notably, myofibroblast-transformed REPs (MF-REPs) recover their original physiological properties after resolution of the disease insults, suggesting that renal anemia and fibrosis could be reversible to some extent. Therefore, understanding the plasticity of REPs will lead to the development of novel targeted therapeutics for both renal fibrosis and anemia. This review summarizes the regulatory mechanisms how hypoxia-inducible *Epo* gene expression is attained in health and disease conditions.

Keywords: erythropoietin, fibrosis, hypoxia, plasticity, renal Epo-producing cell (REP)

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Introduction

Erythropoietin (Epo) is an indispensable erythropoietic glycoprotein hormone that induces red blood cell production (Haase, 2010; Bunn, 2013; Suzuki, 2015). Circulating Epo concentration is dynamically altered by the presence of hypoxia or anemia; up to 1000-fold increase in the circulating Epo concentration is reported in anemic patients (Bunn, 2013). Epo exerts its erythropoietic function through binding to Epo receptor (EpoR) (Remy et al., 1999). The EpoR expression level in erythroid progenitor cells is dependent on their differentiation stage; EpoR is most highly expressed in late-stage erythroid progenitors to early erythroblasts (Suzuki et al., 2003; Yamazaki et al., 2013). The Epo–EpoR signaling primarily mediates survival signaling in these progenitor cells and prevents their apoptosis, causing proliferation and differentiation, and thereby activating erythropoiesis (Wu et al., 1995).

Regarding its application to medicine, Epo represents a prototypical success of molecular biology. The presence of Epo was first suggested in the nineteenth century based on the high blood viscosity of people living in or returning from high altitude areas (Koury, 2005; Bunn, 2013). Experimentally, the presence of Epo as an erythropoietic humoral factor was discovered in the early twentieth century due to the erythropoietic property of serum from phlebotomized rabbits. Most notably, in 1977, Miyake et al. purified Epo from 2550 liters of urine from patients of aplastic anemia and determined its amino acid sequence (Miyake et al., 1977). Based on this finding,

the human *Epo* genes were cloned in 1985 (Jacobs et al., 1985; Lin et al., 1985). Then, recombinant human Epo (rHuEpo) was successfully used to treat anemic patients with end-stage renal diseases (ESRD) (Bunn, 2013). Furthermore, studies on *Epo* gene regulation led to the identification of hypoxia-inducible transcription factors (HIFs) and hypoxia response elements (HREs) as the HIF-binding consensus sequence on genome (Semenza et al., 1991b), and to the current understanding of the molecular mechanisms of cellular adaptation to hypoxia (Semenza, 2011; Ratcliffe, 2013).

In past decades, many researchers have made rigorous efforts to identify erythropoietin-producing cells in kidneys; however, a uniform understanding of which cells produce Epo in kidneys was not established until the era of genetically modified mice (Suzuki et al., 2007). Using gene targeting and bacterial artificial chromosome (BAC) transgenic methods, we identified nearly all of interstitial fibroblast-like cells in the cortex and outer medulla to be renal erythropoietin-producing cells (REPs) (Obara et al., 2008; Pan et al., 2011; Yamazaki et al., 2013). Furthermore, interests in REPs have markedly increased by the evidence showing the crucial link between fibrosis and anemia *via* the loss of Epo-producing ability of myofibroblast-transformed REPs (MF-REPs) (Maxwell et al., 1997; Asada et al., 2011; Souma et al., 2013). Importantly, this direct link indicates that renal fibrosis and anemia could be simultaneously treated by targeting or regulating the cellular properties of REPs. In this review, we provide a summary of recent lines of evidence regarding the role of REPs in health and disease and discuss future research directions for regulating REP functions to treat both fibrosis and anemia.

Identification of Renal Erythropoietin-Producing Cells

To identify Epo-producing cells in the kidneys, *in situ* hybridization and/or immunohistochemistry have been utilized to detect Epo in tissue sections. However, these strategies have limited sensitivity and specificity for detecting Epo, hampering the establishment of a consensus as to which cells produce Epo. By using a transgenic mouse technology, SV40 T antigen cDNA was integrated into the *Epo* gene locus to identify renal Epo-producing cells (REPs) with anti-T antigen antibodies (Maxwell et al., 1993), and the results showed that renal fibroblasts are the top candidates among the proposed Epo-producing cells including tubular epithelial cells, glomerular mesangial cells, and interstitial fibroblasts.

To unequivocally determine the identity of REPs, we have utilized two complimentary strategies, BAC transgenic mice and green fluorescent protein (GFP) reporter knock-in mice (Obara et al., 2008; Pan et al., 2011). The BAC transgenic mouse lines (Tg-EpoGFP) harbor transgene constructs, which direct GFP expression under the control of the 180-kb regulatory region around the mouse *Epo* gene, and label Epo-producing cells by GFP expression with high sensitivity. The other strategy using a genetically modified mouse line (KI-EpoGFP, $Epo^{GFP/wt}$), in which the GFP cDNA is knocked-in to the endogenous *Epo* gene, assures a higher specificity than the Tg-EpoGFP strategy because the GFP expression in the knock-in mice is directed by endogenous *Epo* gene regulation. Only a few renal interstitial cells are labeled by both strategies under normal conditions, whereas GFP-positive cells robustly emerge and increase in the kidneys under anemic or hypoxic stress. These GFP-positive cells in the interstitium are fibroblast-like cells expressing neural genes [e.g., microtubule-associated protein 2 (Map2), nerve growth factor receptor (Ngfr), and neurofilament light peptide (Nefl)]. We named these cells renal erythropoietin-producing cells, or REPs (Suzuki et al., 2007; Obara et al., 2008).

Epo Gene Modification for Further Analyses of REPs

Epo-knockout mice die at approximately embryonic day 12.5 (E12.5) due to severe anemia (Wu et al., 1995), hampering the analyses of *Epo* gene function in adults. We assumed that the embryonic lethality of *Epo*-null mice could be rescued by transgenic *Epo* gene expression in the livers of embryos because hepatocytes are the Epo-producing cells at the lethal time point (Suzuki et al., 2011). To this end, we have utilized the knowledge regarding *Epo* gene regulation; i.e., the proximal downstream region of the *Epo* gene transcription end site (*EpoHE*, hepatic enhancer) is sufficient for *Epo* gene expression from hepatocytes, but is dispensable for renal *Epo* gene expression (Suzuki et al., 2011). An 8-kb *Epo* transgene ($Tg\text{-}Epo^{3.3}$) containing *EpoHE* but not the kidney regulatory elements has been constructed. This transgene successfully rescues the embryonic lethality of *Epo*-null mice ($Epo^{GFP/GFP}$) (Yamazaki et al., 2013). In the transgene-rescued mice (named as Inherited Super-Anemic Mice, ISAM; $Epo^{GFP/GFP}$:$Tg\text{-}Epo^{3.3}$), Epo is mainly produced in their liver, and the plasma Epo concentrations are repressed to undetectable levels by the weaning age, which is the ontogenetic time point at which the Epo-producing site switches from the liver to the kidney. ISAM develop adult onset severe chronic anemia due to defect in renal Epo production, and Epo-producing cells are efficiently labeled with GFP expression from the *Epo* gene locus in adult ISAM kidneys (Yamazaki et al., 2013).

The numbers of GFP-expressing REPs are markedly higher in ISAM with chronic severe anemia than those of Tg-EpoGFP or KI-EpoGFP mice with bleeding-induced acute anemia (Yamazaki et al., 2013). Thus, it appears that the EpoGFP-expressing cells represent a small portion of the total REPs. To identify the total REPs, we have conducted fate-tracking assays of cells with a history of Epo production using newly generated Tg-EpoCre mice in which the Cre-recombinase is expressed under the regulation of the 180-kb transgene containing the *Epo* gene regulatory region (Souma et al., 2013; Yamazaki et al., 2013). To detect EpoCre-labeled cell lineages, we have utilized the R26RtdTomato mouse line in which cells that have expressed Cre at least once are marked by tdTomato red fluorescence. When *EpoCre* transgene expression is enhanced by chronic severe anemia in ISAM (ISAM:R26RtdTomato:Tg-EpoCre, ISAM-REC), tdTomato fluorescence is detected in

almost all fibroblast-like interstitial cells (PDGFRβ$^+$CD73$^+$ cells) in the cortex and outer medulla (both the outer stripe and the inner stripe) (**Figure 1**). The results indicate that cells capable of producing Epo (total REPs) are far more abundant in kidneys than we previously expected in Tg-EpoGFP mice. Interestingly, we have observed REPs around the peritubular capillaries, but not in the regions surrounding the larger vessels (**Figure 1**). Furthermore, in ISAM-REC kidneys, approximately 10% of tdTomato-positive cells express EpoGFP (ON-REPs), indicating that most REPs (OFF-REPs) are resting from Epo production even under severe anemic conditions (**Figure 1**) (Yamazaki et al., 2013; Souma et al., 2013).

Regulatory Mechanisms Governing Epo Synthesis

The balance between oxygen supply and demand precisely controls renal Epo production. A salient physiological study using isolated rat kidneys with hypoxic perfusate shows that renal Epo secretion is regulated by tissue oxygen tension (Pagel et al., 1990). Distant organs, such as skin have been found to participate in regulation of renal Epo synthesis through sensing a hypoxic atmosphere (Boutin et al., 2008). Recently, Dimke et al. demonstrated that the importance of the renal oxygen levels for renal *Epo* gene expression using a mouse model with tubular-specific *Vegfa* (vascular endothelial growth factor a) gene deletion. In this mouse model, the kidney has scarce vascularization, resulting in renal hypoxia, increased Epo secretion, and severe polycythemia (Dimke et al., 2015), indicating that the oxygen supply in the kidneys, but not in other organs or tissues, is the most important factor to determine renal Epo synthesis.

Induction of hypoxia/anemia widely spread the distribution of currently Epo-producing cells (ON-REPs: defined by EpoGFP expression) from the juxta-medullary region, which is physiologically hypoxic, to the entire cortex (Koury et al., 1989; Eckardt et al., 1993; Obara et al., 2008; Souma et al., 2013; Yamazaki et al., 2013). Interestingly, the ratio of ON-REPs to the total REPs in the kidney correlates well with plasma Epo levels, indicating that renal Epo secretion is regulated by "ON–OFF switch" for *Epo*-gene expression in each REP in a hypoxia-inducible manner (**Figure 2**). In other words, the conversion of OFF-REP to ON-REP is governed by transcriptional control of the *Epo* gene, which is primarily regulated by HIF transcription factors (Haase, 2010; Suzuki, 2015).

Understanding of the *Epo* gene regulation has been advanced by the discovery of Epo-producing hepatoma cell lines (Hep3B and HepG2) (Goldberg et al., 1987). Analyses of these cells led to the discovery of HIF proteins and their binding sequences, HREs (**Figure 3**) (Semenza et al., 1991b). In order to understand renal *Epo* gene regulation, transgenic mouse lines with different regulatory regions have been generated. For instance, *EpoHE* containing an HRE is proved to be necessary and sufficient to direct hepatic *Epo* gene expression *in vivo* (Suzuki et al., 2011). However, this enhancer is dispensable for renal *Epo* gene expression, indicating that other *cis*-elements direct renal

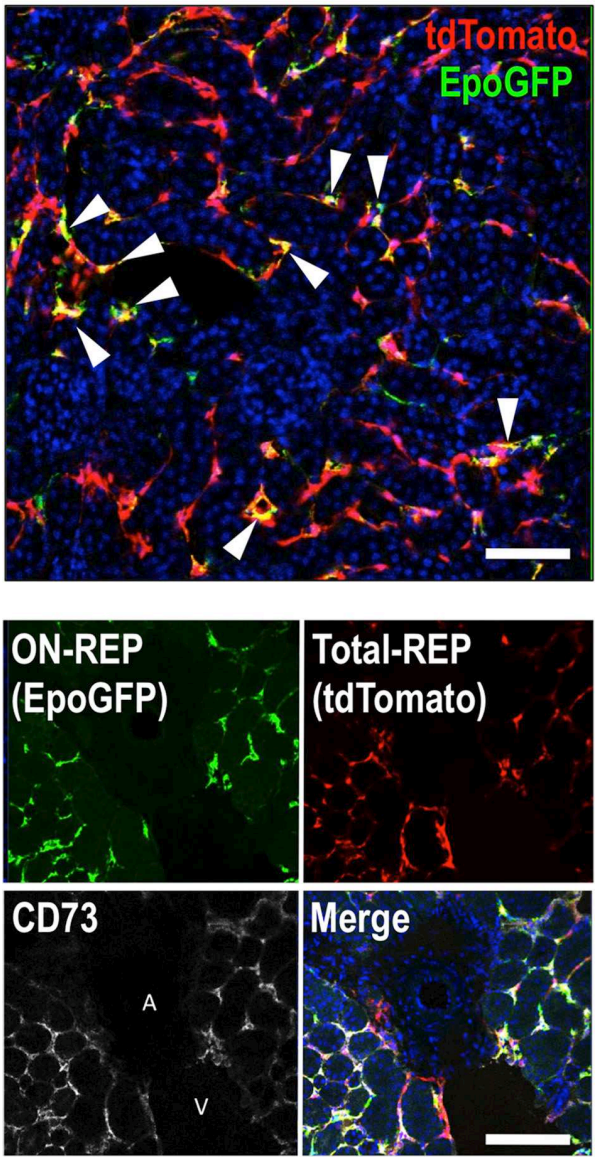

FIGURE 1 | REPs are peri-capillary CD73-positive fibroblast-like cells. A kidney section from ISAM-REC (ISAM:R26RtdTomato:Tg-EpoCre) shows ON-REPs expressing EpoGFP (arrowheads, positive for both green and red) in the total REP population (positive for tdTomato fluorescence, red). The nuclei are stained by DAPI (blue). A kidney section from ISAM-REC shows that REPs or CD73-positive fibroblasts are distributed in peri-capillary interstitial spaces but not in peri-arterial interstitial areas of the kidneys. DAPI (blue) is used for nuclear staining in the merged image. Abbreviations: A, artery; V, vein. Scale bar, 100 μm.

Epo production (**Figure 3**). *EpoHE* also contains a direct repeat sequence that is considered a binding site for hepatic nuclear factor 4 (HNF4) and/or retinoid X receptor (RXR); together with HIFs, these factors synergistically regulate liver-specific, hypoxia-inducible *Epo* gene expression (Galson et al., 1995; Makita et al., 2001).

One of the important lingering questions about *Epo* gene regulation is the location and characteristics of the regulatory

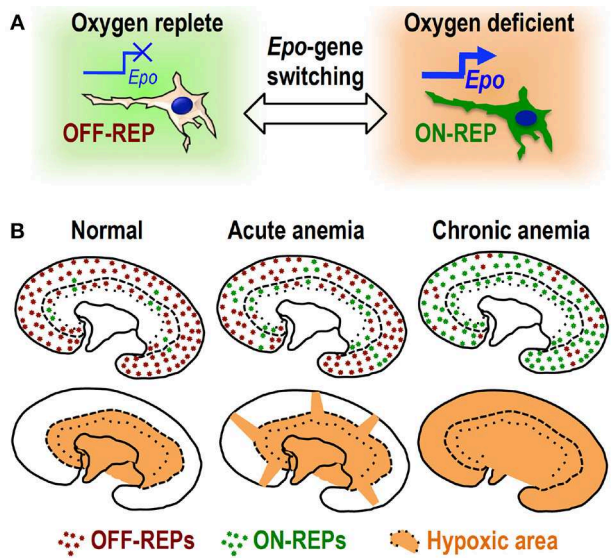

FIGURE 2 | ON–OFF regulation of Epo synthesis in REPs. (A) Oxygen deficiency turns OFF-REPs into ON-REPs by turning on "*Epo* gene switch." **(B)** REPs are primarily in the "OFF" state (OFF-REPs, brown dots) under normal oxygen conditions. Under acute anemic conditions, some clusters of OFF-REPs turn into ON-REPs (green dots). The distribution of ON-REPs extends toward hypoxic cortical areas (orange). Strikingly, most OFF-REPs turn into ON-REPs under chronic anemia. This recruitment of ON-REPs determines the total amount of Epo secreted by the kidneys.

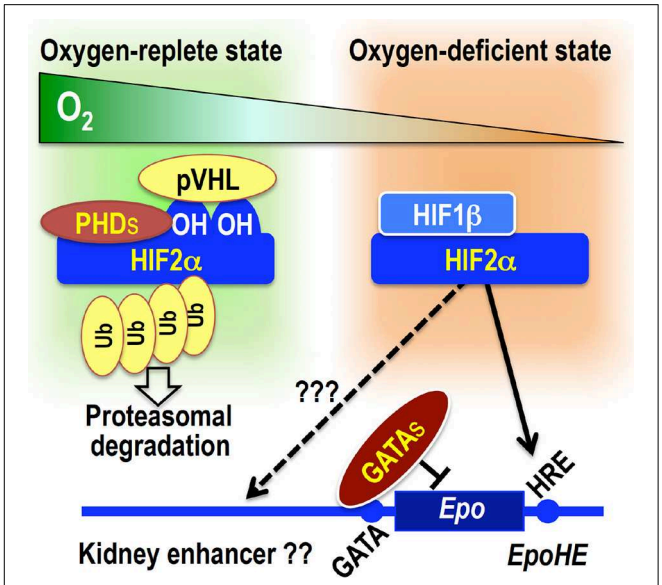

FIGURE 3 | Overview of *Epo* gene transcriptional regulation. Under oxygen-replete conditions, PHDs hydroxylate HIF2α proteins, leading to pVHL-mediated ubiquitination and degradation of HIF2α. Under oxygen-depleted condition, PHDs are inactivated, and HIF2α escapes degradation. The HIF2α–HIF1β heterodimer activates *Epo* gene transcription by binding to the HRE. The functional HRE for hepatic *Epo* gene expression (*EpoHE*) is in the proximal downstream region of the transcription end site, whereas a kidney enhancer has been suggested to be located in a region far upstream of the transcription start site. GATA factors constitutively suppress ectopic *Epo* gene expression in epithelial lineage cells by binding to the promoter GATA sequence.

regions for renal *Epo* gene expression. It has been surmised that an essential regulatory region may lie far upstream from the transcription start site (−14 to −6 kb; **Figure 3**) (Semenza et al., 1991a; Madan et al., 1995; Suzuki et al., 2013). We also have utilized a series of BAC transgenic reporter lines that direct GFP expression from the transgenic *Epo* locus, and the regulatory region for renal *Epo* gene expression has been narrowed down to the region from −17 to +15 kb (Obara et al., 2008; Suzuki et al., 2013).

The *Epo* gene promoter lacks the typical TATA box, but has a negative regulatory element with a GATA box (Imagawa et al., 1991). By mutating the GATA box in *EpoGFP* transgene constructs, the GATA box is proved to be not necessary for inducible *Epo* gene expression in either REPs or hepatocytes (Obara et al., 2008). However, the GATA box is turned out to be indispensable for repressing ectopic *Epo* gene expression in epithelial lineage cells, including distal tubular cells, bronchial epithelial cells, and cholangiocytes. In renal distal tubular cells, the GATA box is occupied by GATA transcription factors. In this manner, the GATA box contributes to the tissue-specific *Epo* gene expression (**Figure 3**) (Obara et al., 2008).

Of the transcription factors interacting with these *Epo* gene regulatory regions, HIFs play the central role in hypoxia-inducible *Epo* gene expression (Haase, 2010; Suzuki, 2015). HIFs are heterodimeric complexes comprising one α subunit (HIF1α, HIF2α, and HIF3α) and one β subunit (HIF1β, also known as ARNT) (Semenza, 2011; Ratcliffe, 2013). Under oxygen-replete conditions, the proline residues of HIFα subunits are hydroxylated by HIF prolyl hydroxylases (or prolyl hydroxylase domain proteins, PHDs: PHD1, PHD2, and PHD3), leading to their proteasomal degradation *via* pVHL (von Hippel Lindau protein)-mediated ubiquitination (**Figure 3**) (Tanimoto et al., 2000). Under oxygen-deficient conditions, the enzymatic activities of PHDs are inhibited, and HIFα proteins escape from degradation. Stable HIFα proteins dimerize with HIF1β, and the heterodimeric complexes bind to HRE in regulatory regions of target genes, including *Epo* (**Figure 3**). PHD2 and HIF2α are the most important factors among those related to PHD-HIF signaling in the renal Epo production (**Table 1**) (Souma et al., in press).

In addition to PHDs, another HIF-hydroxylase, FIH-1 (factor inhibiting HIF-1), is involved in the cellular responses to hypoxia (Mahon et al., 2001). FIH-1 negatively regulates HIF-dependent transactivation by inhibiting CREB-binding protein (CBP)/p300 recruitment to HIFα *via* the asparaginyl hydroxylation of HIFα proteins. The asparaginyl hydroxylation is more resistant to suppression by hypoxia than the prolyl hydroxylation (Tian et al., 2011). FIH-1-dependent hydroxylation is more prone to be inhibited by oxidative stresses than by hypoxic stresses (Masson et al., 2012). Consistent with these findings, the systemic knockout of FIH-1 does not result in defects in the *Epo* gene regulation directly (**Table 1**) (Zhang et al., 2010).

TABLE 1 | Summary of gene targeting studies of hypoxia-related factors on renal *Epo* gene regulation.

Deleted factor	Effects on renal *Epo* gene expression	References
HIF1α	Inducible KO in adult: no change	Gruber et al., 2007
HIF2α	Inducible KO in adult: decrease Kidney-specific KO: decrease	Gruber et al., 2007; Rankin et al., 2006
PHD1 and PHD3	Systemic double KO: no change	Takeda et al., 2008
PHD2	Inducible KO in adult: increase REP-specific KO: increase	Takeda et al., 2008; Souma et al., in press
pVHL	Kidney-specific KO: increase	Rankin et al., 2006

KO, knockout.

REPs as Major Contributors to Renal Fibrosis

All chronic nephropathies progress with tubular atrophy and interstitial fibrosis along with the relative loss of Epo production (Quaggin and Kapus, 2011). While fibrosis is an essential biological process for repairing tissue injuries, uncontrolled and persistent injuries lead to sustained fibrogenesis, followed by destruction of tissue architecture and organ failure (Quaggin and Kapus, 2011; Friedman et al., 2013). Therefore, the identification of therapeutics controlling the pathological fibrogenic response would be beneficial for many devastating diseases, such as chronic kidney disease (CKD), cirrhosis, and pulmonary fibrosis (Friedman et al., 2013). Since a strong correlation between tubulo-interstitial injury and decreased glomerular filtration rate was first described, many researchers have sought the origin of scar-forming cells, i.e., myofibroblasts that are fibroblast-like cells with contractile properties, in the renal interstitium (Quaggin and Kapus, 2011; Boor and Floege, 2012). Using genetic lineage tracing, various cellular sources have been postulated as the origins of myofibroblasts, including pericytes, resident fibroblasts, tubular cells, endothelial cells, fibrocytes, and bone marrow-derived cells, but exact contributions of the sources to renal fibrosis still remain under debate (summarized in **Table 2**) (Quaggin and Kapus, 2011; Mack and Yanagita, 2014).

A possible direct link between the loss of Epo production and progression of fibrosis was first proposed in 1997 (Maxwell et al., 1997). Maxwell et al. showed that REPs, which are tagged by integrated SV40 T antigen cDNA in the *Epo* gene locus, turn into desmin-positive myofibroblasts following ureteral obstruction injury, and that the number of T-antigen-expressing cells decreases to less than 5% of control kidneys in 9 days after the obstruction (Maxwell et al., 1997). Interestingly, tamoxifen, a selective estrogen receptor modulator, is found to improve the Epo-producing ability of myofibroblasts (Asada et al., 2011).

To better understand the contribution of REPs to renal fibrosis and the link between fibrosis and anemia, ISAM have been utilized as the most efficient reporter mouse model for the Epo-producing ability. The Epo-producing ability of REPs is lost in kidneys within 24 h after ureteral obstruction, and strong α-smooth muscle actin (αSMA) expression is observed in REPs from 2 days following ureteral obstruction onward (**Figure 4**). These results indicate that the renal fibrogenic milieu strongly represses *Epo* gene transcription in REPs during their myofibroblast transformation process (Souma et al., 2013). Gene expression analyses of isolated MF-REPs show that MF-REPs produce inflammatory cytokines, chemokines, and extracellular matrix; all of which drive renal fibrosis. Consistent with the finding that renal myofibroblasts contribute to the inflammatory milieu, damage-associated molecular patterns (DAMPs) induce IL-6 and MCP1 productions in myofibroblasts (Campanholle et al., 2013). Additionally, the loss of local Epo production might have deteriorating effects on fibrogenesis and inflammation in the kidneys, because cytoprotective function of Epo beyond erythropoiesis has been predicted (Noguchi et al., 2008).

Functional lineage tracing using the *EpoCre* transgene shows that the cortical and outer medullary interstitium of ISAM-REC kidneys are primarily replaced by MF-REPs in unilateral ureteral obstruction (UUO) model, indicating that REPs are the major source of the myofibroblasts (**Table 2**) (Souma et al., 2013). MF-REPs lose their Epo-producing ability and persist in scar tissues. These results indicate that the transformation of REPs to MF-REPs or myofibroblasts directly links both fibrosis and anemia. Interestingly, the product of the hemoglobin (Hb) concentration times the Epo concentration in the peripheral blood of patients with diabetic nephropathy correlates well with the stages of diabetic nephropathy and predicts future chronic renal failure in overt diabetic nephropathy (Inomata et al., 1997). Although confirmation of this argument waits for larger studies, we surmise that Epo would be a good biomarker to estimate the severity of interstitial injury and to predict the prognosis of damaged kidneys based on the short half-life (4–8 h) of Epo (Jelkmann, 2002).

One aspect that makes the determination of the origins of myofibroblasts difficult is the complexity regarding the identity of the interstitial cells, i.e., pericytes, fibroblasts, and REPs. It has been shown that FoxD1-tagged pericytes are the major source of renal myofibroblasts (Humphreys et al., 2010), whereas another groups argue that resident fibroblasts are the major source of renal myofibroblasts (**Table 2**) (Asada et al., 2011; LeBleu et al., 2013). As mentioned above, we found that REPs are the major source of renal myofibroblasts through EpoCre-based functional lineage tracing (Souma et al., 2013). Because these three cell types (pericytes, resident fibroblasts, and REPs) share similar cellular surface markers (PDGFRβ and CD73), locations, and morphology, we believe that these cells are largely overlapping populations. Recently, Kramann et al. reported that a small subset of pericytes ($Gli1^+PDGFR\beta^+CD73^-$ cells; 0.2% of renal $PDGFR\beta^+$ cells), which displays mesenchymal stem cell features, is the major contributor of renal fibrosis through rigorous proliferation upon injury (Kramann et al., 2015). These evidence raise a possibility

TABLE 2 | Summary of the origins of renal myofibroblasts.

Cell fate tracking method	Target cell type	Injury model	αSMA$^+$ cells	References
TUBULAR EPITHELIAL CELLS (EPITHELIAL–MESENCHYMAL TRANSITION, EMT)				
γGT-Cre	Cortical tubular cells	UUO	36%	Iwano et al., 2002
γGT-Cre	Cortical tubular cells	UUO	5%	LeBleu et al., 2013
Pax8-rtTA:TetON-Cre	All tubular cells	TGFβ O/E	0%	Koesters et al., 2010
Ksp-Cre	Distal tubular cells	UUO	0%	Li et al., 2010
Six2-Cre/Hoxb7-Cre	All tubular cells	UUO	0%	Humphreys et al., 2010
INTERSTITIAL MESENCHYMAL CELLS (FIBROBLASTS/PERICYTES/REPS)				
FoxD1-Cre	Pericytes, fibroblasts	UUO	>90%	Humphreys et al., 2010
P0-Cre	Fibroblasts	UUO	93%	Asada et al., 2011
EpoCre	REPs	UUO	>80%	Souma et al., 2013
ENDOTHELIAL CELLS (ENDOTHELIAL-MESENCHYMAL TRANSITION, ENDMT)				
Tie2-Cre	Endothelial cell	UUO/STZ/Alport	30–50%	Zeisberg et al., 2008
Cdh5-Cre	Endothelial cell	UUO	10%	LeBleu et al., 2013
BONE MARROW-DERIVED CELLS				
Fsp1-EGFP, BMT	Myeloid cells, fibroblasts	UUO	15%**	Iwano et al., 2002
Col1a1-GFP, BMT	Collagen-producing cells	UUO	<0.1%	Lin et al., 2008
Y chromosome, BMT	Bone marrow-derived cells	UUO	8.6%*	Roufosse et al., 2006
Y chromosome, BMT	Bone marrow-derived cells	UIRI	Detected	Lin et al., 2009
R26-hPAP, BMT	Bone marrow-derived cells	UIRI	32%**	Broekema et al., 2007
αSMA-RFP, BMT	αSMA-positive cells	UUO	35%	LeBleu et al., 2013

*UUO, unilateral ureteral obstruction; UIRI, unilateral ischemia reperfusion injury; STZ, streptozotocin-induced diabetic nephropathy; Alport, Col4a3 knockout mice; O/E, overexpression. *No collagen I production; **collagen I production; **12% of αSMA-positive cells in normal kidneys were derived from the bone marrow.*

that renal myofibroblasts are originated from a Gli1$^+$ subset of EpoCre-tagged REPs, and this hypothesis waits for future confirmation.

Plasticity of REPs

Some clinical and experimental reports have shown that renal structural damage, including fibrosis, is reversible (Zeisberg et al., 2003; Fioretto et al., 2006). Clinical observations have shown that more than 10% of patients on dialysis become anemia free and rHuEpo independent (Takeda et al., 2002; Kuo et al., 2005; Schwartz et al., 2005). These lines of evidence suggest that MF-REPs retain functional reversibility even in ESRD. Indeed, a short-term reversible UUO model demonstrates that MF-REPs regain their physiological characteristics, morphology, and Epo-producing ability following disease resolution (**Figure 5**) (Souma et al., 2013). Furthermore, dexamethasone facilitates this reversion, possibly through enhancing the resolution of inflammation in injured kidneys (Souma et al., 2013). These results indicate that REPs possess plasticity in response to environmental cues. Consistent with this observation, hepatic myofibroblasts can revert to their normal cellular character (hepatic stellate cells) during the regression of fibrosis (Kisseleva et al., 2012).

Environmental Cues for Myofibroblast Transformation

A genome-wide transcriptome analysis of sham-treated kidneys, obstructed kidneys, and recovering kidneys indicates that the atherosclerotic and acute phase response signals are the top two up-regulated pathways and that valine, leucine, and isoleucine degradation and fatty acid metabolisms are the top two down-regulated pathways (Souma et al., 2013). Recent transcriptome analyses using kidney samples from human CKD patients reveals that the gene expressions of fatty acid metabolism are decreased and inflammatory signaling is increased in kidney diseases (Kang et al., 2015). Based on the fact that metabolic intermediates play an important role in gene regulation, it is of great interest to determine whether deranged fatty acid metabolism would affect Epo production and whether correcting the metabolism, e.g., by PPARα activation (Kang et al., 2015), would restore Epo-producing ability of MF-REPs.

Uremic toxins are a group of compounds that are normally excreted by healthy kidneys, but accumulate upon kidney injuries. Of the toxins, indoxyl sulfates cause renal inflammation and repress Epo production by decreasing HIF-α accumulation in response to hypoxic stimuli (Chiang et al., 2011), and the NO antagonist N^G-monomethyl-L-arginine (L-NMMA) represses *Epo* gene transcription through GATA2 upregulation

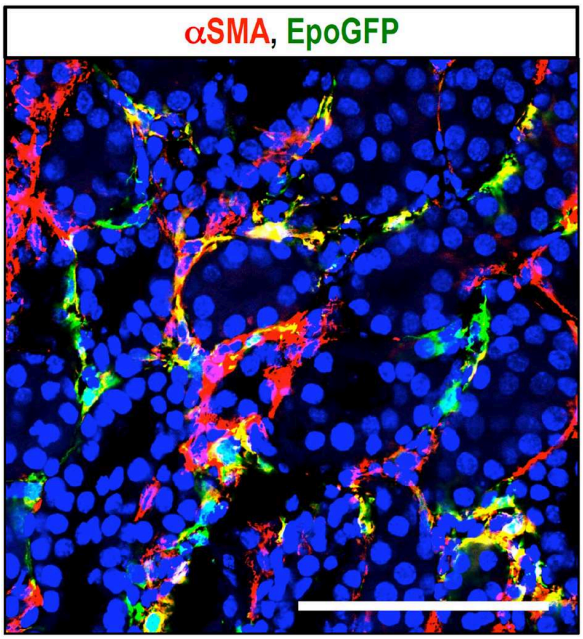

FIGURE 4 | Myofibroblast transformation of REPs. Immunohistochemical detection of myofibroblasts with αSMA antibody (red) in ureteral obstructed kidney of ISAM. EpoGFP-positive REPs (green) transform into myofibroblasts (MF-REPs) upon kidney injury induced by ureteral obstruction for 3 days. Blue: DAPI for nuclear staining. EpoGFP protein expression does not reflect ongoing *Epo* gene transcription due to its longer half-life. *EpoGFP* mRNA expression is immediately silenced by urinary obstruction (Souma et al., 2013). Scale bar, 100 μm.

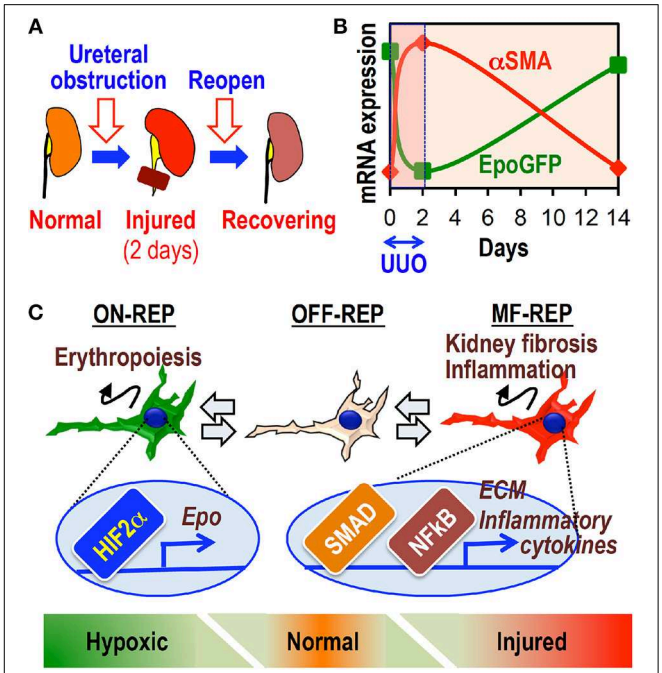

FIGURE 5 | The cellular plasticity of REPs governs both fibrosis and anemia. (A) Schematic diagram of the reversible UUO model. The left ureter is obstructed by a vascular clip for 2 days and then re-opened afterwards. **(B)** The inverse relationship between EpoGFP and αSMA mRNA expression in whole kidneys of ISAM during reversible UUO treatment. **(C)** Schematic summary showing the plasticity of REPs. MF-REPs produce extracellular matrix (ECM) and inflammatory cytokines in injured kidneys through SMAD and NFκB signaling cascades. After the resolution of environmental cues, MF-REPs revert to their original and physiological Epo-producing phenotype. Switching between ON-REPs and OFF-REPs is determined in a hypoxia-dependent manner *via* HIF2α activation.

(Tarumoto et al., 2000). These results further emphasize the importance of correcting the unbalanced microenvironment in injured kidneys.

The TGFβ and NFκB signaling pathways are two major signaling pathways involved in kidney fibrosis. Of note, TGFβ but not LPS injection leads to up-regulation of genes for extracellular matrix, while LPS but not TGFβ injection represses *Epo* gene expression. These results support the notion that TGFβ signaling is the master regulator of the fibrogenic response, while the inflammatory signals are the primary regulators of Epo repression (Souma et al., 2013).

Recently, cellular communication upon kidney injuries is gaining attention, particularly communication between renal tubular cells and fibroblasts/pericytes (Grgic et al., 2012; Humphreys et al., 2013), pericytes and endothelial cells (Schrimpf et al., 2012), and infiltrating leukocytes and resident renal cells such as distal tubular cells (Fujiu et al., 2011). However, fewer efforts have been made to decipher the nexus of environmental cues by quantifying the contribution of each cell type to the fibrogenic cues. Gene expression analyses of leukocytes isolated from injured kidneys demonstrate that expressions of TNFα, TGFβ, and MMP9 are enriched in leukocytes, whereas IL-6 and MMP3 expressions are not, suggesting that the infiltrating leukocytes and other resident renal cells collaboratively create deleterious inflammatory microenvironments (Souma et al., 2013).

Epigenetic alterations, including DNA methylation, play an important role in cellular transformation. TGFβ-mediated activation of DNA methyl transferase 1 (DNMT1) has been shown to fundamentally regulate the perpetuation of fibrosis (Bechtel et al., 2010), and DNMT1 (primarily catalyzes maintenance methylation) and DNMT3b (primarily catalyzes *de novo* methylation) expression is increased in MF-REPs (Souma et al., 2013). Interestingly, the *Epo* gene locus is highly methylated in cell lines lacking Epo-producing abilities, indicating the importance of DNA methylation for the epigenetic silencing of *Epo* gene expression (Yin and Blanchard, 2000). Thus, one would be easy to surmise that the sustained activation of inflammatory and fibrogenic signaling may alter the epigenetic code of REPs and limit the potential of reversing MF-REPs to their original state.

Effect of Hypoxia on Epo Production and Kidney Diseases

Kidney injuries and subsequent fibrosis disrupt oxygen delivery through vascular rarefactions and excessive extracellular matrix accumulation. Increased oxygen demands also cause kidney cells to be vulnerable to hypoxia. Renal anemia further compromises

the delivery of oxygen, and the anemic hypoxia has been suggested to promote kidney diseases (Nangaku, 2006). Because HIFs are the major regulators of hypoxic adaptation, augmenting HIF signaling has been attempted to treat kidney diseases (Miyata et al., 2013). A clinical trial has been reported that the pharmacological activation of HIF signaling successfully augmented Epo production in ESRD (Bernhardt et al., 2010), underscoring the feasibility of this therapeutic strategy. Actually, REP-specific knockout of PHD2 in mice induces Epo production both in healthy and fibrotic kidneys through HIF2α activation (Souma et al., in press).

Genetic activation of HIF signaling in podocytes and/or proximal tubular cells results in the worsening of kidney diseases (Ding et al., 2006; Higgins et al., 2007), whereas deletions of HIF signaling in myeloid cells, endothelial cells, and/or whole body (Kobayashi et al., 2012; Kapitsinou et al., 2014) result in improvement of renal inflammation and fibrosis. Adequacy of hypoxia signaling upon kidney injuries has been questioned recently. Immunohistochemical analyses of kidneys in a rodent AKI model have revealed that HIF1α expression is impaired in proximal tubular cells (Fahling et al., 2013). Similarly, EpoGFP expression in ISAM is repressed by ureteral obstruction despite the presence of severe anemia (Souma et al., 2013). Furthermore, the successful augmentation of Epo production in diseased kidneys by PHD inhibitors implies that the primary cause of Epo insufficiency is the inappropriately high PHD activity in diseased kidneys, despite a severely hypoxic milieu (Bernhardt et al., 2010). Collectively, we posit that the response to hypoxia is impaired or insufficient upon kidney injury through inappropriately high PHD activity (Souma et al., in press).

Epo production is inappropriately repressed despite the presence of severe hypoxia in injured kidneys. Interestingly, TNFα-treated rodents show decreased Epo-producing ability under hypoxic or anemic conditions (Nakano et al., 2004). Consistent with this observation, inflammatory cytokines elicited in damaged kidneys repress the Epo-producing ability, emphasizing the important effect of sterile inflammation on repressing the Epo-producing ability of REPs (Souma et al., 2013). Considering these data, we posit that inflammatory signals redistribute cellular oxygen levels and activate PHDs in severely hypoxic kidneys, resulting in HIF2α degradation and impairment of the *Epo* gene expression (Souma et al., in press).

Perspectives

We propose that identifying signals that restore physiological characteristics of REPs in fibrotic kidneys will open a new avenue for treating CKD. To accomplish this goal, several barriers must be overcome. One of the highest barriers is the difficulty in handling REPs. To screen candidate pathway to treat CKD, a good readout that reflects both diseased and recovered states of kidneys is necessary. For tubular cells, KIM-1 and NGAL (Paragas et al., 2011; Humphreys et al., 2013) are the representative readouts for the injured state and are available in clinics. For myofibroblast activation, αSMA and collagen expression is currently used for monitoring fibrogenic activity. However, these markers are not perfect for testing whether cells regain their original character upon treatment. The Epo-producing ability is the important physiological feature of renal fibroblast-like cells, and its loss is the hallmark of renal myofibroblasts. Thus, developing a methodology to culture REPs *ex vivo* and to monitor their Epo-producing ability by reporter genes would provide an opportunity to perform high-throughput screening to identify novel target signals to restore MF-REPs to normal REPs.

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REVIEW
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Role of Bone Marrow-Derived Fibroblasts in Renal Fibrosis

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Renal fibrosis represents a common pathway leading to progression of chronic kidney disease. Renal interstitial fibrosis is characterized by extensive fibroblast activation and excessive production and deposition of extracellular matrix (ECM), which leads to progressive loss of kidney function. There is no effective therapy available clinically to halt or even reverse renal fibrosis. Although activated fibroblasts/myofibroblasts are responsible for the excessive production and deposition of ECM, their origin remains controversial. Recent evidence suggests that bone marrow-derived fibroblast precursors contribute significantly to the pathogenesis of renal fibrosis. Understanding the molecular signaling mechanisms underlying the recruitment and activation of the bone marrow-derived fibroblast precursors will lead to novel therapy for the treatment of chronic kidney disease. In this review, we summarize recent advances in our understanding of the recruitment and activation of bone marrow-derived fibroblast precursors in the kidney and the development of renal fibrosis and highlights new insights that may lead to novel therapies to prevent or reverse the development of renal fibrosis.

Keywords: chemokine, cytokine, circulating fibroblast precursors, fibroblasts, renal fibrosis, extracellular matrix, chronic kidney disease, monocyte-to-fibroblast transition

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INTRODUCTION

Chronic kidney disease (CKD) is a global public health problem. Worldwide, over 1 million people die from CKD yearly. It is estimated that more than 20 million Americans have CKD and more than 450,000 Americans suffer from end-stage renal disease (ESRD) requiring renal replacement therapy. CKD has become the eighth leading cause of death in the United States. Regardless of various etiologies, a common pathological feature of CKD is renal fibrosis, which is characterized by accumulation of fibroblasts/myofibroblasts with increased production and deposition of extracellular matrix (ECM) including collagen type I, III, IV, fibronectin, vimentin, and proteoglycans (Zeisberg and Neilson, 2010; Conway and Hughes, 2012; Farris and Colvin, 2012). It is generally agreed that activated fibroblasts termed myofibroblasts are the principle cells responsible for the increase ECM production and deposition, the origin of these matrix-producing cells remains debatable. They were traditionally thought to arise from resident interstitial fibroblasts (Qi et al., 2006; Picard et al., 2008). Recent evidence indicate that they may originate from epithelial/endothelial-to-mesenchymal transition (Iwano et al., 2002; Zeisberg and Kalluri, 2004; Zeisberg et al., 2008; Liu, 2010), pericytes (Lin et al., 2008), and bone marrow-derived fibroblast precursors termed fibrocytes (Grimm et al., 2001; Sakai et al., 2006; Broekema et al., 2007; Niedermeier et al., 2009; Chen et al., 2011).

In response to kidney injury, multiple cell types in the circulation are recruited to the site of injury to participate in a wound healing response. A dysregulated wound healing process causes

fibrosis, where ECM and fibroblasts replace normal renal parenchyma and lead to kidney dysfunction. Therefore, investigations of underlying mechanisms and identification of novel therapeutic targets of renal fibrosis will have huge impact on patient survival, global health, and economic burden. Fibrosis is a complex and progressive pathological process involving infiltration of mononuclear cells including bone marrow-derived fibroblast precursors (fibrocytes), macrophages, and immune cells, which suggest that interaction and communication among these cell types regulates the development of fibrotic disorders (Kisseleva and Brenner, 2008; Wynn, 2008). Because activated fibroblasts are the principal effector cells that mediate ECM production in the fibrotic kidney disease, their activation is regarded as an important event in the pathogenesis of renal fibrosis (Eddy, 2005; Neilson, 2006). However, the origin of the activated fibroblasts has been controversial. They are traditionally thought to arise from resident fibroblasts within the kidney. Recent studies have shown that the activated fibroblasts may originate from bone marrow-derived fibroblast precursors (Grimm et al., 2001; Iwano et al., 2002; Sakai et al., 2006; Broekema et al., 2007; Chen et al., 2011). In a clinical study of mismatched kidney transplantation in humans, the proportion of host-derived smooth muscle actin (SMA)-positive cells is ∼30% in allografts undergoing chronic rejection compared with 10% in those without rejection (Grimm et al., 2001). In rodent models of renal fibrosis, we and others have shown that bone marrow-derived fibroblasts migrate into the kidney in response to injury (Iwano et al., 2002; Roufosse et al., 2006; Broekema et al., 2007; Chen et al., 2011; Xia et al., 2014b). For example, one study using bone marrow transplantation of transgenic mice that express enhanced green fluorescence protein (GFP) under the control of the fibroblast specific protein 1 (FSP1) promoter has demonstrated that 15% of bone marrow-derived fibroblasts are present in the kidney 10 days after obstructive injury (Iwano et al., 2002). Using bone marrow transplantation of transgenic rats that express human placental alkaline phosphatase, Broekema et al. have shown that more than 30% α-SMA positive myofibroblasts are derived from bone marrow 7 days after ischemia-reperfusion injury (Broekema et al., 2007). Using chimeric mice transplanted with GFP-expressing bone marrow cells, Li et al have reported that more than 30% of renal α-SMA+ myofibroblasts are derived from the bone marrow in a mouse adriamycin-induced fibrosis model (Li et al., 2007). Recently, Lebleu et al. have demonstrated that 35% α-SMA positive myofibroblasts are derived from bone marrow 10 days after obstructive injury using bone marrow transplantation of transgenic mice express red fluorescence protein (RFP) driven by α-SMA (Lebleu et al., 2013). Using bone marrow transplantation of transgenic mice that express GFP driven by collagen 1A1 promoter, we have shown that bone marrow-derived hematopoietic fibroblasts migrate into the kidney, proliferate, and differentiate into α-SMA$^+$ myofibroblasts (Xia et al., 2014b). In contrast, one study reported that bone marrow-derived GFP$^+$ fibroblasts contribute to a minor fraction of myofibroblasts (Lin et al., 2008). Another study reported that bone marrow-derived cells do not contribute significantly to collagen synthesis using bone marrow transplantation of transgenic mice that express luciferase and β-galactosidase driven by collagen 1A2 promoter (Roufosse et al., 2006). One potential pitfall of bone marrow transplantation is the bone marrow engraftment rate. Furthermore, detection of GFP, luciferase, or β-galactosidase in tissue can be technically demanding. Therefore, it is difficult to interpret negative data obtained with these reporter mice. To objectively quantify the number of hematopoietic fibroblasts in the kidney, we stained freshly-isolated kidney cells with CD45, a hematopoietic cell marker, and collagen I, a mesenchymal cell marker, and examined with flow cytometry. The results have shown that CD45$^+$ and collagen I$^+$ cells constituted 45% of total collagen I$^+$ cells in the kidney 7 days after obstructive injury (Xia et al., 2014b). Using lineage tracing and adoptive transfer, Wang et al. have recently reported that monocytes/macrophages from bone marrow can transform into myofibroblasts via macrophage-to-myofibroblast transition (Wang et al., 2015). Therefore, compelling evidence indicate that bone marrow-derived fibroblasts contribute significantly to the pathogenesis of renal fibrosis and suggest targeting bone marrow-derived fibroblasts may represent a novel therapeutic strategy for the treatment of fibrotic kidney disease and possible fibrotic disorders of other organs.

RECRUITMENT OF BONE MARROW-DERIVED FIBROBLAST PRECURSORS

The recruitment of circulating cells into sites of injury is mediated by locally produced chemokines. Chemokines are classified based on the relative position of cysteine residues near the NH2 terminus into four major families: CC, CXC, C, and CX3C (Rollins, 1997; Mackay, 2001). Recent studies have shown that chemokines and their receptors play an important role in the recruitment of bone marrow-derived fibroblast precursors into the kidney in response to injury. CCL21/CCR7, CXCL16/CXCR6, CCR2 are involved in recruiting circulating fibroblast precursors into the kidney (**Table 1**) (Sakai et al., 2006; Chen et al., 2011; Reich et al., 2013; Xia et al., 2013a,b, 2014a,b).

The chemokine CXCL16 is a recently discovered cytokine belonging to the CXC chemokine family (Matloubian et al., 2000; Wilbanks et al., 2001). It was originally described as a scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein (SR-PSOX; Shimaoka et al., 2000). There are two forms of CXCL16. The transmembrane form is a type I transmembrane glycoprotein consisting of an extracellular N-terminal chemokine domain, glycosylated mucin-like stalk, transmembrane-spanning region, and a short cytoplasmic domain, with a YXPV motif that is a potential tyrosine-phosphorylation and SH2-protein-binding site (Izquierdo et al., 2014). The transmembrane form functions as an adhesion molecule for CXCR6 expressing cells and scavenger receptor for oxidized low-density lipoprotein (Shimaoka et al., 2000, 2004). The soluble form generated by its cleavage at the cell surface functions as a chemoattractant to recruit circulating cells (Gough et al., 2004). CXCL16 is induced in kidney tubular epithelial cells *in vivo* in a murine model of renal fibrosis induced by obstructive injury (Okamura et al., 2007; Chen et al., 2011). Tumor necrosis

TABLE 1 | Chemokines and their receptors in the recruiment of bone marrow-derived fibroblast precusors or fibrocytes.

Chemokines/Receptors	Methods	Models	Effect on fibrosis	References
CCL21/CCR7	Anti-CCL21 antibody CCR7 knockout mice	UUO	40–50% reduction	Sakai et al., 2006
CXCL16	CXCL16 knockout mice	UUO	40–50% reduction	Chen et al., 2011
CCR2	CCR2 knockout mice, depletion of fibrocytes, BMT	UUO	20–30% reduction	Reich et al., 2013
CCR2	CCR2 knockout mice	UUO	30–40% reduction	Xia et al., 2013a
CXCL16	CXCL16 knockout mice	Angiotensin II infusion	40–50% reduction	Xia et al., 2013b
CXCR6	CXCR6 knockout mice, BMT	Angiotensin II infusion	40–50% reduction	Xia et al., 2014a
CXCR6	CXCR6 knockout mice, BMT	UUO, IRI	40–50% reduction	Xia et al., 2014b

BMT, bone marrow transplantation; IRI, ischemia-reperfusion injury; UUO, unilateral ureteral obstruction.

factor (TNF)-α and interferon (IFN)-γ upregulate CXCL16 expression in tubular epithelial cells *in vitro* (Xia et al., 2013a). In addition, the TNF superfamily cytokine TNF-like weak inducer of apoptosis (TWEAK) increases CXCL16 expression in kidney tubular epithelial cells *in vitro* and *in vivo* (Izquierdo et al., 2012). Interestingly, angiotensin II, a key promoter of kidney injury, also enhances CXCL16 expression in tubular epithelial cells via activation of NF-kB, a master regulator of inflammation (Xia et al., 2013b). We have recently studied the functional role of CXCL16 in the pathogenesis of renal fibrosis in a murine model of obstructive nephropathy using CXCL16 knockout mice. Our results have shown that bone marrow-derived fibroblast precursor infiltration, myofibroblast activation, and ECM protein deposition are reduced in the obstructed kidneys of CXCL16-knockout mice (Chen et al., 2011). These data indicate that CXCL16 promotes renal fibrosis by recruiting bone marrow-derived fibroblast precursors. More recently, we have examined the functional role of CXCL16 in angiotensin II-induced renal injury and fibrosis. Our results have demonstrated that genetic deletion of CXCL16 protects the kidney from angiotensin II infusion-induced renal dysfunction, inhibits renal fibrosis, reduces proteinuria, suppresses bone marrow-derived fibroblast accumulation, myofibroblast formation, macrophage, and T cell infiltration and pro-inflammatory cytokine expression without affecting blood pressure at baseline or in response to angiotensin II infusion (Xia et al., 2013b). In support of clinical relevance of these observations in experimental animal models, clinical studies have shown that circulating CXCL16 is elevated in patients with CKD and diabetic nephropathy and high levels of CXCL16 are associated with CKD progression and development of proteinuria (Lin et al., 2011; Zhao et al., 2014).

CXCR6 is the receptor for CXCL16. CXCR6 was first cloned as an orphan receptor by three independent groups and was termed STRL33, BONZO, or TYMSTR (Alkhatib et al., 1997; Deng et al., 1997; Loetscher et al., 1997). We have recently shown that both circulating fibroblast precursors and bone marrow-derived fibroblasts in the kidney express CXCR6 (Chen et al., 2011; Xia et al., 2014b). Genetic disruption of CXCR6 reduces the recruitment of bone marrow-derived fibroblast precursors into the kidney and the development of renal fibrosis induced by ureteral obstruction, ischemia-reperfusion, and angiotensin II infusion (Xia et al., 2014a,b).

In these studies, we have observed that genetic deficiency of CXCL16 or CXCR6 does not completely block bone marrow-derived fibroblast precursor infiltration into the kidney and renal fibrosis development, suggesting that other chemokine/receptor pairs may be involved in the process of recruiting bone marrow-derived fibroblast precursors into the kidney. Consistent with this notion, CCR2 and CCL21/CCR7 have been reported to play a role in the recruitment of bone marrow-derived fibroblast precursors into the kidney and the development of renal fibrosis (Sakai et al., 2006; Reich et al., 2013; Xia et al., 2013a). Interestingly, the expression of CXCL16 in the kidney is reduced in CCR2 knockout mice in response to obstructive injury, suggesting the interaction of two distinct chemokine systems modulates renal tubular epithelial cell-initiated fibrosis (Xia et al., 2013a).

ACTIVATION OF BONE MARROW-DERIVED FIBROBLAST PRECURSORS

TGF-β1/Smad3

The activation of bone marrow derived fibroblast precursors plays a crucial role in the pathogenesis of renal fibrosis (Yang et al., 2013; Chen et al., 2014). Myofibroblasts are a population of smooth muscle-like fibroblasts that express α-smooth muscle actin (α-SMA; Powell et al., 1999). The activation of myofibroblasts is generally considered a main event in the pathogenesis of renal fibrosis (Nath, 1992; Eddy, 2013). Furthermore, experimental and clinical studies have shown that the number of interstitial myofibroblasts is associated closely with the severity of tubulointerstitial fibrosis and the rapidity of kidney disease progression (Zhang et al., 1995; Essawy et al., 1997; Roberts et al., 1997). The activation of bone marrow-derived fibroblast precursors are regulated by locally produced cytokines (Yang et al., 2013; Chen et al., 2014). TGF-β1 is a key profibrotic cytokine that play an important role in the pathogenesis of renal fibrosis through activation of a cascade of intracellular signaling pathways (Border et al., 1990; Border and Noble, 1994; Böttinger and Bitzer, 2002; Lan, 2011). Evidence suggests that activation of the canonical Smad signaling cascade plays an important role in stimulating ECM protein expression and tissue fibrosis (Verrecchia et al., 2001; Zhao et al., 2002; Sato et al., 2003; Latella et al., 2009; Huang et al., 2010; Meng et al., 2015). We have recently examined the functional role of Smad3 in the activation of bone marrow-derived fibroblast precursors *in vitro* and *in vivo* (Chen et al., 2014). In cultured monocytes,

TGF-β1 activates Smad3. Smad3 deficient monocytes express less amount ECM proteins at baseline and Smad3 deficiency completely abolished TGF-β1-induced expression of α-SMA and extracellular matrix proteins in cultured monocytes *in vitro*. Smad3-knockout mice accumulate significantly fewer bone marrow-derived fibroblasts in the kidney after obstructive injury, exhibit less myofibroblast activation, and express less α-SMA in the obstructed kidney. Furthermore, genetic deletion of Smad3 reduces total collagen deposition and suppresses expression of extracellular matrix proteins. Additionally, wild-type mice engrafted with $Smad3^{-/-}$ bone marrow cells displayed fewer bone marrow-derived fibroblasts in the kidney with obstructive injury and showed less severe renal fibrosis compared with wild-type mice engrafted with $Smad3^{+/+}$ bone marrow cells. These results indicate that Smad3 of bone marrow-derived cells plays an important role in bone marrow-derived fibroblast activation. However, Samd3 deficiency does not completely abolish bone marrow-derived fibroblast activation, collagen deposition, and ECM protein expression *in vivo*. These results suggest that other factors may be involved in bone marrow-derived fibroblast activation.

JAK3/STAT6

The activation of bone marrow-derived fibroblasts is modulated by inflammatory cells in the microenvironment. T cells plays an important role in the pathogenesis of renal fibrosis (Tapmeier et al., 2010), which have been reported to regulate bone marrow-derived fibrocyte activation (Niedermeier et al., 2009). Naïve $CD4^+$ T cells can differentiate into two major distinct phenotypes, Th1 and Th2 cells, which are characterized by specific cytokine expression patterns (Wynn, 2004). Th2 cells produce Th2 cytokines such as IL-4 and IL-13, which induce alternative activation of macrophage and promotes monocyte-to-fibroblast transition, Th1 cells produces Th1 cytokines such as IFN-γ and IL-12, which promote classical activation of macrophages and inhibit fibrocyte differentiation (Wynn, 2004; Shao et al., 2008). However, the molecular signaling mechanisms by which Th2 cytokines promote bone marrow-derived fibroblast activation are not known. We have found that JAK3/STAT6 signaling pathway is activated during the development of renal fibrosis and plays an important role in bone marrow-derived fibroblast activation, extracellular matrix protein production, and interstitial fibrosis development (Yan et al., 2015). Specifically, our results have shown that Th2 cytokine—IL-4 or IL-13 induces STAT6 activation and stimulates bone marrow monocytes to express ECM proteins and α-smooth muscle actin (α-SMA). CP690550, a JAK3-specific inhibitor, or STAT6 deficiency inhibits IL-4/IL-13-indcued STAT6 activation and expression of ECM proteins and α-SMA in bone marrow monocytes *in vitro*. Furthermore, CP690550 treatment or STAT6 deficiency inhibits bone marrow-derived fibroblast activation and ECM protein production in the kidney in response to obstructive nephropathy. To further confirm the role of bone marrow STAT6 signaling in myeloid fibroblast activation, we performed bone marrow chimeric experiments. Wild-type mice transplanted with STAT6 null bone marrow cells exhibit fewer bone marrow-derived fibroblasts and develop a lesser degree of renal fibrosis. These results suggest that inhibition of JAK3/STAT6 signaling may serve as a novel therapeutic target for fibrotic kidney disease.

Adiponectin/AMPK

Adiponectin is a multifunctional cytokine and an important regulator of lipid and carbohydrate metabolism. Emerging evidence suggests that circulating adiponectin levels are elevated in patients with CKD, and a high level of adiponectin is linked to increased cardiovascular mortality (Zoccali et al., 2003; Shimotomai et al., 2005; Iwashima et al., 2006; Zoccali and Mallamaci, 2011; Mills et al., 2013). We have discovered that adiponectin is induced following ischemia-reperfusion and obstructive injury (Yang et al., 2013). Genetic deletion of adiponectin inhibits bone marrow-derived fibroblast accumulation and myofibroblast activation. Furthermore, adiponectin deficiency also reduces the expression of profibrotic chemokines and cytokines and the production of ECM protein in the kidneys following obstructive injury or ischemia-reperfusion. These results indicate that adiponectin plays a significant role in the activation and maturation of bone marrow-derived fibroblasts and the development of renal fibrosis. Mechanistically, adiponectin stimulated α-SMA and extracellular matrix protein expression in bone marrow-derived monocytes via activation of adenosine monophosphate-activated protein kinase (AMPK). AMPK inhibition with a pharmacological inhibitor (compound C) or dominant negative AMPK-α1 attenuated adiponectin-induced expression of α-SMA and extracellular matrix proteins. Furthermore, AMPK activation with 5-aminoimidazole-4-carboxamide-riboside (AICAR), a cell permeable adenosine analog (Corton et al., 1995), resulted in increased expression of α-SMA and extracellular matrix proteins. These results indicate that adiponectin is a critical regulator of monocyte-to-fibroblast transition and renal fibrosis. Therefore, inhibition of adiponectin/AMPK signaling may represent a novel therapeutic target for fibrotic kidney disease.

It is generally thought that macrophages do not produce ECM proteins. These cells promote fibrosis indirectly by producing profibrotic cytokines that activate fibroblasts (Wynn and Barron, 2010). Recently, a model of two major macrophage classes has been proposed. Classically activated macrophages exhibit a Th1-like phenotype and promote inflammation in response to Th1 cytokines; while alternatively activated macrophages or M2 macrophages display a Th2-like phenotype and stimulate ECM production in response to Th2 cytokines (Gordon and Martinez, 2010). M2 macrophages are characterized by expressing MHC class II, mannose receptor (CD206), Fizz1/Relm-α, and arginase. Alternatively activated macrophages are implicated in the fibrogenesis of other organs (Gordon and Martinez, 2010). The functional role of macrophages has been intensively investigated using a variety of depletion/blocking strategy in the UUO model of renal fibrosis. Kitagawa et al. reported that genetic deletion of CCR2 or treatment with CCR2 inhibitors reduces the infiltration of F4/80-positive macrophages into the kidney and attenuates the development of renal fibrosis (Kitagawa et al., 2004). Depletion of macrophages using CD11b-DTR mice reduces myofibroblast accumulation and the degree of renal fibrosis through regulation of galectin-3 expression in the kidney (Henderson et al.,

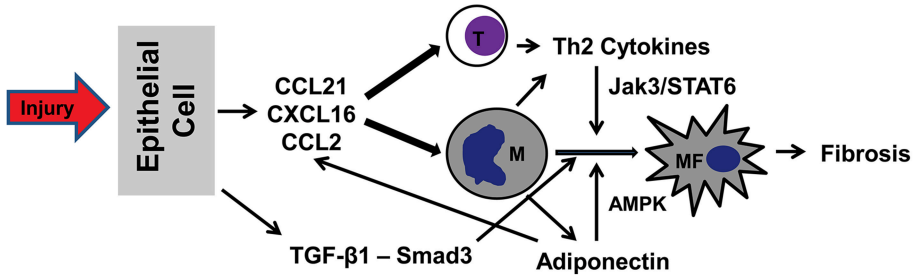

FIGURE 1 | A proposed model of signaling mechanisms underlying recruitment and activation of bone marrow-derived fibroblasts in renal fibrosis. In response to injury (urinary tract obstruction, ischemia-reperfusion, hypertension), tubular epithelial cells produce chemokines CCL21/CXCL16/CCL2 and cytokine TGF-β1. Chemokines act concertedly to recruit bone marrow-derived cells (T cells, monocytes, and fibrocytes) via interaction with their respective receptors. TGF-β1 activates Smad3 to stimulate monocyte-to-fibroblast transition. Th2 cells produce Th2 cytokines, which activate JAK3/STAT6 signaling pathway to promote monocyte-to-fibroblast transition. Finally, adiponectin produced by infiltrating inflammatory cells regulates chemokine and cytokine production and stimulates monocyte-to-fibroblast transition through activation of AMPK. T, T cells; M, Monocytes; MF, Myeloid fibroblasts.

2008). Interestingly, Galectin-3 has been shown to promote M2 macrophage polarization (MacKinnon et al., 2008). Selectively depletion of F4/80-positive cells with liposome clodronate attenuates the development of renal fibrosis, which is associate with a reduction of TNF-α and TGF-β gene expression (Kitamoto et al., 2009). However, not all macrophage depletion/inhibition strategies result in a reduction of renal fibrosis in the UUO model. Depletion of leukocyte with cyclophosphamide increases the degree of renal fibrosis, which is attenuated by adoptive transfer of macrophages (Nishida et al., 2005). Furthermore, inhibition of macrophage colony-stimulating factor receptor kinase reduces macrophage accumulation and tubular apoptosis without affecting the development of renal fibrosis (Ma et al., 2009). These studies may reflect the functional heterogeneity of the macrophage subsets. We have recently demonstrated for the first time that alternatively activated macrophages produce procollagen I, suggesting a link between M2 macrophage polarization and monocyte-to-fibroblast transition (Yang et al., 2013). Consistent with this novel concept, we have shown that adiponectin deficiency suppresses M2 macrophage polarization and inhibits the number of collagen-expressing M2 macrophages in the injured kidneys (Yang et al., 2013). Therefore, macrophages can participate in the pathogenesis of renal fibrosis via direct and indirect mechanisms (Meng et al., 2014; Nikolic-Paterson et al., 2014).

Renin Angiotensin System

Recent studies have shown that renin angiotensin system plays an important role in the development of fibrotic kidney disease (Mezzano et al., 2001). Inhibition of renin angiotensin system with ACE inhibitors or angiotensin type 1 blockers attenuates experimental renal fibrosis development in animals and retards CKD progression in humans (Ishidoya et al., 1995; Brenner et al., 2001). Sakai et al. have examined the role of renin angiotensin system in the regulation of fibrocytes in murine models of renal fibrosis (Sakai et al., 2008). Their results have shown that inhibition of angiotensin II type 1 receptor (AT1R) with valsantan reduces the number of fibrocytes in the kidney and bone marrow and inhibits the development of renal fibrosis. In contrast, angiotensin II type 2 receptor (AT2R) knockout mice exhibit increased number of fibrocytes in the kidney and bone marrow and develop more severe renal fibrosis. Furthermore, inhibition of AT1R with valsantan decreases angiotensin II-induced collage type I and TGF-β1 expression while inhibition of AT2R increases angiotensin II-induced collage type I and TGF-β1 expression in cultured fibrocytes. This study indicates that angiotensin II receptors play opposite role in the development and activation of fibrocytes. Further studies are needed to dissect the downstream signaling mechanisms underlying angiotensin II-induced fibrocyte activation.

CONCLUSION

Recent studies have demonstrated that bone marrow derived fibroblast precursors contribute significantly to the pathogenesis of renal fibrosis. Recruitment and activation of bone marrow-derived fibroblasts are mediated through the interaction between chemokines/cytokines and their receptors (**Figure 1**). Therefore, targeting the signaling machinery of these chemokines/cytokines could represent novel therapeutic strategy for the treatment of renal fibrosis and possible fibrotic disorders of other organs.

FUTURE PERSPECTIVE

Although chemokines are involved in recruiting bone marrow-derived fibroblasts into the kidney in response to injury, the molecular signaling mechanisms underlying chemokines-induced bone marrow-derived fibroblast recruitment remains to be defined. Ras proteins are members of a family of small GTPase that control signaling pathways involved in cell migration, proliferation, differentiation, and survival (Rodríguez-Pena et al., 2005). There are three Ras proteins, H-Ras, K-Ras, and N-Ras. These Ras proteins are ubiquitously expressed including the kidney. Recent studies have shown that Ras proteins are involved in the development of renal fibrosis. Grande et al. have reported that genetic gelation of H-Ras inhibits myofibroblast activation and renal fibrosis development following ureteral obstruction in mice (Grande et al., 2010). Future studies are needed to define

the role of Ras signaling in the regulation of fibrocyte migration, proliferation, and differentiation.

AUTHOR CONTRIBUTIONS

JY drafted the manuscript. ZZ, LJ, and YW reviewed and edited the manuscript.

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