TGF-β IN HUMAN DISEASE: FRIEND OR FOE?

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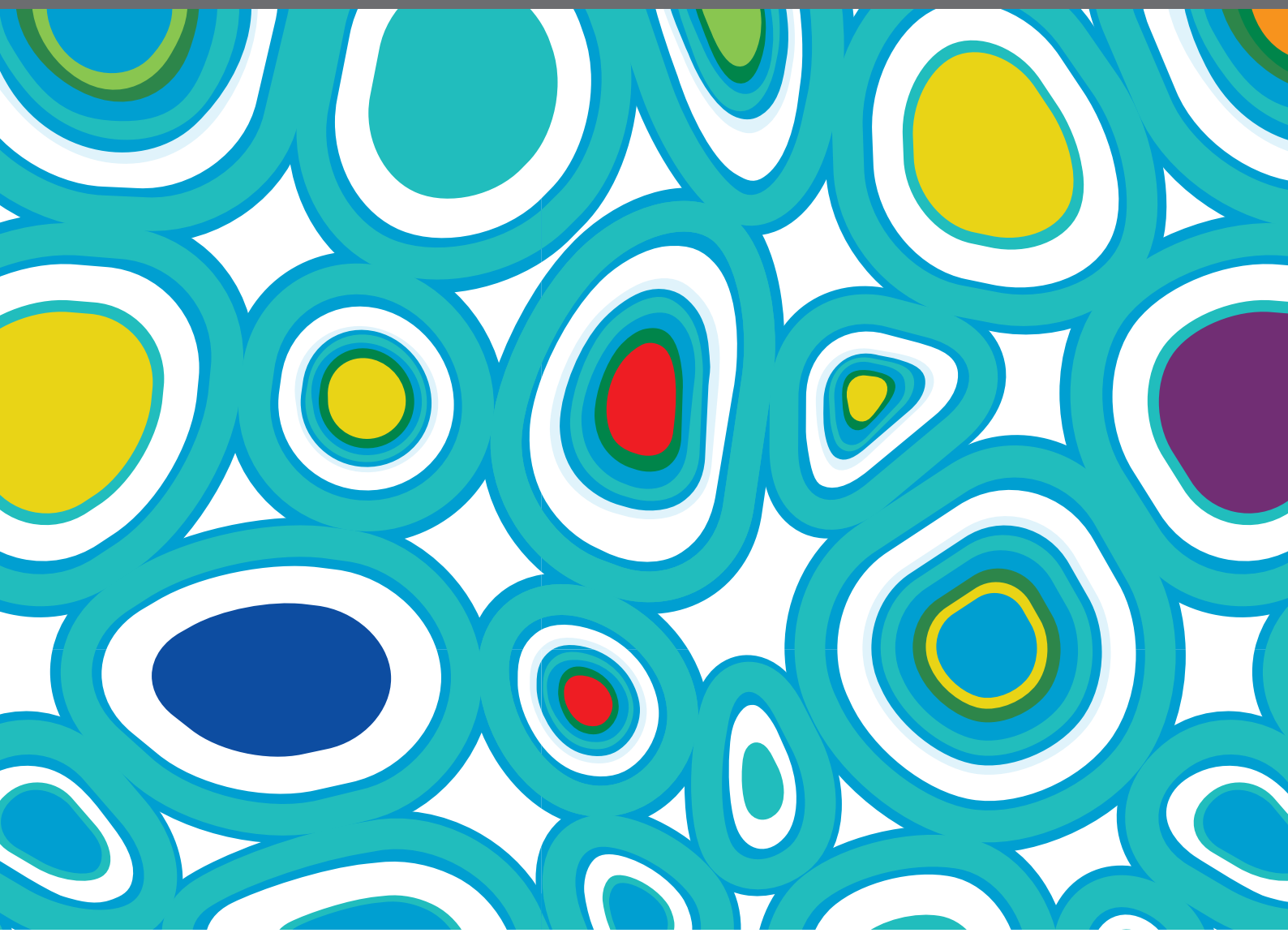

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TGF-β IN HUMAN DISEASE: FRIEND OR FOE?

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EDITORIAL
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Editorial: TGF-β in Human Disease: Friend or Foe?

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Keywords: TGF-β, inflammation, fibrosis, cancer, TCF, Foxo (forkhead box protein O), p53, SMAD

Editorial on the Research Topic

TGF-β in Human Disease: Friend or Foe?

TGF-β is a family of essential multifunctional cytokines regulating (1) cell growth & development at both embryonic and adult stages; (2) inflammation and maintenance of host resistance mechanisms; and (3) remodeling and repair processes including angiogenesis and regeneration. However, conflicting roles of TGF-β in human diseases create a major challenge to the therapeutic targeting of this multifunctional cytokine.

In this issue, the protean roles of TGF-β in humans are discussed in chronic inflammatory diseases characterized with inflammation and fibrosis, in cerebrovascular disease and in cancer. The chronic inflammatory diseases included are chronic kidney disease, diabetic kidney disease, cardiac fibrosis, pulmonary arterial hypertension, atherosclerosis, and fibrosis of other organs. TGF-β-induced fibrosis of the diseased organs is one of the most prominent features of these diseases. TGF-β contributes to organ fibrosis by induction of mesenchymal transition, namely epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition (EndoMT), although their direct contribution to the origin of myofibroblasts in organ fibrosis has been debated (Ma et al.). The potential of TGF-β-induced EndoMT in tissue engineering is reviewed. In addition, TGF-β's induction of mesenchymal transition is discussed with regard to its contribution to development of cerebrovascular diseases such as cerebral cavernous malformation due to maladaptive dysfunction of TGF-β (Zhang and Yang). Post-translational modification of SMAD ubiquitin regulatory factor 2 (Smurf2) is reviewed for its role in regulating TGF-β signaling and its potential for targeting TGF-β signaling in human fibrotic diseases and cancer (Bai and Ying). p53, a tumor suppressor, was found to complex with SMADs to transcriptionally regulate genomic TGF-β1 fibrotic-response gene profiles. SMADs/p53 targeted genes and cross-talking pathways are discussed as targets for treating kidney fibrosis (Higgins et al.). The protective anti-inflammatory roles of TGF-β via differential functions of the SMAD family members are reviewed in kidney disease (Gu et al.). New candidate targets for treatment of kidney fibrosis, receptor interacting protein kinase 3 (RIPK3) (Shi et al.) and calcium-activated potassium channel KCa3.1 (Huang et al.) are discussed. An important review article discusses in depth the lack of success of on-target anti-TGF-β therapies in clinical cancer treatment as well as remaining challenges (Teixeira et al.).

The conflicting roles of TGF-β, which may slow or accelerate progression of various human diseases, render it unsuitable as a therapeutic target. Indeed, anti-TGF-β therapies have proven unsuccessful in clinical trials for fibro-inflammatory kidney disease (Vincenti et al., 2017), and in cancer clinical trials (Ahmadi et al., 2019).

To resolve the conflicting anti-inflammatory and profibrotic roles of TGF-β in inflammatory diseases, rebalancing of Smad3/Smad7 signaling or specifically targeting Smad3-dependent non-coding RNAs that regulate kidney fibrosis or inflammation suggested as better therapeutic approaches for kidney fibrosis (Gu et al.).

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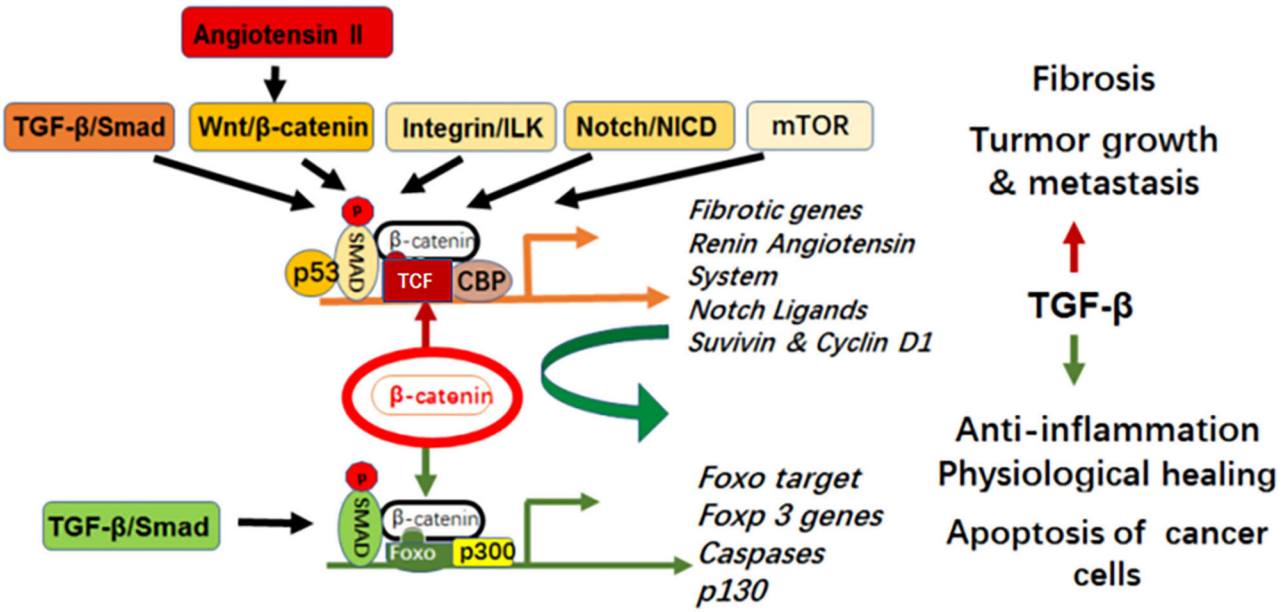

FIGURE 1 | Cell specific cross-talking pathways in fibro-inflammatory disease and cancer.

Similarly in cancer, TGF-β inhibits proliferation and induces apoptosis of cancer cells, but it also promotes metastasis by inducing the invasive phenotype of tumor cells through induction of epithelial-mesenchymal transition (EMT). Furthermore, it causes immuno-suppression, including the induction of immunosuppressive checkpoint molecule PD-1, which inactivates the anti-tumor function of immune cells. Combination of anti-TGF-β therapies with immune checkpoint inhibitors, or more spatio-temporal controlled interventions, are suggested by Teixeira *et al* for improved treatment of cancer (Teixeira et al.).

Previously, a mechanism to explain the multiple functions of TGF-β had been proposed by Massagué. He suggested that the net outcome of TGF-β signals is determined by a cell specific complex network of cross-talking signals. The combined DNA-binding specificity of a SMAD-cofactor complex dictates the choice of target gene, whereas SMAD affinity for DNA is too low to do that alone (Massague, 2000). β-catenin binding to SMAD as a cofactor enables a cell specific cross-talk between TGF-β and Wnt pathways. Foxo has been identified as another co-factor of β-catenin competing with TCF.

β-catenin binds to either TCF or Foxo to produce opposite signaling outcomes: proliferation (β-catenin/TCF) or cell cycle arrest for survival under oxidative stress (β-catenin/Foxo). TGF-β mediates pro-fibrotic signaling via cross-talk with multiple pathways including Wnt/β-catenin, integrin/integrin linked kinase (ILK), the renin angiotensin system (RAS), Notch/NICD and mROR, all converging at activation of β-catenin/TCF (**Figure 1**). In this context, Qiao and colleagues revealed in murine models of kidney fibrosis that the inhibition of β-catenin/TCF prevented the profibrotic effects of TGF-β while promoting its anti-inflammatory function via concomitant β-catenin/Foxo1-induction of regulatory T cells (Tregs) (Qiao et al., 2018, Rao et al., 2019, Rao et al., 2021; **Figure 1**). This has been confirmed by other labs; the β-catenin/Foxo complex induces Tregs (Sumida et al., 2018) and protects against kidney fibrosis (Nlandu-Khodo et al., 2020).

In summary, the papers in this issue illustrate how the various paradoxical functions of TGF-β signaling may be targeted to design and optimize therapeutic approaches for patients suffering from diseases associated with TGF-β. More importantly, dissection of the unique and often conflicting roles of transcription cofactor complexes holds great promise for targeting TGF-β pathways in human disease (Emami et al., 2004).

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GZ contributed to conception and drafting and final approval of the manuscript. DH contributed to conception and final approval of the manuscript. All authors contributed equally to the article and approved the submitted version.

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REVIEW
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Diverse Role of TGF-β in Kidney Disease

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Inflammation and fibrosis are two pathological features of chronic kidney disease (CKD). Transforming growth factor-β (TGF-β) has been long considered as a key mediator of renal fibrosis. In addition, TGF-β also acts as a potent anti-inflammatory cytokine that negatively regulates renal inflammation. Thus, blockade of TGF-β inhibits renal fibrosis while promoting inflammation, revealing a diverse role for TGF-β in CKD. It is now well documented that TGF-β1 activates its downstream signaling molecules such as Smad3 and Smad3-dependent non-coding RNAs to transcriptionally and differentially regulate renal inflammation and fibrosis, which is negatively regulated by Smad7. Therefore, treatments by rebalancing Smad3/Smad7 signaling or by specifically targeting Smad3-dependent non-coding RNAs that regulate renal fibrosis or inflammation could be a better therapeutic approach. In this review, the paradoxical functions and underlying mechanisms by which TGF-β1 regulates in renal inflammation and fibrosis are discussed and novel therapeutic strategies for kidney disease by targeting downstream TGF-β/Smad signaling and transcriptomes are highlighted.

Keywords: TGF-β, Smads, fibrosis, inflammation, mechanisms, therapy

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INTRODUCTION

Increasing evidence shows that chronic kidney disease (CKD) is a global-burden-disease (Romagnani et al., 2017). The prevalence and incidence of CKD have risen by almost 90% over last 30 years (Provenzano et al., 2019). During the progression of CKD, renal function is impaired with a loss of nephrons and the development of renal fibrosis characterized by the excessive accumulation of extracellular matrix (ECM) components, reduction in glomerular filtration rate (GFR), and abnormal albuminuria (Glassock et al., 2017). CKD eventually leads to the development of end-stage renal disease (ESRD) (Eddy and Neilson, 2006; Liu, 2011). Fibrosis and inflammation are the two major features of CKD and prolonged renal inflammation promotes renal fibrosis as well (Meng et al., 2014; Li et al., 2017). Physiologically, fibrosis is a repair and healing process in response to the initial renal insults. However, as the pathological condition prolongs, unresolved renal inflammation turns into a major driving force to promote renal scar formation via a progressive process of renal fibrosis (Meng et al., 2014; Mihai et al., 2018).

Transforming growth factor-β has been long considered as a master cytokine in the pathogenesis of renal inflammation and fibrosis (Meng et al., 2016). The TGF-β superfamily contains

members of TGF-βs, activins, inhibins, growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs), and glial-derived neurotrophic factors (GDNFs) (Zhang and Newfeld, 2013). It is well established that there are three isoforms of TGF-β in mammals, the TGF-β1, 2 and 3 (Roberts et al., 1991). Of these, TGF-β1 has been considered as a profibrotic mediator in various kidney diseases (Sureshbabu et al., 2016). Newly synthesized TGF-β1 releases and binds to the latency-associated peptide (LAP) to form a latent complex which later binds to the TGF-β binding protein (LTBP) to form a larger complex (Ando et al., 1995; Kusakabe et al., 2008). The latent complex is inactive and stored in the ECM until it is released by reactive oxygen species (ROS) and plasmin or acid. Once TGF-β1 is released from LAP and LTBP, it becomes active (Saharinen et al., 1999; Annes et al., 2003). Active TGF-β1 binds to Type II TGF-β receptor (TβRII), which recruits and activates Type I TGF-β receptor (TβRI) and downstream receptor-associated Smads (R-Smads), Smad2, and Smad3. The phosphorylated Smad2/3 then form an oligomeric complex with Smad4 (Derynck and Zhang, 2003; Lan and Chung, 2012). Subsequently, the Smad2/3/4 complex translocate into the nucleus to regulate transcription of target genes, inducing α-smooth muscle actin (α-SMA), collagens, and inhibitory Smad7 (Nakao et al., 1997; Miyazawa and Miyazono, 2017). Interestingly, Smad7 can antagonize TGF-β-mediated fibrosis, carcinogenesis and inflammation in various diseases (Yan et al., 2009; Troncone et al., 2018; Zhou G. et al., 2018). Smad7 negatively regulates TGF-β/Smad signaling by competing with the R-Smad binding to the TβRI (Yan et al., 2016; **Figure 1**). Moreover, Smad7 also induces the IκBα, a NF-κB inhibitor, to suppress NF-kB-driven inflammatory response (Bitzer et al., 2000; Wang et al., 2005a; Chen et al., 2018).

In this review, the diverse roles of canonical TGF-β signaling, the distinct roles of downstream Smad proteins, and the potential therapeutic strategies for renal fibrosis and inflammation by targeting downstream TGF-β/Smad signaling are discussed.

DIVERSE ROLES OF TGF-β1 IN RENAL FIBROSIS AND INFLAMMATION

It is well accepted that TGF-β is a master regulator in renal inflammation and fibrosis (Meng et al., 2016). TGF-β exerts multifunctional effects on cell proliferation, apoptosis, migration,

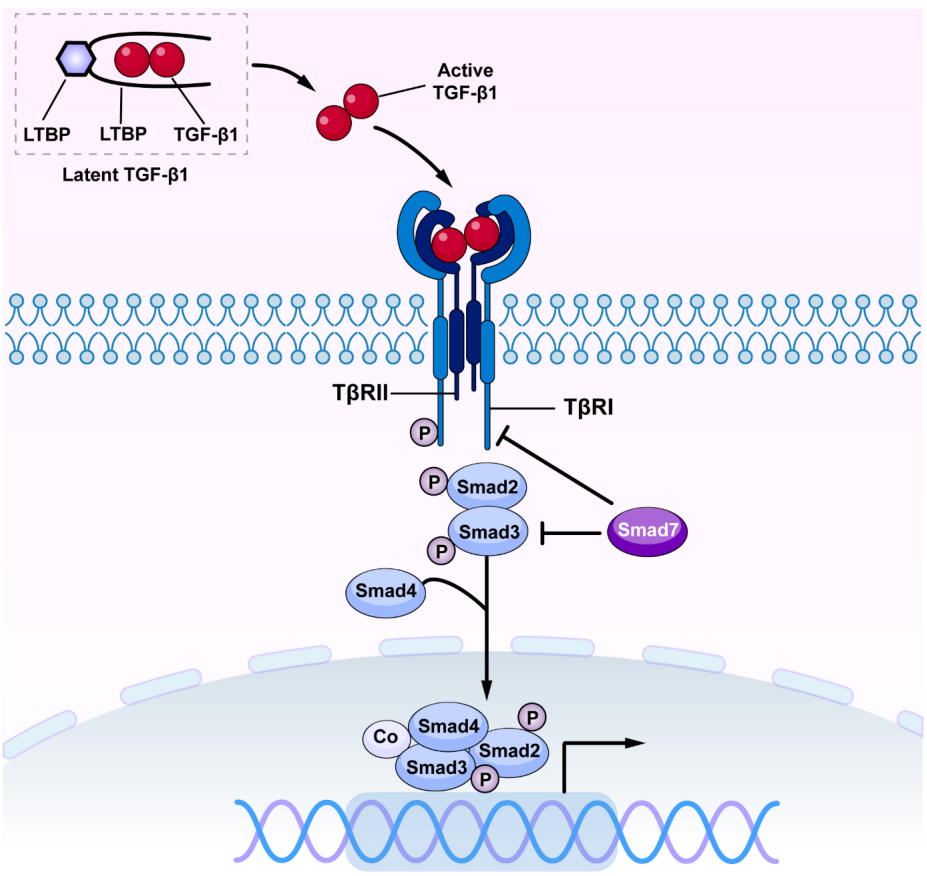

FIGURE 1 | The canonical TGF-β/Smad signaling in fibrosis. Once released, active TGF-β1 binds TβRII and activates TβRI and R-Smads (Smad2 and Smad3), resulting in formation of a complex with Smad4. The Smad2/3/4 complex then translates into the nucleus and binds to the target genes to induce fibrosis and inflammation. TGF-β, transforming growth factor β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II.

differentiation, and ECM production (Massagué, 2012). TGF-β1 induces tubular and glomerular epithelial cell-to-mesenchymal transition (EMT) and excessive ECM production and deposition in glomeruli and tubulointerstitium (Fan et al., 1999; Ng et al., 1999). TGF-β1 is highly expressed in a wide range of kidney diseases associated with fibrosis (Lopez-Hernandez and Lopez-Novoa, 2012; Wang et al., 2017; Isaka, 2018). The functions of TGF-β1 on renal fibrosis and EMT were further confirmed by the findings that overexpression of active TGF-β1 in liver causes the development of severe renal fibrosis in mice (Bottinger et al., 1996; Kopp et al., 1996). Whereas, anti-TGF-β treatments by using neutralizing antibodies (Border et al., 1990), inhibitors against the TβRII (Sutaria et al., 1998; Liu et al., 2018), or antisense oligonucleotides to TGF-β1 (Akagi et al., 1996; Miyajima et al., 2000; Ziyadeh et al., 2000; Chen et al., 2003) halt the progression of renal fibrosis, suggesting a vital pathological role of TGF-β in CKD.

Renal inflammation is driven by NF-κB-dependent mechanism (Sanz et al., 2010; Ernandez and Mayadas, 2016). TGF-β is considered to be one of anti-inflammatory cytokines during the renal repair process in response to the injuries (Meng et al., 2014; Nikolic-Paterson et al., 2014; Meng, 2019; Tang et al., 2019). A number of studies have reported that mice deficient TGF-β1 suffer from the lethal inflammation and the early death (Kulkarni et al., 1993; Yaswen et al., 1996), suggesting a protective role for TGF-β in renal inflammation. Consistently, conditional deletion of TβRII from mice results in protection against TGF-β/Smad3-mediated renal fibrosis while enhancing NF-κB-driven renal inflammation (Meng et al., 2012a). More importantly, TGF-β is also a master regulator of T cell immune responses in a variety of immune diseases (Li and Flavell, 2008), which makes TGF-β as a key regulator in renal inflammation.

It should be pointed out that TGF-β signaling is not the sole pathway mediating the fibrotic process (Luo, 2017). Increasing evidence shows that TGF-β signaling can interact with other signaling pathways to mediate fibrosis. Among TGF-β signaling, both canonical and non-canonical TGF-β/Smad signaling pathways play a role in the renal fibrosis (**Figure 2**). Importantly, under disease conditions, Smad signaling can also be activated independently TGF-β1 by many stress molecules such as angiotensin II, and advanced glycation end products (AGE) via the ERK/p38/MAPK-Smad crosstalk pathway (Wang et al., 2005b, 2006; Yang et al., 2009; Meng et al., 2016). TGF-β/Smad can also interact with other signaling pathways such as Wnt/β-catenin, Jagged1/Notch, and Hedgehog to regulate epithelial dedifferentiation, myofibroblast transformation and proliferation (Edeling et al., 2016). In addition, TGF-β can induce renal fibrosis by transactivating epidermal growth factor receptor (EGFR) and p53 via proto-oncogene tyrosine-protein kinase Src (c-Src) and ROS-dependent mechanisms

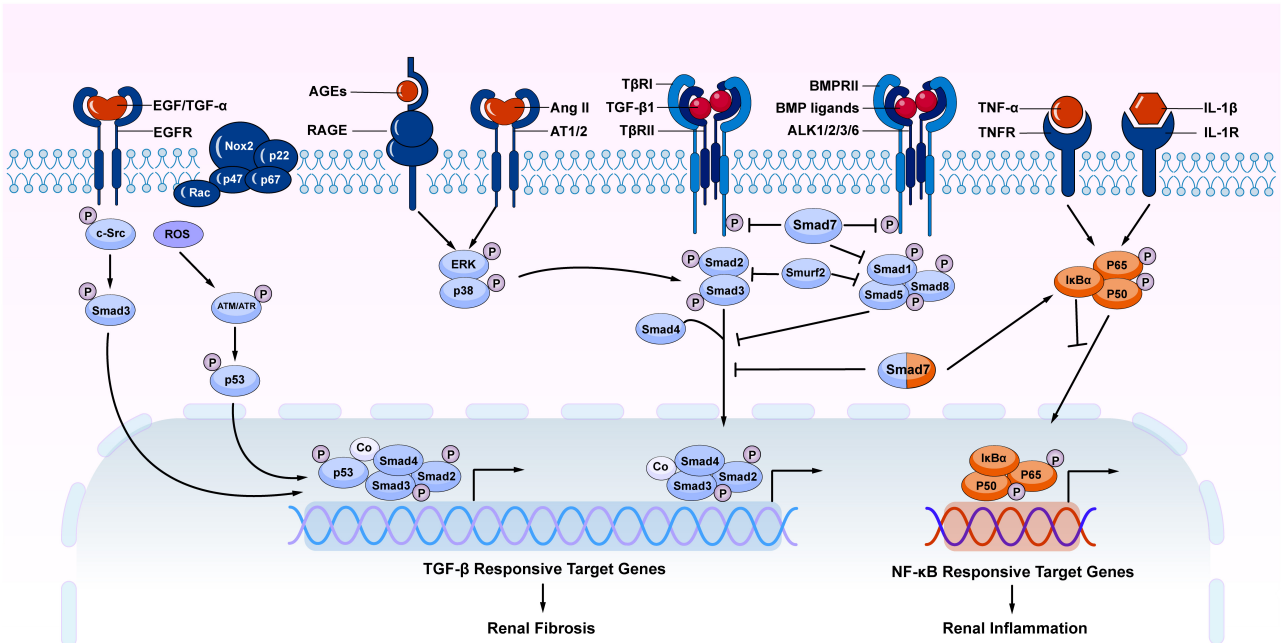

FIGURE 2 | The overview of crosstalk pathways associated with renal fibrosis and inflammation. Many stress molecules such as TGF-β1, EGF, TGF-α, ROS, AGEs, and Ang II can activate individual pathways and interact with TGF-β/Smad signaling pathway to regulate renal fibrosis and inflammation. Among TGF-β super family, the BMP signaling negatively regulates TGF-β/Smad signaling. In TGF-β/Smad signaling, Smad7 inhibits the phosphorylation of TβRI and R-Smads via ubiquitin degradation mechanism. Meanwhile, Smad7 also alleviates renal inflammatory by inducing IκBα, therefore inhibiting NF-κB-driven inflammation. AGEs, advanced glycation end products; RAGE, receptor for AGE; Ang II, angiotensin II; AT1/2, Ang II receptor 1 and 2; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; EGF, epidermal growth factor; EGFR, EGF receptor; c-Src, proto-oncogene tyrosine-protein kinase Src; ROS, reactive oxygen species; BMP, bone morphogenic protein; ALK, activin receptor-like kinases; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; IL-1, Interleukin 1; IL-1R, IL-1 receptor; Nox, NADPH oxidase.

(Samarakoon et al., 2013; Harskamp et al., 2016). TGF-β1 also induces phosphorylation and acetylation of p53 and promote formation of p53/Smad3 complexes during renal fibrosis (Higgins et al., 2018; Rane et al., 2019). By contrast, BMP signaling via Smad1/5/8 complex is able to counter regulate TGF-β/Smad-mediated renal fibrosis (Weiskirchen et al., 2009; Meng et al., 2013; Munoz-Felix et al., 2015). Thus, TGF-β may exert its diverse role in renal inflammation and fibrosis by interacting with many other signaling pathways and molecules.

DISTINCT ROLES OF Smad2 AND Smad3 IN RENAL FIBROSIS

In canonical TGF-β signaling, Smad2, and Smad3 are two key downstream mediators that are highly activated in the fibrotic kidney (Wang et al., 2006; Chung et al., 2010b; Zhou et al., 2010; Loeffler et al., 2018). Although Smad2 and Smad3 bind together, their functional roles are distinct. In the context of fibrosis, Smad3 is pathogenic while Smad2 is protective (Meng et al., 2010, 2016; Duan et al., 2014). Smad3 can induce matrix deposition by directly binding to the promoter region of collagen-producing genes and tissue inhibitor of matrix metalloproteinases (TIMP) while reducing the activity of MMP-1 to inhibit ECM degradation (Hall et al., 2003). By contrast, role of Smad2 in fibrosis is not fully elucidated due to a lack of Smad2 knockout (KO) mice which is embryonic lethal (Ju et al., 2006). However, a recent finding that conditional deletion of Smad2 from TECs accelerates renal fibrosis reveals a protective role of Smad2 in renal fibrosis (Meng et al., 2010). In addition, FSP1-specific Smad2 knockout in renal tubular, endothelial, and interstitial cells is also reported to reduce renal fibrosis and epithelial-to-mesenchymal transition in murine streptozotocin (STZ)-induced diabetic nephropathy (Loeffler et al., 2018).

TABLE 1 | MicroRNAs regulated by TGF-β/Smad signaling in renal fibrosis.

Micro RNA	Target genes/Mechanisms
Antifibrotic	
miR-15b	TβR1
miR-19b	TβR2
miR-26a	Smad4
miR-29	TGF-β1/2, Col, MMP, Fos, Adams, HDAC4
miR-30	TGF-β2, Snail
miR-101	TβR1
miR-130b	TβR1
miR-let-7	TβR1
Antifibrotic or profibrotic	
miR-145	TβR2, latent TGF-β1, KLF4
miR-192	P53, Zeb1/2E-cadherin
miR-200	TGF-β2, Zeb1/2E-cadherin
Profibrotic	
miR-17-5p	Smad7
miR-216a	PTEN
miR-217	PTEN
miR-377	SIRT1
miR-382	HSPD1, SOD2
miR-491-5p	Par-3
Profibrotic and pro-inflammatory	
miR-21	Smad7, PPARα, PTEN, ERK/MAPK, Spry1

DIVERSE ROLE OF Smad4 IN RENAL FIBROSIS AND INFLAMMATION

Smad4 is a common Smad associated with nuclear translocation of Smad2/3 and Smad1/5/8 complexes in response to TGF-β and BMP signaling (Gomez-Puerto et al., 2019). Limited evidence has shown a direct role of Smad4 in renal fibrosis due to the lethality of Smad4 knockout mice. However, conditional deletion of Smad4 from TECs significantly reduces renal fibrosis in the obstructive kidney (Meng et al., 2012b). Mechanistically, deletion of Smad4 inhibits renal fibrosis by suppressing Smad3 promoter activity and blocking the binding of Smad3 to the collagen promoter without affecting its phosphorylation and nuclear translocation (Meng et al., 2012b). This finding is consistent with studies in Smad4 knockout mesangial cells and in the folic acid-induced rodent model (Tsuchida et al., 2003; Morishita et al., 2014). It is also reported that the formation of Smad3/Smad4/CDK9 complex drives renal fibrosis during ureteral obstruction (Qu et al., 2015). In contrast, conditional deletion of Smad4 promotes renal inflammation by impairing Smad7-mediated inhibition of NF-κB activation (Meng et al., 2012b). Thus, Smad4 may play a diverse role in renal fibrosis and inflammation and may not be a specific therapeutic target for CKD.

Smad7 AS AN INHIBITORY PROTEIN OF RENAL FIBROSIS AND INFLAMMATION

Smad7 is a vital negative regulator of both TGF-β/Smad and NF-κB signaling pathways (Lan, 2008, 2011; Yan and Chen, 2011; Meng et al., 2016). Indeed, although TGF-β1 induces Smad7 transcriptionally, Smad7 inhibits TGF-β signaling by directly binding to the TβRI and blocking the activation of R-Smads (Hayashi et al., 1997). Mechanistically, Smad7 interacts with E3 ubiquitin ligases, such as arkadia, Smurf1 or Smurf2 (Smad ubiquitination regulatory factors), and recruit them to the TRβI to cause its degradation, hence resulting in the inhibition of TGF-β/Smad signaling (Ebisawa et al., 2001; Chong et al., 2006; Liu et al., 2008). Under fibrosis conditions, Smad7 is reduced while Smad3 is highly activated as seen in diabetic nephropathy, hypertensive nephropathy, and aristolochic acid-induced nephropathy (Chen et al., 2011; Liu et al., 2012; Chung et al., 2013a; Tian et al., 2015). Thus, the imbalance between Smad3 and Smad7 signaling may be a key mechanism in fibrogenesis and rebalancing this pathway by overexpressing Smad7 and inactivating Smad3 may represent as a better therapeutic strategy for CKD.

Smad7 can also induce expression of IκBα, an inhibitor of NF-κB, to negatively regulate NF-κB-driven renal inflammation (Wang et al., 2005a,b; Lan, 2008, 2011). Furthermore, Smad7

can interact with NF-κB directly as Smad7 promoter contains a putative NF-κB regulatory site (Nagarajan et al., 2000). Under CKD conditions, loss of renal Smad7 is associated with activation of NF-κB signaling and severe renal inflammation as reported in hypertensive nephropathy (Liu et al., 2013, 2014) and aristolochic acid-induced nephropathy (Dai et al., 2015). In contrast, overexpression of Smad7 suppresses both renal fibrosis and inflammatory in these disease models, making Smad7 as an promising therapeutic strategy for CKD (Lan, 2008).

DIVERSE ROLE OF TGF-β/Smad SIGNALING IN REGULATION OF NON-CODING RNAs EXPRESSION AND FUNCTIONS DURING RENAL FIBROSIS AND INFLAMMATION

MicroRNAs (miRNAs) are small (approximately 20–22 nucleotides in length) non-coding single stranded RNAs. More than 200 miRNAs have been identified in renal cells and tissues so far (Jelencsics and Oberbauer, 2015). These miRNAs regulate a wide range of biological processes, including fibrosis and inflammation. Increasing evidence has demonstrated that TGF-β1/Smad3 signaling regulates various miRNAs during the renal pathological processes (Meng et al., 2016; Tang et al., 2018). As a transcriptional factor, Smad3 can bind and upregulate or downregulate miRNAs to promote renal inflammation and fibrosis. It is now clear that Smad3, but not Smad2, regulates these miRNAs by physically interacting with Smad binding site (SBE) located in their promoters to either increase (such as miR-21 and miR-192) or inhibit their transcription (such as miR-29 and miR-200 families) (Chung and Lan, 2015). In addition, Smad7 may inactivate Smad3 to protect kidneys from fibrosis by upregulating renal miR-29b but suppressing miR-192 and miR-21 (Chung and Lan, 2015). Among these miRNAs, miR-21 is well characterized as a profibrotic miRNA. miR-21 is upregulated in renal fibrosis in the patients with CKD as well as AKI (Zarjou et al., 2011; Chau et al., 2012; Glowacki et al., 2013). Mice deficient miR-21 or administration of anti-miR-21 oligonucleotides are able to protect against renal fibrosis (Zhong et al., 2011, 2013). Expression of miR-21 is positively regulated by Smad3 but negatively by Smad7 (Chung et al., 2013a). Overexpression of miRNA-21 promotes renal fibrosis by targeting PTEN and Smad7 (Zhou et al., 2013; McClelland et al., 2015). Thus, knockdown of miR-21 restores renal Smad7 levels and blocks both TGF-β/Smad3 and NF-κB signaling, thereby inhibiting progressive renal fibrosis and inflammation in mouse models of obstructive and diabetic nephropathy (Zhong et al., 2013). However, miR-21 may be also protective in kidney disease as miR-21-deficient TGF-β(1)-transgenic mice show increased proteinuria and glomerular injury in streptozotocin-induced diabetic mice, suggesting a diverse role of miR-21 as a feedback inhibitor of TGF-β/Smad3 signaling (Lai et al., 2015).

MiR-29 family is another well-documented miRNA in fibrotic diseases (He et al., 2013). The miR-29 family consists of miR-29a, b, c. All family members are encoded by two distinct genomic loci in both human and rodent genomes. As all members have the same seed binding sequence, they all bind to the same set of target genes (Kriegel et al., 2012). Renal miR-29b is decreased in association with activation of TGF-β/Smad3 signaling and progressive renal fibrosis in kidney diseases (Qin et al., 2011; Wang et al., 2012; Chen et al., 2014; Meng et al., 2016). miR-29b is negatively regulated by Smad3, but not Smad2, in response to TGF-β1, AGE, and angiotensin II (Qin et al., 2011; Wang et al., 2012; Chen et al., 2014; Yu et al., 2014; Zhang et al., 2014). Overexpression of miR-29 inhibits renal fibrosis and inflammation by targeting TGF-β and Sp1/NF-κB signaling (Chen et al., 2014; Zhang et al., 2014). Interestingly, miR-29b can also target T-bet, a master transcriptional factor for Th-1 T cell immune response. Therefore, overexpression of miR-29b is also capable of inhibiting T cell-mediated type-2 diabetic nephropathy in db/db mice (Chen et al., 2014). Notably, miR-29 also acts as a urinary exosome biomarker of renal fibrosis (Lv et al., 2013). Intramuscular injection of exosome-encapsulated miR-29 has been shown to inhibit renal fibrosis and muscle atrophy (Wang et al., 2019).

Moreover, miR-93, miR-216a, miR-217, miR-377, miR-382, miR-491-5p, miR-433 and miR-17-5p are also demonstrated to be TGF-β1/Smad3-regulated profibrotic miRNAs (Chung and Lan, 2015), whereas miR-let-7, miR-15b, miR-101, and miR-130b exert their antifibrotic effects by inhibiting the expression and activity of TβRI, thus limiting transduction of downstream TGF-β-mediated signals (Wang et al., 2014; Tang et al., 2018). Other miRNAs such as miR-19b, miR-26a, miR-29, and miR-30 inhibit the TGF-β1/Smad signaling by targeting Smads or fibrotic transcriptional factors (Tang et al., 2018). All these findings imply that TGF-β may regulate miRNAs to exert its diverse roles in renal inflammation and fibrosis as shown in **Table 1** and **Figure 3**.

However, the off-target effects, non-specificity, and toxicity of miRNAs are unavoidable. Thus, research into long non-coding RNAs (lncRNAs) is more promising for a better understanding of the pathogenic mechanisms of kidney diseases (Moghaddas Sani et al., 2018). Compared to miRNAs, lncRNAs are transcripts with lengths exceeding 200 nucleotides without protein-coding functions and are highly tissue-and-cell-type-specific. lncRNA regulates both target DNAs/RNAs and proteins transcriptionally or post-transcriptionally (Dykes and Emanueli, 2017). By using the high-throughput RNA sequencing, 21 TGF-β/Smad3-dependent lncRNAs have been identified in an immunologically induced anti-glomerular basement membranous glomerulonephritis (anti-GBM GN) and obstructive nephropathy (Zhou et al., 2014). Of these, the Arid-IR is a novel and Smad3-related lncRNA as a Smad3 binding site is found in its promoter region. It has been proven that knockdown of Arid2-IR in TECs improves renal inflammation *in vivo* and *in vitro* by inhibiting NF-κB-dependent inflammatory transduction without affecting Smad3-mediated fibrosis (Zhou et al., 2015). In contrast, Erbb4-IR is another novel Smad3-dependent lncRNA capable of inhibiting renal fibrosis by targeting miR-29b and Smad7 in both obstructive nephropathy and type II diabetic nephropathy, respectively

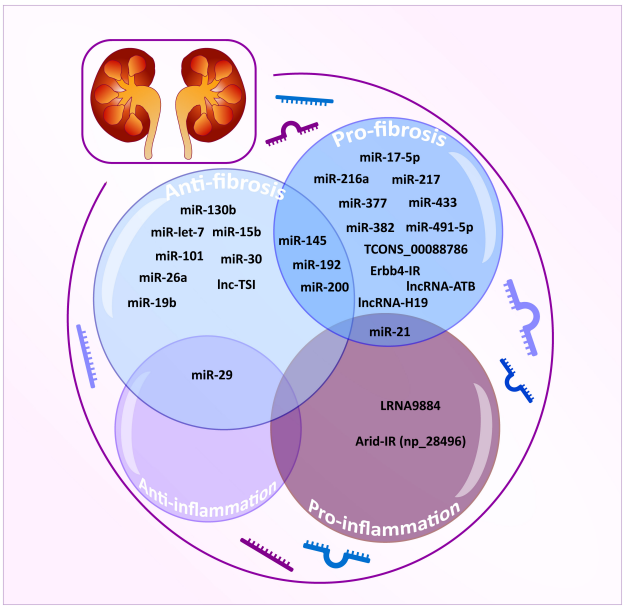

FIGURE 3 | TGF-β/Smad3-dependent miRNAs and lncRNAs related to renal fibrosis and inflammation. TGF-β/Smad3-dependent miRNAs and lncRNAs are classified as anti-fibrotic (powder blue), pro-fibrotic (sky blue), anti-inflammatory (lavender), and pro-inflammatory effect (plum). The integrated area indicates multiple functions for each miRNA/lncRNA.

(Feng et al., 2018; Sun et al., 2018). A recent study also reveals the pathogenic role and mechanism of LRNA9884 in type II diabetic nephropathy (Zhang et al., 2019). LRNA9884 is tightly regulated by Smad3 in response to TGF-β and AGEs and functions to trigger MCP-1 production by directly binding to the MCP-1 promoter, thereby promoting inflammation-driven type II diabetic nephropathy (Zhang et al., 2019). In addition, several TGF-β/Smad3-associated lncRNAs are found to be associated with renal fibrosis. TCONS_00088786 and TCONS_01496394 are TGF-β/Smad3-associated lncRNAs as they contain potential binding sites for Smad3 and silencing TCONS_00088786 inhibits renal interstitial fibrosis by targeting miR-132 (Sun et al., 2017; Zhou S.G. et al., 2018). lncRNA-ATB is highly upregulated in patients with acute renal allograft rejection and renal carcinoma and is able to promote EMT (Qi et al., 2017; Qiu et al., 2017; Zhou and Jiang, 2019). lncRNA uc.412 is able to induce mesangial cell proliferation *in vitro* although the underlying mechanisms are unclear (Yu et al., 2019). Lnc RNA-H19 is associated with TGF-β2-induced fibrosis *in vivo* and *in vitro* (Xie et al., 2016). lncRNA ENST00000453774.1 (LncRNA 74.1) is significantly down-regulated in TGF-β-treated TECs and in fibrotic kidney (Xiao et al., 2019). Interestingly, a recent study also revealed that decreased human lnc-TSI (TGF-β/Smad3-interacting long non-coding RNA) correlates with the degree of renal fibrosis in patients with IgA nephropathy and treatment with lnc-TSI inhibits renal fibrosis by blocking its binding to the MH2 domain of Smad3 (Wang et al., 2018).

TABLE 2 | Long non-coding RNAs regulated by TGF-β/Smad signaling in renal fibrosis.

Non-coding RNA	Target genes/Mechanisms
Antifibrotic	
Lnc-TSI	Smad3
Antifibrotic or profibrotic	
TCONS_01496394	Unclear
Profibrotic	
Erbb4-IR (np_5318)	miR-29b, Smad7
lncRNA-H19	miR-17
lncRNA-ATB	Livin
TCONS_00088786	miR-132
Pro-inflammatory	
LRNA9884	MCP-1
Arid2-IR (np_28496)	NF-κB

Taken together, TGF-β may diversely regulate renal fibrosis and inflammation via Smad3-dependent miRNAs/lncRNAs as shown in **Table 2** and **Figure 3**.

CLINICAL TRIALS OF ANTI-TGF-β THERAPY

Theoretically, TGF-β is a key mediator for renal fibrosis and thus targeting TGF-β signaling could be a good therapeutic strategy for CKD. There are many approaches to develop anti-TGF-β treatment for CKD clinically (**Table 3**). It has been shown that treatment with Pirfenidone, a non-specific antifibrotic effect of TGF-β, can improve eGFR in the trials of DN and focal segmental glomerulosclerosis (FSGS) (Lancaster et al., 2017). Disappointingly, a recent clinical trial study using a humanized monoclonal neutralizing antibody against TGF-β1 (LY2382770) for treatment of patients with diabetic nephropathy has been proven no efficacy on the improvements of serum creatinine, estimated GFR (eGFR), and proteinuria (Voelker et al., 2017). In addition, the use of another humanized monoclonal antibody, Fresolimumab that inhibits all three isoforms of TGF-β, also fails to achieve the endpoints of proteinuria reduction in patients with FSGS (Trachtman et al., 2011; Vincenti et al., 2017), demonstrating targeting on the upstream of TGF-β signaling may not be a good therapeutic strategy for CKD. It is possible that blockade of the general effect of TGF-β1, including latent form of TGF-β1, may attribute to the failure of these clinical trials. Our previous studies in latent TGF-β transgenic mice explain this notion since mice overexpressing latent TGF-β1 are protected against renal inflammatory and fibrosis in unilateral ureteral obstructive (UUO) nephropathy and anti-GBM glomerulonephritis model (Huang et al., 2008a,b). Thus, the latent form of TGF-β1 is renal protective while its active form is pathogenic. As most circulating TGF-β1 is latent form, thus, the use of anti-TGF-β1 antibodies may largely block the protective effect of latent TGF-β1, resulting in progressive renal injury as seen in these clinical trials. Results from these studies also suggest that treatment against renal fibrosis in patients with CKD should

TABLE 3 | Therapeutic drugs and clinical trials for treatment of CKD by targeting TGF-β.

Drug and trials	Mechanisms	Disease	Drug administration and period	Results	Side effects	References
LY2382770						
NCT01113801	TGF-β1	DN	Subcutaneous injection given monthly for 12 months	No efficacy on improvements in eGFR, Scr and proteinuria	Risk of toxicity and loss of renal efficacy	Voelker et al., 2017
Fresolimumab						
NCT01665391	TGF-β1,2,3	FSGS	Administered intravenously at 1 mg/kg or 4 mg/kg for 112 days, followed double-blind for 252 days	No efficacy in proteinuria reduction; non-significant trend on eGFR decline	Herpes zoster; skin lesions, bleeding events and cancers	Vincenti et al., 2017
NCT00464321	TGF-β1,2,3	FSGS	Administered intravenously at one of four single-dose (0.3,1,2 and 4 mg/kg), followed for 112 days	Less eGFR decline (non-significant)	Pustular rash	Trachtman et al., 2011
Pirfenidone						
NCT02689778	TGF-β1,2,3	DN	Administrated orally 600 mg with breakfast and 1200 mg with dinner for 12 months	Phase 3 ongoing	N/A	
NCT00063583	TGF-β1,2,3	DN	Administered orally at a dose of 1200 mg or 2400 mg per day for 12 months	eGFRs increased significantly in the 1200 mg/d pirfenidone group compared with placebo	Gastrointestinal disorders, fatigue and photosensitivity rash	Sharma et al., 2011
NCT02408744	TGF-β1,2,3	CKD	Prolonged-released tablets, orally administered 2 time per day for 36 months	Phase 2 ongoing	N/A	
NCT02530359	TGF-β1,2,3	Septic AKI	Pirfenidone extended release 600 mg per month every 12 h for 7 days	Phase 4 ongoing	N/A	
NCT00001959	TGF-β1,2,3	FSGS	Orally administrated 3 times daily for 12 months	Improved eGFR decline; no effect on BP or proteinuria	Dyspepsia, sedation, and photosensitive dermatitis	Cho et al., 2007

DN, diabetic nephropathy; FSGS, focal and segmental glomerulosclerosis; CKD, chronic kidney disease; AKI, acute kidney disease.

specifically target the downstream TGF-β signaling molecules, rather than to block the general effect of TGF-β1.

TREATMENT OF CKD BY TARGETING DOWNSTREAM TGF-β/Smad SIGNALING MOLECULES AND NON-CODING RNAs

Given the diversity and the complexity of TGF-β in renal fibrosis and inflammation, direct targeting TGF-β or receptors may not be an ideal tactic due to its involvement in various vital biological processes (Trachtman et al., 2011; Vincenti et al., 2017; Voelker et al., 2017). Although general blockade of the upstream TGF-β signaling may reduce fibrosis, it can also promote renal inflammation and cause unexpected renal injuries (**Figure 4a**). Because the imbalance of TGF-β/Smad3 signaling with overreactive Smad3 and reduced Smad7 is a key mechanism leading to renal fibrosis and inflammation, rebalancing Smad3/Smad7 signaling may serve as effective strategies to treat renal fibrosis and inflammation (**Figure 4b**). SIS3, a specific Smad3-inhibitor, has been shown to inhibit renal fibrosis in STZ-induced diabetic nephropathy (Li et al., 2010) and in obstructive nephropathy (Zhang et al., 2018). Overexpression of renal Smad7 is also capable of inhibiting Smad3-mediated renal fibrosis and NF-κB-driven renal inflammation in various kidney diseases, including diabetic and hypertensive nephropathy

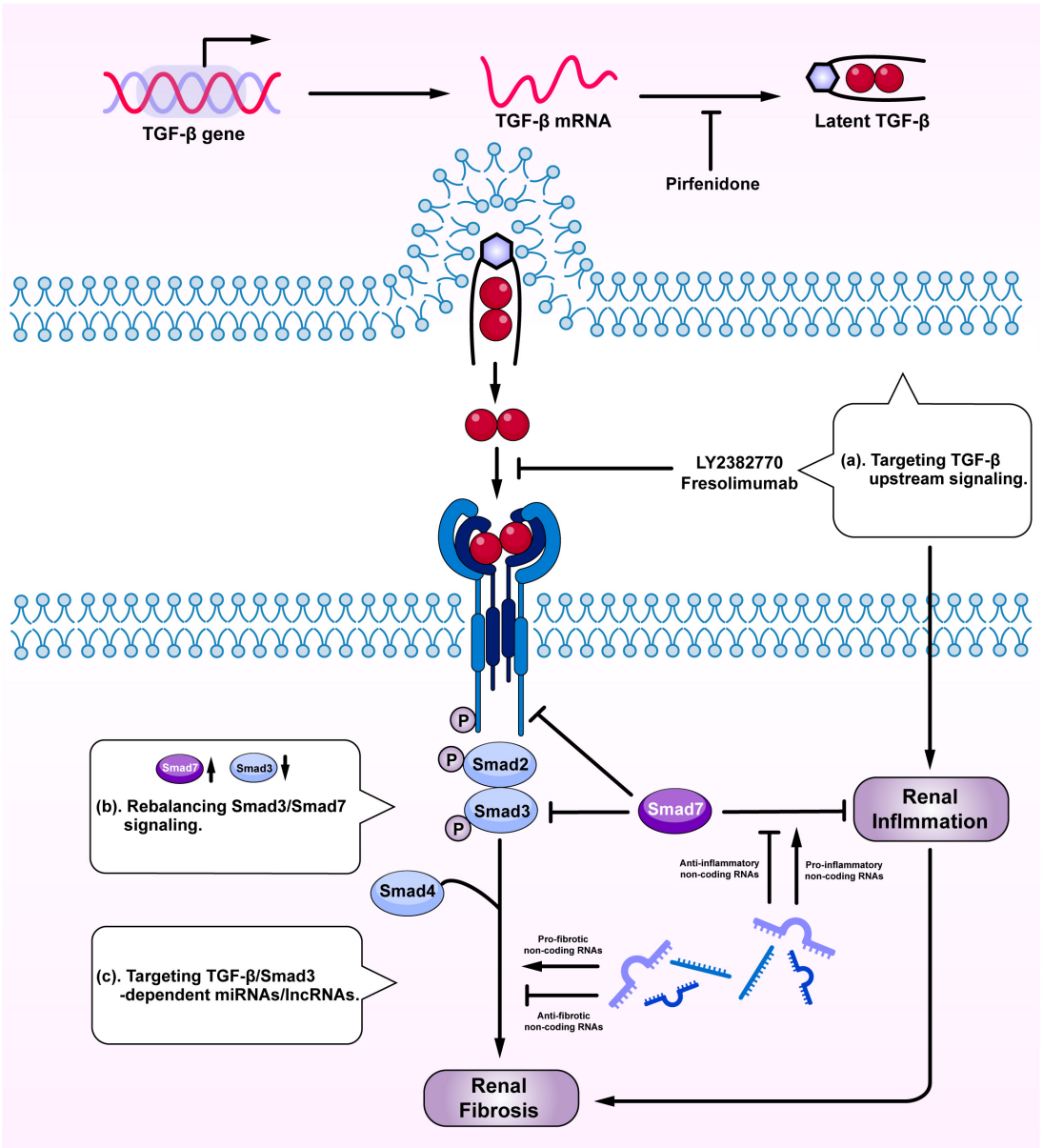

FIGURE 4 | Therapeutic potentials by targeting TGF-β signaling. Anti-TGF-β treatment by: (a) targeting upstream signaling; (b) rebalancing Smad3/Smad7 signaling; and (c) targeting Smad3-dependent miRNAs/lncRNAs.

(Chen et al., 2011; Lan, 2011; Ka et al., 2012; Liu et al., 2014), obstructive nephropathy (Li et al., 2002; Lan et al., 2003; Lan, 2008; Chung et al., 2013a), remnant kidney disease (Hou et al., 2005; Ng et al., 2005), crescentic glomerulonephritis (Ka et al., 2007), and chronic aristolochic acid nephropathy (Dai et al., 2015). Interestingly, treatment of CKD with two Traditional Chinese Medicine compounds, Naringenin from fruits as a Smad3 inhibitor and Asiatic acid derived from *Centella asiatica* as a Smad7 agonist, is capable of restoring the balance of Smad3/Smad7 signaling and thus additively inhibits renal fibrosis in rodent obstructive nephropathy (Meng et al., 2015). Similarly, the combination of Ginsenoside Rg1 from *Panax ginseng C. A. Mey* and Astragaloside IV from *Radix astragali* have also improved fibrosis and inflammation in STZ-induced diabetic nephropathy by inhibiting TGF-β/Smad2/3 while enhancing Smad7 signaling (Du et al., 2018). Asperulosidic acid, a bioactive iridoid glycoside, can also exert renal protective effects by inactivating both TGF-β/Smad and NF-κB signaling pathways (Xianyuan et al., 2019). Similar therapeutic effects are also found in other studies with herbal medicines (Nie et al., 2014; Wan et al., 2014; Zhao et al., 2016).

Targeting Smad3-dependent non-coding RNAs could be another therapeutic approach to treat renal fibrosis and inflammation (**Figure 4c**). Of Smad3-dependent miRNAs

(**Figure 3**), inhibition of miR-21, miR-192, miR-433, and overexpression of miR-29 and miR-200 have been shown to have therapeutic effects on obstructive nephropathy (Chung et al., 2010a, 2013b; Oba et al., 2010; Qin et al., 2011; Zhong et al., 2011; Li et al., 2013) and diabetic nephropathy (Zhong et al., 2013; Chen et al., 2014). However, the off-target effect of anti-miRNA therapies raises concern and new therapeutic approach by targeting Smad3-dependent lncRNAs is sought. Targeting Arid2-IR and LRNA9884 can specifically inhibit renal inflammation while targeting Erbb4-IR can specifically inhibit renal fibrosis in obstructive and diabetic nephropathy (Zhou et al., 2015; Feng et al., 2018; Sun et al., 2018; Zhang et al., 2019). Furthermore, delivery of a human lncRNA lnc-TSI into the UUO kidney also inhibits Smad3-mediated renal fibrosis (Wang et al., 2018). All these findings highlight the therapeutic potentials by targeting downstream TGF-β signaling molecules including Smad3, Smad7, and non-coding RNAs in renal fibrosis and inflammation.

CONCLUSION

Transforming growth factor-β plays diverse roles in renal fibrosis and inflammation. Blockade of upstream TGF-β signaling may not be a good therapeutic strategy, which has been proved by unsatisfied clinical trials. TGF-β may specifically regulate renal fibrosis and inflammation via downstream Smad-dependent mechanisms involving Smad3, Smad4, Smad7, and particularly Smad3-dependent non-coding RNAs. Targeting downstream TGF-β/Smad signaling by rebalancing Smad3/Smad7 or by specifically inhibiting or overexpressing Smad3-dependent non-coding RNAs related to fibrosis or inflammation may be a better therapeutic approach. Further studies to understand the diverse role of TGF-β signaling in kidney diseases may promote the translation from bench into clinical settings.

AUTHOR CONTRIBUTIONS

Y-YG, X-SL, and X-RH wrote and revised the manuscript. X-QY and H-YL revised and edited the manuscript. All authors contributed to the manuscript conception development, data collection and analysis, and discussion on the manuscript writing and revising.

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MINI REVIEW
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Transforming Growth Factor-Beta1 in Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease (ESRD) worldwide. Renin-angiotensin-aldosterone system (RAAS) inhibitors and sodium-glucose co-transporter 2 (SGLT2) inhibitors have shown efficacy in reducing the risk of ESRD. However, patients vary in their response to RAAS blockades, and the pharmacodynamic responses to SGLT2 inhibitors decline with increasing severity of renal impairment. Thus, effective therapy for DKD is yet unmet. Transforming growth factor-β1 (TGF-β1), expressed by nearly all kidney cell types and infiltrating leukocytes and macrophages, is a pleiotropic cytokine involved in angiogenesis, immunomodulation, and extracellular matrix (ECM) formation. An overactive TGF-β1 signaling pathway has been implicated as a critical profibrotic factor in the progression of chronic kidney disease in human DKD. In animal studies, TGF-β1 neutralizing antibodies and TGF-β1 signaling inhibitors were effective in ameliorating renal fibrosis in DKD. Conversely, a clinical study of TGF-β1 neutralizing antibodies failed to demonstrate renal efficacy in DKD. However, overexpression of latent TGF-β1 led to anti-inflammatory and anti-fibrosis effects in non-DKD. This evidence implied that complete blocking of TGF-β1 signaling abolished its multiple physiological functions, which are highly associated with undesirable adverse events. Ideal strategies for DKD therapy would be either specific and selective inhibition of the profibrotic-related TGF-β1 pathway or blocking conversion of latent TGF-β1 to active TGF-β1.

Keywords: diabetic kidney disease, transforming growth factor-β1, fibrosis, inflammation, Smad signaling

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INTRODUCTION

Diabetic kidney disease (DKD), the most common cause of end-stage renal disease (ESRD) worldwide, accounts for about 40% of new cases of ESRD each year in the United States and China (Zhang et al., 2016; Alicic et al., 2017). With the increasing incidence of diabetes, there is a heightened need for therapy to delay progression of DKD. Existing therapies have had limited success. Renin-angiotensin-aldosterone system (RAAS) inhibitors, such as losartan and irbesartan, have been effective in reducing the risk of ESRD for patients with DKD (Brenner et al., 2001; Lewis et al., 2001; Parving et al., 2001). However, patients exhibited great variation in their responses to RAAS blockades. In the past two decades, there has been a decline in the rate of acute myocardial infarction and death from hyperglycemic crisis, but no change has occurred in the rate of ESRD (Gregg et al., 2014). Although sodium-glucose co-transporter 2 (SGLT2) inhibitors have conferred cardiovascular and renal protection (Perkovic et al., 2019), effective therapy for DKD is still unavailable. An epidemiological study revealed that the 5-year mortality rate of DKD was

approximately 40%, as high as many cancers (Abdel Aziz et al., 2017). Transforming growth factor-β1 (TGF-β1) signaling contributes to DKD progression, and inhibiting TGF-β1 signaling has shown potential renoprotective properties in animal and human studies. In this mini-review, we discuss the pleiotropic and the potential therapeutic effects of TGF-β1 in DKD.

TGF-β1 AND TGF-β1 SIGNALING PATHWAY

TGF-βs exist as five isoforms, but only TGF-β1, TGF-β2, and TGF-β3 are present in mammals; the three isoforms elicit similar responses *in vitro*. TGF-β1, the most abundant isoform, is synthesized by all types of resident renal cells and infiltrating inflammatory cells (Aihara et al., 2010). TGF-β1 is secreted into the extracellular matrix (ECM) in an inactive complex (latent TGF-β1) with TGF-β-latency-associated peptide (LAP) and latent TGF-β binding proteins (LTBP) (Munger et al., 1997). The activation of latent TGF-β1 is mediated by proteolytic cleavage in the presence of the serine protease plasmin, reactive oxygen species (ROS), thrombospondin-1 (TSP-1), or integrins (Khalil, 1999; Kim et al., 2018). Integrins bind to the arginine-glycine-aspartic acid sequence in LAP. This binding appears to change the conformation of the latent TGF-β1 complex by tractional force (Munger et al., 1999). This conformational change presents the latent TGF-β1 complex to transmembrane metalloproteinases, such as membrane-type-1-matrix metalloproteinase (MT-1-MMP), which cleave the latent TGF-β1 complex and release active TGF-β1 (Mu et al., 2002; Sheppard, 2004; Araya et al., 2006; Wipff and Hinz, 2008). Active TGF-β1 interacts with its receptors to activate Smad-dependent and Smad-independent downstream signaling (Lan, 2011; Sutariya et al., 2016).

Smad-Dependent Signaling Pathway

Active TGF-β1 binds to a Type II membrane receptor, TGF-β Type II receptor (TβRII). This binding results in the phosphorylation and recruitment of the TGF-β Type I receptor (TβRI). The activated complex of TGF-β1-TβRI-TβRII phosphorylates Smad2 and -3. Then, the phosphorylated Smad2 and -3 bind to Smad4 to form the Smad complex (Lan, 2011). This Smad complex translocates into the nucleus and binds to Smad-binding elements (SBEs) or Smads-containing complexes (Nakao et al., 1997b; Meng et al., 2013), in turn, regulating transcription of genes encoding, e.g., collagen, fibronectin, α-smooth muscle actin (Chakravarthy et al., 2018), and Smad7 (Yan et al., 2009).

Smad proteins are classified into three subgroups. Smad2 and -3 comprise the receptor-regulated Smads (R-Smads) for TGF-β1 signaling, and Smad1, -5, and -8 for bone morphogenetic protein (BMP) signaling. Smad2 and -3 are key downstream mediators of TGF-β1, and they are highly activated in animal renal tissues in DKD (Isono et al., 2002; Høj Thomsen et al., 2017). Smad2 and -3 may have distinct functions in renal fibrosis. Either a Smad3 knockout or a Smad3-specific inhibitor delayed de-differentiation of proximal tubular cells and alleviated renal fibrosis in a streptozotocin-induced model of diabetes (Fujimoto et al., 2003; Li et al., 2010). These findings suggested that TGF-β1/Smad3 signaling has critical activities in renal fibrosis. Conversely, unlike Smad3, the function of Smad2 in DKD is unclear. Overexpression of Smad2 attenuated TGF-β1-induced phosphorylated Smad3 and collagen expression, whereas deletion of Smad2 promoted renal fibrosis via substantially enhanced Smad3 signaling (Meng et al., 2010; Loeffler et al., 2018). Although Smad2 interacts with Smad3 physically, Smad2 and -3 may compete for phosphorylation in response to TGF-β1 stimulation. Thus, Smad2 may competitively inhibit phosphorylation of Smad3 in response to TGF-β1 (Meng et al., 2010). Besides TGF-β1 signaling, Smad2 nuclear translocation and phosphorylation can also be mediated by advanced glycation end-products in DKD (Li et al., 2004). Thus, the activity of Smad2 is complicated in DKD.

The second Smad subgroup is the common-partner Smad (co-Smad), Smad4, which forms a heterotrimeric complex with phosphorylated R-Smads. The Smad4-containing complex translocates into the nucleus and regulates expression of the genes indicated earlier. Furthermore, Smad4 is implicated in suppressing nuclear factor-κB (NF-κB)-driven inflammation by inducing Smad7 expression (Ka et al., 2012).

The third Smad subgroup is the inhibitory Smads (I-Smads). Members of this Smad family have a conserved carboxy-terminal MH2 domain. I-Smads inhibit TGF-β1 family signaling via interaction with type I receptors, and I-Smads compete with R-Smads for receptor activation (Miyazawa and Miyazono, 2017). Smad7, one of the most investigated I-Smad in DKD, can cause degradation of TβRI and Smads activity in a negative feedback process. Smad7 inhibits Smad2/3 during renal fibrosis. In chronic kidney disease, TGF-β1 signaling upregulated the Smurfs and caused ubiquitin-dependent degradation of Smad7, which led to a decrease in Smad7 protein level (Kavsak et al., 2000; Ebisawa et al., 2001; Fukasawa et al., 2004; Liu et al., 2008). Smad7 knockout mice progressed to more severe interstitial fibrosis and enhanced inflammation (Cheng et al., 2013; Chung et al., 2013), and overexpression of Smad7 in kidney was effective in reducing collagen matrix expression and in alleviating inflammatory infiltration in DKD (Ka et al., 2012). These findings revealed anti-fibrotic and anti-inflammatory functions of Smad7 in DKD.

Smad-Independent Signaling Pathway

In addition to Smad-mediated transcription, TGF-β1 directly activates other signal transduction pathways in the pathophysiology of kidney disease. These other pathways include the mitogen-activated protein kinases (MAPK) pathway (Meng, 2019), growth and survival kinases phosphatidylinositol-3-kinase (PI3K)/Akt (Lu et al., 2019), small GTP-binding proteins such as Ras, RhoA, Rac1, and Cdc42, the Notch signaling pathway (Atfi et al., 1997; Sweetwyne et al., 2015), Integrin-linked kinase (ILK), and the Wnt/β-catenin pathway (Xu et al., 2017; Zhang and Huang, 2018). These non-canonical, non-Smad pathways can indirectly participate in de-differentiation of proximal tubular cells (Lu et al., 2019), apoptosis (Matoba et al., 2017), and matrix formation (Meng, 2019), thereby mediating signaling responses

either as stand-alone pathways or as pathways that converge onto Smads to control Smad activities.

TGF-β1 PROMOTES RENAL FIBROSIS IN DKD

Diabetic kidney disease pathology is characterized by thickening of the glomerular basement membrane, mesangial expansion, segmental glomerulosclerosis or global glomerulosclerosis, tubulointerstitial fibrosis, and afferent and efferent arteriole hyalinosis (Najafian et al., 2015). The TGF-β1 signaling pathway is activated in DKD, and the inhibition of TGF-β1 attenuates fibrosis in animal models of diabetes (Meng, 2019). Pathogenic stimuli in DKD activate TGF-β1 signaling. Angiotensin-II, which was elevated in mesangial cells and glomerular endothelial cells, has been implicated in activating TGF-β1 by generation of ROS from nicotinamide adenine dinucleotide phosphate oxidases (Lee, 2011; Morales et al., 2012) or by activating protein kinase C- and p38 MAPK-dependent pathways (Weigert et al., 2002). Hyperglycemia, mechanical stretch, and advanced glycation end products were found to upregulate TGF-β1 in DKD (Gruden et al., 2000; Chuang et al., 2015). TSP-1, a prototypic matricellular ECM protein, was heavily deposited in glomeruli of patients with DKD (Hohenstein et al., 2008). TSP-1 binds to the latent TGF-β1 complex, and, by a non-proteolytic mechanism, converts latent TGF-β1 to the active form, which leads to upregulation of TGF-β1 signaling (Murphy-Ullrich and Suto, 2018). Direct evidence for the importance of TSP-1 in regulating TGF-β signaling in DKD comes from two different models of type 1 diabetes. Streptozotocin-treated TSP-1 knockout mice showed decreased glomerular TGF-β signaling as measured by phosphorylated Smad2, and attenuated glomerulosclerosis (Daniel et al., 2007). In another type 1 diabetic animal model, uninephrectomized Akita mice treated with TSP-1 blocking peptide LSKL were protected from tubulointerstitial fibrosis and had reduced phosphorylation of Smad2 and -3 (Lu et al., 2011).

Mechanisms of TGF-β1 regulated fibrosis in DKD are multifactorial and involve (1) overexpression of ECM, (2) decreased degradation of ECM, (3) enhanced cross-linking between collagen and elastin fibers, and (4) overactivation of proximal tubular and endothelial cell de-differentiation. Both canonical TGF-β1/Smads-dependent signaling pathways and alternative signaling by TGF-β1 are involved in stimulating collagen expression and accumulation. Neutralizing all three mammalian TGF-β isoforms (-β1, -β2, and -β3) with antibodies reduced ECM gene (fibronectin and type IV collagen) expression and attenuated renal fibrosis in mice with type 1 or type 2 diabetes (Sharma et al., 1996; Ziyadeh et al., 2000). Thus, TGF-β1 has a critical signaling function in ECM accumulation in DKD.

TGF-β1 expression greatly inhibited ECM degradation by promoting the synthesis of plasminogen activator inhibitor-1 (PAI-1) which resulted in renal fibrosis (Shihab et al., 1997). The abundance of matrix metalloproteinase-9 (MMP-9), an ECM-degradation MMP, was decreased in transgenic mice that overexpressed TGF-β1 (Zechel et al., 2002; Ueberham et al., 2003). In addition, TGF-β1 augmented the expression of tissue inhibitor of metalloproteinases-1 (Ueberham et al., 2003; Abdel Aziz et al., 2017), which inhibited the ECM-degrading MMPs.

TGF-β1 promotes formation of the cross-linking between collagen and elastin fibers by upregulating lysyl oxidase (Boak et al., 1994; Di Donato et al., 1997). *In vitro*, TGF-β1 significantly increased (∼5 times) lysyl oxidase expression in tubular epithelial cells (Di Donato et al., 1997). In addition, TGF-β1 stimulated expression of procollagen lysyl hydroxylase 2, an enzyme that hydroxylates lysyl residues of collagen telopeptides and stabilizes collagen cross-linking (Gjaltema et al., 2015). Crosslinking increases ECM resistance to degradation by MMPs (El Hajj et al., 2018).

De-differentiation of the proximal tubular cells and endothelial cells contributes to renal fibrosis in diabetic mice. Extensive studies confirmed that TGF-β1 contributes to renal fibrosis by stimulating proximal tubular de-differentiation (Zeisberg et al., 2003) and endothelial de-differentiation (Li et al., 2009; Pardali et al., 2017). Hypoxia-inducible factor 1α (HIF-1α) accumulated in DKD and HIF-1α enhanced de-differentiation of murine proximal tubular epithelial cells *in vitro* (Higgins et al., 2007). Conditional HIF-1α ablation decreased interstitial collagen deposition and inhibited the development of tubulointerstitial fibrosis (Higgins et al., 2007). Although TGF-β1 stimulation increased HIF-1α expression, blocking TGF-β1 signaling inhibited HIF-1α activity, and, conversely, blocking HIF-1α activity decreased TGF-β1 signaling (Basu et al., 2011). These studies suggested cross-talk between TGF-β1 and HIF-1α signaling in regulating proximal tubular de-differentiation (Basu et al., 2011). As to endothelial de-differentiation, in animal models of folic acid nephropathy or unilateral ureteral obstruction, curtailed TGF-β signaling in the endothelium by endothelium-specific heterozygous TβRII knockout reduced endothelial de-differentiation and led to less tubulointerstitial fibrosis (Xavier et al., 2015). The mechanism by which TGF-β1 regulates endothelial de-differentiation is unknown. TGF-β1 stimulated endothelial de-differentiation in mouse endothelial cells by activating Snail expression (Kokudo et al., 2008).

In summary, the active TGF-β1 system promotes renal fibrosis, and it is involved in elevating collagen synthesis, suppressing ECM degradation, promoting collagen cross-linking, and fostering proximal tubular or endothelial cell de-differentiation (**Figure 1**).

DIVERSE INFLAMMATORY FUNCTIONS OF TGF-β1 IN DKD

TGF-β1 is a critical factor in the pathophysiological progression of DKD, having both pro- and anti-inflammatory properties (Sureshbabu et al., 2016).

TGF-β1 control of innate immune cells can have severe pathological consequences. Leukocytes and fibroblasts are recruited by the activation of resident kidney immune cells in DKD. This recruitment stimulates the expression of pro-inflammatory and chemotactic cytokines, which further drives the infiltration of monocytes and macrophages (Lv et al., 2018). TGF-β1 recruited macrophages and dendritic cells by stimulating

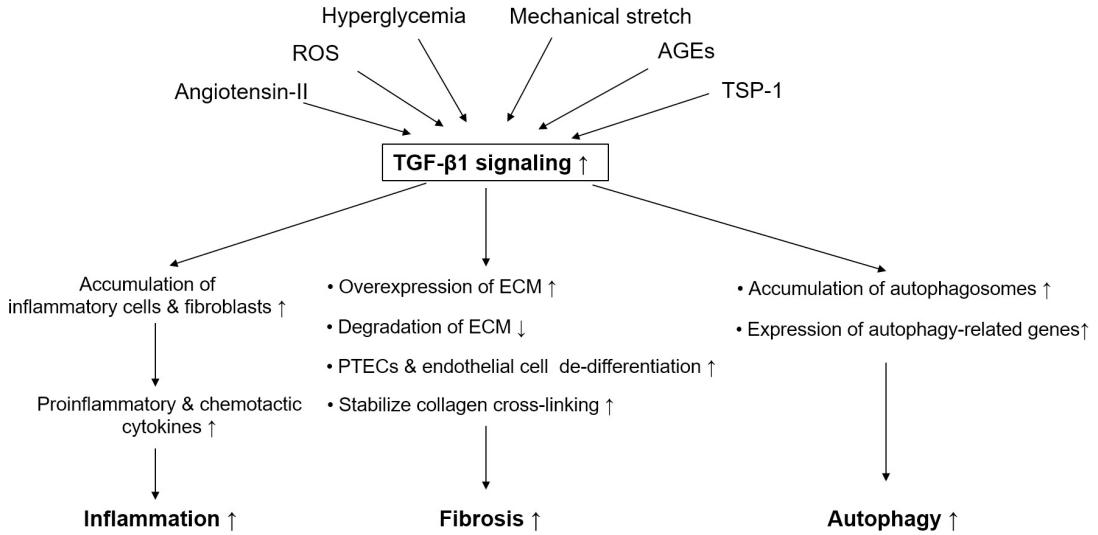

FIGURE 1 | Simplified schematic diagram of pathological role of TGF-β1 signaling in diabetic kidney disease. Pathogenic stimuli in diabetic kidney disease like hyperglycemia, angiotensin-II, reactive oxygen species, mechanical stretch, advanced glycation end products, and thrombospondin-1 are able to active TGF-β1 signaling. TGF-β1 signaling plays an important role in mediating renal fibrosis, inflammation, and autophagy in proximal tubular epithelial cells in diabetic kidney disease. TGF-β, transforming growth factor-beta; ROS, reactive oxygen species; PTECs, proximal tubular epithelial cells; AGE, advanced glycation end products; TSP-1, thrombospondin-1; ECM, extracellular matrix.

the production of chemokines, including tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and inducible nitric oxide synthase. Furthermore, the secreted chemokines induced TGF-β1 expression in a positive feedback loop (Cheng et al., 2005), which sustained the high levels of TGF-β1 in the microenvironment. TGF-β1 induced the expression and release of other proinflammatory cytokines such as interleukin-8 (IL-8) and MCP-1 (Qi et al., 2006) in proximal tubular cells. In addition, TGF-β1 drove the differentiation of T helper 17 cells, which were activated in various proinflammatory conditions. In the presence of IL-6, TGF-β1 promoted the differentiation of naive T lymphocytes into proinflammatory T helper cells that produced IL-17 and augmented autoimmune conditions, which were enhanced by IL-1β and TNF-α (Korn et al., 2009; Sanjabi et al., 2009). In this way, TGF-β1 propagates and amplifies the proinflammatory and profibrotic processes that contribute to renal insufficiency in DKD (**Figure 1**).

Nevertheless, TGF-β1 also possesses anti-inflammatory properties, which was suggested by the findings that targeted deletion of the TGF-β1 gene resulted in profound multifocal inflammatory disease in mice (Shull et al., 1992). Additionally, TGF-β1 knockout mice developed severe inflammatory responses that were evidenced by massive lymphocytes, macrophages, immunoblasts, and plasma cell infiltration in many organs (Kulkarni et al., 1993). Tubular epithelial cell-specific TβRII knockout mice showed massive leukocytes or macrophages infiltration, increased proinflammatory cytokine release, and enhanced renal inflammation (Meng et al., 2012). Direct evidence for the importance of TGF-β1 in anti-inflammation comes from two studies. First, Ma et al. (2004) used animal studies to investigate the effect of different doses of TGF-β antibodies on glomerulosclerosis. Only low dose TGF-β antibody decreased macrophage infiltration, and reduced sclerosis, indicating that the amount of TGF-β may influence the inflammatory process. Second, regulatory T cells appeared to ameliorate DKD, and nude mice, which lacked all T-cell subtypes, had more severe DKD (Lim et al., 2010; Eller et al., 2011). In the presence of IL-2, TGF-β1 converted naive T cells into Foxp3 + regulatory T cells and inhibited the progression of DKD (Davidson et al., 2007; Kanamori et al., 2016).

Thus, the effects of TGF-β1 activation in renal inflammation may be protective or harmful depending on concentration or the presence of IL-6. However, the underlying mechanism by which TGF-β1 exerts its anti-inflammatory properties in DKD requires further investigation.

OTHER ACTIVITIES OF TGF-β1 IN DKD

Recent studies illustrated that TGF-β1 promoted autophagy (Ding et al., 2010; Koesters et al., 2010). Autophagy, a system for removing protein aggregates and damaged organelles to maintain cellular homeostasis, is impaired in glomeruli and tubules in DKD (Yang et al., 2018). However, persistent activation of autophagy in kidney tubular epithelial cells induced tubular degeneration and promoted renal fibrosis (Livingston et al., 2016). Overexpression of TGF-β1 in renal tubules induced the accumulation of autophagosomes and stimulated expression of autophagy-related genes (Koesters et al., 2010; Xu et al., 2012). In proximal tubular cells, TGF-β1 promoted autophagy by generation of ROS, which contributed to the proapoptotic effect of TGF-β1 (Xu et al., 2012). Koesters et al. (2010) proposed TGF-β1-driven autophagy as a novel mechanism of tubular degeneration that led to renal interstitial fibrosis. On the

contrary, TGF-β1 induced autophagy had positive effects. In a study by Ding et al. (2010), TGF-β1 induced autophagy in mesangial cells, and autophagy enhanced cell survival by preventing mesangial cells from undergoing apoptosis. Whether TGF-β1 driven autophagy has protective or deleterious effects on kidney depending upon the amount. In the study by Koesters et al., TGF-β1 level was higher than its level in pathological disease states, which triggered violent autophagy and promoted kidney injury. Thus, we need further clarification of the functions of TGF-β1 signaling-induced autophagy in the pathogenesis of DKD.

TGF-β1 also suppresses reabsorption of glucose by proximal epithelial cells. A dose-dependent increase in TGF-β1 expression by genetic manipulation increased urinary output of glucose in Akita mice, whereas genetic insufficiency of TGF-β1 decreased glucose output (Hathaway et al., 2015). Moreover, SGLT2 was directly regulated by TGF-β1 via Smad3 (Panchapakesan et al., 2013) and TGF-β1 showed decreased expression of SGLT1 and SGLT2 (Lee and Han, 2010). Thus, these results support the notion that TGF-β1 suppresses urinary glucose reabsorption in proximal tubular epithelial cells (**Figure 1**).

TABLE 1 | Pre-clinical and clinical studies aimed to TGF-β signaling in diabetic kidney disease.

Authors	Target	Method	Subject	Major findings
Preclinical studies				
Sharma et al., 1996	TGF-β1, TGF-β2, TGF-β3	Neutralizing monoclonal antibody	Streptozotocin-induced diabetic mice	Attenuated renal fibrosis
Ziyadeh et al., 2000	TGF-β1, TGF-β2, TGF-β3	Neutralizing monoclonal antibody	*db/db* mice	Decreased glomerular mesangial matrix expansion and attenuated renal fibrosis
Chen et al., 2003	TGF-β1, TGF-β2, TGF-β3	Neutralizing monoclonal antibody	*db/db* mice	Reversed the glomerular basement membrane thickening and mesangial matrix expansion, attenuated renal fibrosis
Benigni et al., 2006	TGF-β1, TGF-β2, TGF-β3	Neutralizing monoclonal antibody	Streptozotocin-induced diabetic mice	Alleviated sclerotic glomerulosclerosis and attenuated renal fibrosis
Petersen et al., 2008	TGF-β type I and type II receptor kinase activity	GW788388, pharmacological inhibitor	*db/db* mice	Decreased epithelial-mesenchymal transitions and attenuated renal fibrosis
RamachandraRao et al., 2009	TGF-β1 promoter activity; other pathways besides TGF-β (suppressing production of reactive oxygen species and downregulating profibrotic cytokine genes)	Pirfenidone, a pharmacological inhibitor	*db/db* mice	Ameliorated mesangial matrix expansion and attenuated renal fibrosis
Hathaway et al., 2015	TGF-β1	Genetic overexpression	Akita mice	Progressively exacerbated thicker glomerular basement membranes and severe podocyte effacement is dose-dependent
Fujimoto et al., 2003	Smad3	Genetic knockout	Streptozotocin-induced diabetic mice	Alleviated glomerular basement membrane thickness and attenuated renal fibrosis
Li et al., 2010	Smad3	SIS3, pharmacological inhibitor	Streptozotocin-induced diabetic mice	Attenuated renal fibrosis
Ka et al., 2012	Smad7	Ultrasound-mediated gene transfer of inducible Smad7 overexpression plasmids	*db/db* mice	Inhibited diabetic kidney injury including fibrosis and inflammation
Loeffler et al., 2018	Smad2	Renal tubular, endothelial, and interstitial cells-specific knockout	Streptozotocin-induced diabetic mice	Reduced epithelial-to-mesenchymal transition and attenuated renal fibrosis
Clinical studies				
Sharma et al., 2011	TGF-β1 promoter activity; other pathways besides TGF-β (suppressing production of reactive oxygen species and downregulating profibrotic cytokine genes)	Pirfenidone, a pharmacological inhibitor	Type 1 and type 2 diabetic patients	Increased estimated glomerular filtration rate level
Voelker et al., 2017	TGF-β1	Neutralizing monoclonal antibody added to renin-angiotensin-aldosterone system inhibitor	Type 1 and type 2 diabetic patients	Failed to slow the progression of diabetic kidney disease

TGF-β1, transforming growth factor-β1.

TGF-β1 SIGNALING AS A THERAPEUTIC STRATEGY FOR DKD

Blockade of TGF-β1 signaling as a therapeutic strategy has been achieved by gene technology and pharmacological drugs (**Table 1**). Inhibition of TGF-β with a pan-neutralizing monoclonal antibody (1D11) against all three isoforms ameliorated renal fibrosis and alleviated kidney structural changes in the rodent models of type 1 and type 2 diabetes mellitus (Sharma et al., 1996; Ziyadeh et al., 2000; Chen et al., 2003; Benigni et al., 2006). Pirfenidone is a low molecular weight synthetic molecule that has antifibrotic properties in animal models; it suppresses production of ROS and downregulates genes encoding profibrotic cytokines, such as α-SMA, collagen I, and collagen IV. Pirfenidone upregulates regulator of G-protein signaling 2 (Xie et al., 2016; Li et al., 2018; Pourgholamhossein et al., 2018). Moreover, RamachandraRao et al. (2009) found that pirfenidone decreased TGF-β promoter activity, blocked TGF-β1 production, and was effective in reducing mesangial matrix expansion and fibrosis in DKD. Switching TGF-β1 expression from low to high by genetic manipulation exacerbated renal injury in Akita mice, a result that further supported the idea that blockade of TGF-β1 was renoprotective for DKD (Hathaway et al., 2015).

The success of TGF-β1 signaling inhibition in animal studies has promoted the strategy in clinical investigations with DKD (Sharma et al., 2011; Voelker et al., 2017). Pirfenidone significantly increased estimated glomerular filtration rates (eGFR) in a cohort of 77 diabetic patients with baseline eGFR of 20–75 ml/min/1.73 m^2 (Sharma et al., 2011). However, a placebo-controlled, phase II study that used a humanized TGF-β1-specific neutralizing monoclonal antibody plus renin-angiotensin system blockades failed to slow the progression of DKD in diabetic patients who had eGFR of 20–60 ml/min/1.73 m^2 (Voelker et al., 2017). Lack of improvement in clinical trials may be explained by the fact that rodent models of diabetes do not recapitulate tubulointerstitial fibrosis to the same degree observed in human disease. Also, inhibiting TGF-β1 fully and indiscriminately may not be wise because of its multiple physiological functions.

Nevertheless, targeting the conversion of latent to active TGF-β1 holds promise as a DKD therapeutic intervention. Animal studies revealed that overexpression of an active form of TGF-β1 in liver led to progressive kidney fibrosis in mice (Kopp et al., 1996), whereas overexpression of latent TGF-β1 in the skin displayed anti-inflammatory and anti-fibrosis effects in obstructive and crescentic glomerulonephritis (Huang et al., 2008a,b). The distinct functions of active and latent TGF-β1 in renal fibrosis and inflammation suggest that a better therapeutic approach would be to block conversion of latent TGF-β to active TGF-β. Wong et al. (2011) showed that inhibiting conversion of latent to active TGF-β1 in human proximal tubular cells reduced matrix protein expression and inhibited fibrosis under hyperglycemia and hypoxia conditions. What is more, the αv-containing integrins with different β-subunits that interact with latent TGF-β1 and activate TGF-β1 have a critical function in kidney fibrosis. A pharmacologic inhibitor of αvβ1 integrin prevented activation of the latent TGF-β complex and ameliorated renal fibrosis in mice fed an adenine diet (Chang et al., 2017). The mechanisms of the distinct functions of latent versus active TGF-β1 may be related to the prevention of Smad7 from Smurf-mediated ubiquitination and degradation in response to higher levels of latent TGF-β1 (Lan, 2011). Smad7 inhibits TGF-β signaling by promoting degradation of the TβRI and inhibiting Smad2/3/4 activity (Nakao et al., 1997a; Miyazawa and Miyazono, 2017). But in chronic kidney disease, active TGF-β1 activates the Smurfs and arkadia-dependent ubiquitin-proteasome pathways, which degrades Smad7 protein by a post-transcriptional modification mechanism (Kavsak et al., 2000; Ebisawa et al., 2001; Fukasawa et al., 2004).

CONCLUSION

On the basis of experimental and clinical studies, modulating TGF-β1, instead of directly inhibiting TGF-β1 ligands/receptors, may be a good antifibrosis tactic for DKD. TGF-β1 promotes wound healing (Wang et al., 2014), tissue regeneration (Borges et al., 2013), anti-inflammation (Kulkarni et al., 1993), autophagy (Koesters et al., 2010), and urinary glucose regulation (Hathaway et al., 2015). Nonetheless, the dose regimen must be considered carefully because a large dose of TGF-β blockade had severe toxicity and poor efficacy in animal experiments (Khanna et al., 2004; Ma et al., 2004). A pan-neutralizing monoclonal antibody could also lead to undesired effects such as tumor formation, even though animal studies have not exhibited such events during prolonged TGF-β1 inhibition. What is more, developing molecules that suppress the activation of latent TGF-β1 would be a potential therapy. Given the central role of TGF-β1 in the pathophysiology of DKD, the TGF-β1 system is an attractive target to retard the progression of DKD, provided that the approach maintains an acceptable balance between renoprotective and harmful effects.

AUTHOR CONTRIBUTIONS

LZ and FL conceptualized this review and decided on the content. LZ, YZ, and FL wrote and revised the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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TGF-β-Induced Endothelial to Mesenchymal Transition in Disease and Tissue Engineering

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Endothelial to mesenchymal transition (EndMT) is a complex biological process that gives rise to cells with multipotent potential. EndMT is essential for the formation of the cardiovascular system during embryonic development. Emerging results link EndMT to the postnatal onset and progression of fibrotic diseases and cancer. Moreover, recent reports have emphasized the potential for EndMT in tissue engineering and regenerative applications by regulating the differentiation status of cells. Transforming growth factor β (TGF-β) engages in many important physiological processes and is a potent inducer of EndMT. In this review, we first summarize the mechanisms of the TGF-β signaling pathway as it relates to EndMT. Thereafter, we discuss the pivotal role of TGF-β-induced EndMT in the development of cardiovascular diseases, fibrosis, and cancer, as well as the potential application of TGF-β-induced EndMT in tissue engineering.

Keywords: cancer-associated fibroblast, cardiovascular disease, EndMT, fibrosis, signal transduction, Smad, TGF-β, tissue regeneration

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INTRODUCTION

The cardiovascular system has the supportive role of supplying oxygen and nutrition to the whole body and simultaneously removes toxic waste products from tissues and organs through an extensive and intricated network of blood vessels. The inner surface of blood vessels consists of a monolayer of endothelial cells (ECs). These ECs, which may be supported by mural cells [i.e., pericytes and vascular smooth muscle cells (SMCs)], regulate the interchange between the luminal blood and the outer tissues (Pober and Sessa, 2007). During the development of the embryonic heart, a specific group of ECs lining the atrioventricular (AV) canal dedifferentiate into mesenchymal cells and migrate into the underlying extracellular matrix (ECM) to form the AV cushion (Markwald et al., 1975). This process of phenotypic switching of cardiac ECs was defined as endothelial to mesenchymal transition (EndMT) and thought to be regulated in part by the paracrine action of ligands secreted by the myocardium. Much of the mechanistic knowledge regarding EndMT has originated through studies focused on epithelial to mesenchymal transition (EMT). EMT is an evolutionarily conserved developmental process, induced by cytokines, mechanical forces, and metabolic factors (Saito, 2013; Wesseling et al., 2018), that has been shown to play a role in tumorigenesis and other pathophysiological processes (Heerboth et al., 2015; Pastushenko and Blanpain, 2018).

Notably, transforming growth factor β (TGF-β), a multifunctional cytokine secreted by the myocardium (among other tissues) with pleiotropic physiological roles, is one of the best studied EndMT (and EMT) inducers (Yoshimatsu and Watabe, 2011; Moustakas and Heldin, 2016).

When ECs undergo EndMT, their tight cell–cell junctions are disrupted, causing ECs to lose their cobblestone-like and well-structured appearance, reorganize their cytoskeleton and turn into spindle-shaped, fibroblast-like cells. During this transitional process, the expression of cell–cell adhesion proteins, such as vascular endothelial (VE)-cadherin, platelet/EC adhesion molecule-1 (CD31/PECAM-1), tyrosine kinase with immunoglobulin-like and epidermal growth factor (EGF)-like domains 1 (TIE1), TIE2, and von Willebrand factor (vWF), are diminished, while mesenchyme-specific factors, including N-cadherin, α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), vimentin, fibronectin, and fibroblast-specific protein-1 (FSP-1), are upregulated. These endothelial-derived mesenchymal cells gain stem cell properties as they can differentiate into different mesodermal cell types under certain conditions. Like EMT, EndMT is a gradual, reversible, and dynamic process. It is therefore difficult to capture in fixed biopsies; the presence of cells that express different levels of both endothelial and mesenchymal markers is suggestive that EndMT does occur. Partial EndMT is considered part of physiological angiogenesis (Welch-Reardon et al., 2015). ECs that have undergone partial EndMT were identified in the mouse heart (CD31/PECAM-1 and FSP-1) during the progression of cardiac fibrosis (Zeisberg et al., 2007b) as well as in the mouse brain (CD31/PECAM-1 and N-cadherin) in cerebral cavernous malformation (CCM) (Maddaluno et al., 2013).

In recent decades, the contribution of EndMT to human disease has been demonstrated in an increasing number of pathologies, including cardiovascular and fibrotic diseases and cancer (Medici et al., 2010; Souilhol et al., 2018; Platel et al., 2019). Increased TGF-β signaling has been suggested as a common underlying mechanism in almost every EndMT-associated disorder. Therefore, blocking TGF-β signaling might be a promising therapy for EndMT-related diseases. In contrast, because EndMT-derived mesenchymal multipotent cells can be used to generate various cell types within the mesodermal lineage, researchers have just begun to explore the potential of EndMT in tissue engineering, by recapitulating the EndMT process that occurs during embryogenesis and in organ development (Susienka and Medici, 2013). In this review, we summarize the mechanisms of TGF-β signaling and its role in driving EndMT. Furthermore, we discuss the role of EndMT in cardiovascular diseases, fibrosis, and cancer, as well as the potential applications of EndMT in tissue engineering.

TGF-β SIGNALING

Ligands

TGF-β signal transduction is involved in regulating a large number of cellular functions, including proliferation, migration and differentiation, and essential biological processes, such as embryonic development, the immune response, wound healing, angiogenesis, and cancer (Batlle and Massagué, 2019; Derynck and Budi, 2019). Since the discovery of TGF-β1 in the early 1980s due to its ability to induce the growth of normal rat kidney cells in soft agar, 33 human genes encoding polypeptide members belonging to the TGF-β family have been identified and characterized (Morikawa et al., 2016). TGF-β family members can be divided into subfamilies according to their sequences and functional similarities: TGF-βs, activins and nodal, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and anti-Müllerian hormone (AMH). Whereas TGF-βs were initially associated with the stimulation and inhibition of cell proliferation, activins (and their antagonists, termed inhibins) were first identified by their activity in the gonads (Xia and Schneyer, 2009). BMPs were discovered as molecules with the potential to induce ectopic cartilage and bone formation in rodents (Urist et al., 1982). These early discoveries have been followed by multiple studies that have unveiled the broad roles of each TGF-β family member in human (patho)physiology.

In response to extracellular stimuli (i.e., inflammation and hypoxia), TGF-βs are transcribed and secreted by cells in an inactive dimeric form. TGF-βs are inactive due to the non-covalent interaction between the amino-terminal pro-peptide sequence, known as latency-associated peptide (LAP), and the carboxy-terminal of the mature TGF-β peptide. When specific enzymes are activated, such as serine protease, plasmin and furin, the pro-peptide is cleaved thereby releasing TGF-β in a mature and active form. TGF-β family members may also be sequestered by binding to extracellular matrix (ECM) proteins or shielded from receptor binding by interacting with soluble antagonists. Together, these mechanisms carefully regulate TGF-β family member bioavailability (Brazil et al., 2015; Robertson and Rifkin, 2016).

Receptors

TGF-β family members trigger biological processes by inducing the formation of cell surface receptor complexes bearing intrinsic serine/threonine kinase activity. Seven human type I receptors [activin receptor-like kinases (ALKs) 1–7] and five human TGF-β family type II receptors, i.e., activin type II A and B receptors (ActRIIA and ActRIIB), BMP type II receptor (BMPRII), TGF-β type II receptor (TβRII), and AMH type II receptor (AMHRII), have been identified. In the case of TGF-βs, their oligomeric receptor complexes comprise the type I (TβRI) and type II (TβRII) receptors (Cheifetz et al., 1990; Lin et al., 1992). Binding of TGF-β to TβRII promotes the recruitment of TβRI (also termed ALK5). While both TβRI and TβRII have intracellular kinase domains, only the type I receptor contains a glycine–serine-rich domain (GS domain) at its juxtamembrane region. Specific serine and threonine residues in the GS domain are phosphorylated by TβRII kinase, resulting in TβRI activation (Wrana et al., 1994). In addition to TβRI and TβRII, there are a number of TGF-β coreceptors (including Endoglin, TβRIII (also termed betaglycan) and Cripto) that contain a short (or lack an) intercellular domain without kinase activity and fine-tune the interaction between extracellular ligands and membrane receptor complexes, thereby modulating cellular responses to TGF-β stimulation (Nickel et al., 2017). While there are differences in how TGF-β family members engage their cell surface receptors, the notion that ligand-induced receptor complex formation mediates type I phosphorylation and activation by type II kinase is common to all TGF-β family members and their signaling receptors.

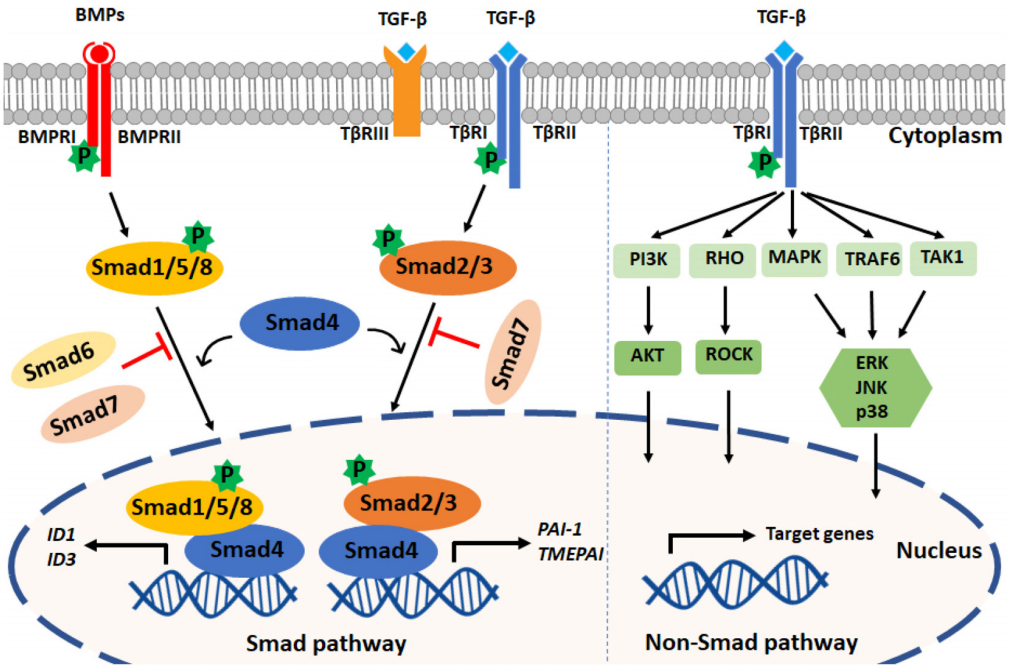

FIGURE 1 | TGF-β family signaling pathways. **Left panel:** TGF-β family ligands signal via type I and type II receptors on the cell surface. Upon ligand engagement, the type II kinases transphosphorylate the type I receptors, which are then activated. TGF-βs and BMPs are shown as an example; TGF-βs bind TβRI and TβRII, and BMPs bind BMPRI and BMPRII. TβRIII (also termed Betaglycan) is a coreceptor that facilitates interaction with TβRI and TβRII. TGF-βs induce the phosphorylation of Smad2/3, and BMPs mediate Smad1/5/8 phosphorylation. By forming complexes with Smad4, phosphorylated Smad2/3 and Smad1/5/8 translocate into the nucleus to regulate target gene expression. *PAI1* and *TMEPAI* are typical target genes induced downstream of Smad3 phosphorylation, and *Id1* and *Id3* are induced after Smad1 and Smad5 activation. Inhibitory Smads (i.e., Smad6 and Smad7) can antagonize the action of signal-transducing R-Smads and Smad4. **Right panel:** TGF-β family members can also activate PI3K, RHO, MAPK, TRAF6, and TAK1 through non-Smad pathways. TGF-β is shown, but these non-Smad signaling pathways can also be activated by BMPs and other family members.

Intracellular Signaling

Upon type I receptor activation, the signal is transduced from the cell membrane into the nucleus by phosphorylation of a specific subset of intracellular transcriptional effector proteins, termed mothers against decapentaplegic and Sma homologs or Smads (Derynck et al., 1996; **Figure 1**). Smad proteins can be classified into three groups: (1) receptor-associated Smads (R-Smads, Smad1/2/3/5/8), (2) common Smad (i.e., co-Smad, also known as Smad4 in vertebrates), and (3) inhibitory Smads (I-Smads, Smad6/7) (Hill, 2016). By using different receptor complexes, ligands of the TGF-β family induce the phosphorylation and activation of specific R-Smads. For example, TGF-βs (via TβRI/ALK5) and activins (via ALK4/7) induce the phosphorylation of Smad2 and Smad3, whereas BMPs, upon activating ALK1/2/3/6, signal via Smad1/5/8. Activated R-Smads then associate with the co-Smad, i.e., Smad4, to form heteromeric complexes. These complexes can translocate into the nucleus, where they regulate specific gene transcriptional responses (Shi and Massagué, 2003; Derynck and Budi, 2019). In general, while Smad1/5/8 promote the induction of genes involved in proliferation and osteogenic differentiation (i.e., *Id-1/3* and *Runx2*), Smad2/3 induce the expression of pro-fibrotic genes (i.e., *Serpine-1* and *Collagen tissue growth factor*). Smad6 and Smad7 antagonize TGF-β family-induced signal transduction by inhibiting the stability or function of the activated receptors or by interacting with Smad4 to prevent the heteromeric complex formation of activated R-Smads and Smad4 (Itoh and ten Dijke, 2007).

In addition to the so-called canonical Smad pathway described above, TGF-β family members can signal via non-Smad pathways, such as the extracellular signal-regulated kinase (Erk) MAP kinase (MAPK), Rho-like GTPase, phosphatidylinositol-3-kinase (PI3K)/AKT, p38 MAPK, Jun amino-terminal kinase (JNK), ubiquitin ligase tumor necrosis factor (TNF)-receptor associated factor 6 (TRAF6), and TGF-β activated kinase 1 (TAK1) pathways. The non-Smad signaling pathways act in a context-dependent manner and will fine-tune cell-specific biological processes (Zhang, 2017). Notably, the Smad and non-Smad pathways engage in crosstalk, e.g., ERK MAPK, which can be activated through the non-Smad pathway, is able to engage in crosstalk with the Smad pathway to regulate Smad2/3 phosphorylation (Hayashida et al., 2003; Zhang, 2009).

TGF-β-INDUCED EndMT

TGF-β Family Members in EndMT

EndMT is a process of pivotal importance for proper cardiac cushion formation during embryonic development (Markwald et al., 1975; Eisenberg and Markwald, 1995; Brown et al., 1999).

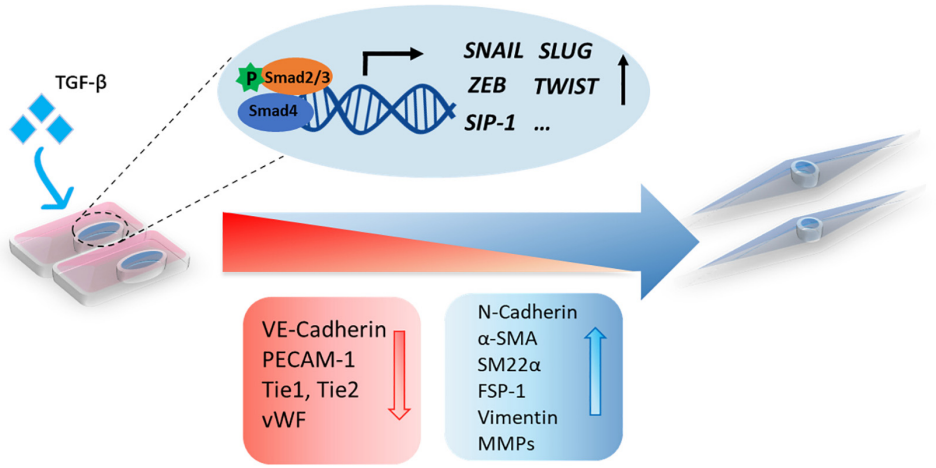

FIGURE 2 | A schematic representation of TGF-β-induced EndMT. The activation of TGF-β signaling leads to the accumulation of nuclear Smad transcription factor complexes. These complexes can induce the expression of transcription factors (*SNAIL*, *SLUG*, *ZEB*, *TWIST*, and *SIP-1*) and trigger EndMT in which cell morphological changes occur, including a switch from the cobblestone-like endothelial morphology to a spindle-like fibroblast morphology. Upon EndMT, endothelial cells lose polarity, and the expression of endothelial markers, such as VE-cadherin, PECAM-1, Tie1, Tie2, and Vwf, is decreased, while the expression of mesenchymal markers, including N-cadherin, α-SMA, SM22α, FSP-1, vimentin, and MMPs, is increased.

Similar to EMT, a variety of autocrine and paracrine signaling molecules can drive EndMT, including TGF-β, Wnt/β-catenin Notch, and inflammatory cytokines (Watabe et al., 2003; Kokudo et al., 2008; Pérez et al., 2017; Wang et al., 2018; Zhong et al., 2018; Sánchez-Duffhues et al., 2019a). In recent years, valuable insights regarding the role of TGF-β family members in controlling the dynamic EndMT process have been obtained (**Figure 2**). All three mammalian isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) can induce EndMT, although different isoform- and species-specific functions have been reported (Goumans et al., 2008; Pardali et al., 2017). Recently, Sabbineni et al. (2018) showed that in human dermal microvascular ECs (HMECs) TGF-β2 is more potent than TGF-β1 or TGF-β3 in inducing the expression of the mesenchymal transcription factors Snail and FoxC2. Treatment with TGF-β1 and TGF-β3 induced the expression of TGF-β2, suggesting that they can act in an indirect manner. Furthermore, TGF-β2-induced EndMT has been reported to increase the pool of cancer-associated fibroblasts (CAFs) in colon cancer (Wawro et al., 2019). The function of TGF-β signaling in regulating EndMT *in vivo* has been interrogated in part by investigating different transgenic and knockout animal models. Both TGF-β2 and TGF-β3 were shown to be required for the EndMT process involved in the formation of AV cushions in chick embryos (Camenisch et al., 2002). By histological examination of cushion morphology in E14.5-specific TGF-β deficient mouse embryos, no obvious valvular defects were observed in *Tgfb1*- or *Tgfb3*-knockout mice. *Tgfb2* deficient mice, however, demonstrated multiple defects in AV cushion formation. This is line with the observation that only TGF-β2 is strongly expressed in the cushion myocardium and invading mesenchymal cells in mice (Brown et al., 1999; Azhar et al., 2009). Furthermore, Jiao et al. (2006) used the Cre/loxp system to specifically inactivate the TβRII in mice. They showed that inactivation of this receptor in either the myocardium or the endothelium of mouse embryos did not prevent EndMT and AV cushion formation, suggesting that other TGF-β family ligands compensate for this pathway (Jiao et al., 2006). While BMPs were found to induce EndMT *ex vivo* and *in vitro*, the specific deletion of different BMPs in mice did not unveil their functions in early cardiac differentiation due to the early lethality of the loss of specific BMPs or functional redundancy. While BMP5- or BMP7-deficient mice survived, without obvious cardiac abnormalities (Kingsley et al., 1992; Dudley and Robertson, 1997), the BMP5/7 double knockout mouse did show defects in AV cushion formation (Solloway and Robertson, 1999). BMP6-deficient mice did not show any cardiac abnormalities, although BMP6/BMP7 double-knockout mice did have cardiac defects (Solloway et al., 1998; Kim et al., 2001). BMP2 plays a vital role in modulating AV canal morphogenesis, as mice with BMP2 specifically inactivated in AV myocardium showed abnormal AV canal morphology at 9.5 days post coitum (dpc) and pericardial effusion and growth retardation at 10.5 dpc (Ma et al., 2005). ALK2 or ALK3 deficiency within the endothelium in mice resulted in AV canal defects, indicating that these two BMP type I receptors are important in inducing EndMT for endocardial cushion formation (Wang et al., 2005; Kaneko et al., 2008). Medici et al. (2010) showed that both TGF-β2 and BMP4 induce EndMT in human umbilical vein ECs (HUVECs) and human cutaneous microvascular ECs (HCMECs) in an ALK2- and TβRI-dependent manner. In summary, both the TGF-β and BMP signaling pathways have pivotal functions in EndMT.

Transcription Factors Involved in TGF-β-Induced EndMT

TGF-β family members mediate EndMT via Smad or non-Smad signaling propagated by inducing the expression of specific

transcription factors, such as Snail, Slug, Twist, ZEB1, SIP-1/ZEB2, and LEF-1 (Yoshimatsu and Watabe, 2011). The Snail family of transcription repressors, including Snail (*SNAI1*) and Slug (*SNAI2*), are the most studied downstream EndMT effectors induced by TGF-β (Kokudo et al., 2008; Medici et al., 2011). Snail family members are proteins containing four to six C2H2-type zinc finger motifs in their carboxy-terminal domain that bind a specific DNA region (E-box) (Nieto, 2002). Snail represses the expression of EC cell–cell adhesion molecules by binding to the promotor of *CDH5* (encoding for VE-cadherin) or *PECAM1* (encoding for CD31) and inducing the expression of mesenchymal markers such as *ACTA2* (encoding for α-SMA) (Kokudo et al., 2008; Cheng et al., 2013). Snail has a higher apparent DNA-binding affinity than Slug, which can result in more potent inhibition of endothelial specific target genes (Bolós et al., 2016). Kokudo et al. (2008) showed that Snail is essential for TGF-β2-induced EndMT in mouse embryonic stem cell-derived ECs (MESECs). Snail expression was upregulated by TGF-β2, whereas Snail knockdown abrogated TGF-β2-induced EndMT in these cells. The transcription factor Forkhead Box M1 (Foxm1), which can be induced by TGF-β, was found to drive EndMT by binding to Snail and enhancing its activity (Song et al., 2019). Twist can be transcriptionally activated in a signal transducer and activator of transcription (STAT)3 dependent manner by the recruitment of the transcriptional modulator megakaryoblastic leukemia (MKL)1 to its promotor region. Depletion of MKL1 or treatment with a Twist small molecule inhibitor attenuated TGF-β-induced EndMT in human vascular ECs (HVEC) and inhibited liver fibrosis in mice (Li et al., 2019). In addition, basic helix–loop–helix (bHLH) transcription factors, such as E2A (including E12 and E47) and ID (DNA-binding protein inhibitor), are master regulators of EMT (Lamouille et al., 2014). ID proteins bind E2A to form heterodimers and thereby regulate E2A activity (Slattery et al., 2008). The E2A protein contributes to EMT by regulating the expression of target genes, such as *ACTA2* (α-SMA) and *CDH1* (E-cadherin). Due to the similarity between the EMT and EndMT processes, the bHLH proteins might also play an important role in regulating EndMT.

Interplay With Other Signaling Pathways That Mediate or Regulate EndMT

In addition to the Smad/non-Smad signaling pathway, TGF-β interacts with other signaling pathways that mediate and/or regulate EndMT, such as the Notch (Fu et al., 2009), fibroblast growth factor (FGF) (Chen and Simons, 2018), Wnt, and Sonic Hedgehog pathways (Horn et al., 2012). As such, Notch signaling is critical for heart formation during embryonic development (MacGrogan et al., 2018). TGF-β and Notch signaling cooperate to induce the expression of Snail, thereby downregulating the expression of VE-cadherin and promoting EndMT (Fu et al., 2009). In contrast, Patel et al. (2018) demonstrated that EC specific deletion of Notch signaling resulted in enhancement of EndMT since more $CD31^{-}FSP^{+}$ cells were detectable in skin wounds of endothelial specific transcription factor Rbpj-deficient mice. Interestingly, TGF-β1 expression was found to be increased in these $CD34^{-}/FSP\text{-}1^{+}$ wound ECs, which suggests that TGF-β is the main driver of EndMT in mice deficient for endothelial specific Notch signaling (Patel et al., 2018).

Several studies indicate that microRNAs (miRNAs) are regulated in response to TGF-β-induced EndMT. For example, Ghosh et al. (2012) reported that several miRNAs are regulated during TGF-β2-induced EndMT in mouse cardiac ECs (MCECs). After promoting EndMT by stimulating MCECs with TGF-β2 for 7 days, miR-125b, Let7C, Let-7g, miR-21, miR-30b, and miR-195 were upregulated while miR-122a, miR-127, miR-196, and miR-375 were downregulated (Ghosh et al., 2012). Correia et al. (2016) found that miR-20a is decreased during TGF-β1-induced EndMT. miR-20a regulates the expression levels of the TGF-β receptors TβRI and TβRII. FGF2 was found to induce miR-20a and antagonize TGF-β1-induced EndMT (Correia et al., 2016).

Fibroblast growth factor (FGF) is known to inhibit TβRI expression (Fafeur et al., 1990). An increasing number of studies have shown that FGF and TGF-β crosstalk in more complex ways. Endothelial specific deletion of *Fgfr1* or *Frs2α* encoding FGF receptors inhibited FGF signaling, resulting in enhanced TGF-β signaling and EndMT induction (Chen et al., 2014). Moreover, let-7 miRNA seems to have a crucial function in establishing a bridge between FGF and TGF-β. FGF signaling activation is necessary for the expression of let-7 miRNA, which binds multiple sites on the untranslated region of human *TβRI*. Antagonizing FGF signaling diminished the expression of let-7 miRNA, which increased TGF-β1 and TβRI expression and thereby promote TGF-β signaling (Chen et al., 2012). Recently, FGF2 was shown to not only inhibit TGF-β-induced endothelial-to-myofibroblast transition (End-MyoT) mediated via the transcription factor ELK1, but also promoted the formation of active fibroblastic cells with migratory and proliferative characteristics. This revealed the opposing and cooperative action between FGF and TGF-β signaling during the modulation of different mesenchymal cell phenotypes (Akatsu et al., 2019). In mouse embryos with ECs deficient in β-catenin, the cardiac cushion had fewer cells, suggesting that β-catenin in ECs is needed for efficient EndMT and invasion of the mesenchymal cells into the cardiac jelly to form cardiac septa and valves. *In vitro*, TGF-β-induced EndMT was strongly inhibited in β-catenin-knock out ECs, as much less α-SMA was expressed after TGF-β2 stimulation and VE-cadherin levels or Snail1 expression did not change (Liebner et al., 2004). Consistent with this notion, we showed that ECs lacking primary cilia expressed high levels of β-catenin, which was needed to induce Slug expression and subsequent BMP-induced osteogenic differentiation (Sánchez-Duffhues et al., 2015). The Sonic Hedgehog pathway cooperates with TGF-β signaling to stimulate fibroblast differentiation (Horn et al., 2012). Furthermore, inflammatory interleukin (IL)-1β and TGF-β synergistically induce EndMT in HUVECs (Maleszewska et al., 2013). Liguori et al. (2019) showed that the IL-1β/TGF-β2-induced EndMT in HUVECs could be reduced by conditioned medium of adipose derived stromal cells. Katsura et al. (2016) demonstrated that TGF-β signaling engages in crosstalk with the tumor necrosis factor (TNF)-α

pathway to enhance EndMT by inducing more miR-31 as a molecular hub, which is required for induction of EndMT. TGF-β suppresses VAV3 and Stk40, which are a negative regulator of MRTF-A (involved in induction of EndMT related gene *ACTA2*) and a suppressor of NF-κB pathway, respectively, in a miR-31-dependent manner. Thus, the lack of Stk40 augments the positive function of miR-31 in EndMT (Katsura et al., 2016). Recently, Glaser et al. (2020) demonstrated that TGF-β2 as well as a combination of IL-1β/TGF-β1 or hypoxia increased the expression of the histone demethylase Jumonji domain-containing protein 2B (JMJD2B) in HUVECs. Interestingly, both siRNA-mediated silencing and pharmacological inhibition of JMJD2B greatly reduced TGF-β2-induced EndMT in HUVECs as demonstrated by a deceased SM22α expression, preserved CDH5 expression and reduced endothelial permeability. The critical function of JMJD2B in EndMT was verified *in vivo*; endothelial specific depletion of JMJD2B in mice resulted in substantial fewer EndMT positive cardiac ECs in the heart after experimentally induced myocardial infarction. However, the reduced EndMT only resulted in a modest rescue of cardiac function 2 weeks after infarction (Glaser et al., 2020).

EndMT-RELATED DISEASES

While EC plasticity and EndMT are important for proper embryonic development, preserving the function of ECs during adult life is an active process and crucial for tissue homeostasis. Endothelial dysfunction can be the consequence of EndMT and can lead to pathological tissue remodeling, thereby contributing to the progression of a variety of diseases, such as fibrotic disorders and tumor development (**Figure 3**).

EndMT in Fibrotic Diseases

Fibrotic disorders are characterized by the excessive deposition of matrix produced by an increased number of activated fibroblasts and/or myofibroblasts, which eventually leads to organ dysfunction and systemic disease (Rosenbloom et al., 2017). Although the contribution of ECs to fibrosis is still debatable, results obtained in the past years suggest that EndMT provides an additional source of fibroblasts in fibrotic organs (Zeisberg et al., 2007b, 2008; Piera-Velazquez and Jimenez, 2019). The origin and composition of these fibrosis associated fibroblasts may vary depending on the affected organs. Due to the lack of effective and safe therapies that do not compromise physiological healing, fibrotic diseases constitute a serious health problem and contribute to high mortality. Therefore, there is an urgent need to gain a deeper understanding of the mechanism underlying fibrotic disease to provide the basis for the development of potential antifibrotic treatments, perhaps through the modulation of EndMT.

Cardiac Fibrosis

Fibrosis in the heart, the accumulation of excessive ECM in the myocardial and perivascular tissues, is an important determinant in the pathogenesis of cardiovascular disorders. Cardiac fibrosis is a response of the heart to stress and injury. Interstitial fibrosis is characterized by unbalanced turnover and excessive deposition of diffuse collagen in the interstitial space and it is often found under conditions of pressure and/or volume overload, in metabolic disorders, or following ischemic insults (Frangogiannis, 2019). Replacement fibrosis mainly occurs after myocardial infarction in the healing ventricle, where dead myocardial cells are substituted by a collagen-based fibrotic scar (Prabhu and Frangogiannis, 2016). Cardiac fibrosis compromises the contractile function of the heart, leading to impaired ventricular relaxation and eventually ventricular hypertrophy, reduced cardiac output, and heart failure (Mocumbi et al., 2019). Whether EndMT contributes to the pool of cardiac fibroblasts remains controversial and depends on the affected tissue. Using a *Tie1Cre;R26RstoplacZ* fate mapping strategy, Zeisberg et al. (2007b) showed an increase in LacZ-positive cells that co-expressed the fibroblast marker FSP1 surrounding the cardiac capillaries. Furthermore, the authors demonstrated how activated Smad2/3 was increased in these cells, and that the knockout of *Smad3* decreased EndMT and reduced cardiac fibrosis (Zeisberg et al., 2007b). Notably, neither Tie1 nor FSP1 are exclusively expressed in ECs or fibroblasts, respectively (Van Amerongen et al., 2008; Kong et al., 2013). Furthermore, whether the labeled Tie1$^+$ fibroblasts are derived from cardiac ECs or whether they are derived from existing fibroblasts that originated during cardiac development, and proliferated in response to tissue damage, remains unknown. Therefore, additional studies using alternative endothelial and fibroblast markers and/or inducible (postnatal) reporter strategies are needed.

Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a disease characterized by progressive thickening and narrowing of the pulmonary arterial walls (Farber and Loscalzo, 2004). This leads to increased resistance in the pulmonary circulation, which negatively impacts the cardiac left ventricle that becomes hypertrophic (Farber and Loscalzo, 2004). Inactivating gene mutations affecting the *BMPRII* have been found in 70% of familial PAH cases and in 10–40% of idiopathic PAH cases (Ranchoux et al., 2015). Moreover, non-genetic cases of PAH exhibit decreased expression of *BMPRII* (Orriols et al., 2017), likely due to an inflammatory environment that negatively affects the expression of BMPRII (Hurst et al., 2017; Sánchez-Duffhues et al., 2019a). Using two different endothelial reporter mice (i.e., Tie2 and VE-cadherin) in combination with immunostaining for α-SMA and MYH11, Qiao et al. (2014) demonstrated the occurrence of EndMT in pulmonary vessels in an experimental animal model of PAH induced by monocrotaline and pneumonectomy. More recent studies combining immunofluorescent labeling and confocal imaging confirmed the presence of EndMT in lung sections from PAH patients (Ranchoux et al., 2015). Furthermore, Good et al. (2015) demonstrated the presence of transitional EndMT cells in the lungs of both hypoxia/SU5416 mice (a murine PAH

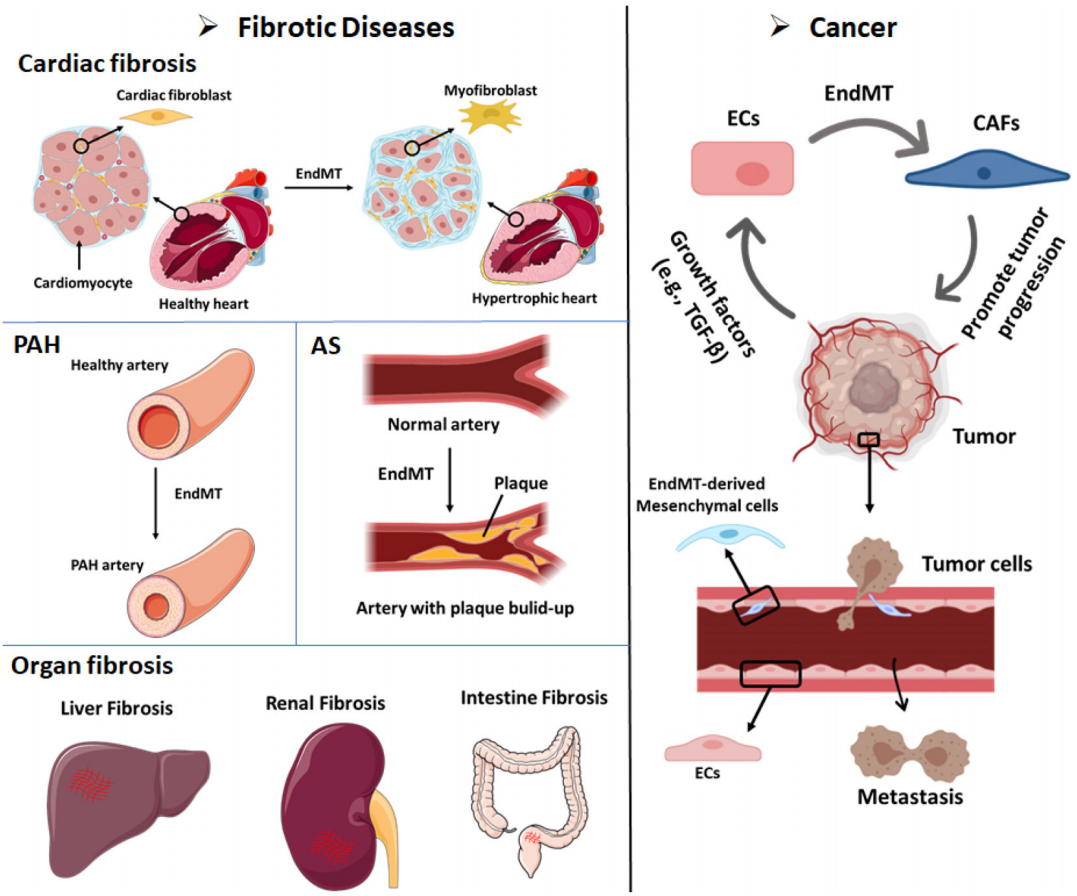

FIGURE 3 | EndMT can contribute to the development of multiple diseases, including fibrotic diseases and cancer. **Cardiac fibrosis:** Endothelial cells that undergo EndMT can differentiate into cardiac fibroblasts that enable cardiac fibrosis. **Pulmonary arterial hypertension (PAH):** The accumulation of EndMT-derived SMA-overexpressing fibrosis cells can thicken and narrow the arterial walls and favor the development of PAH. **Atherosclerosis (AS):** The accumulation of EndMT-derived fibroblasts can lead to plaque growth and facilitate the thickening of AS plaques. **Organ fibrosis:** EndMT-induced fibroblasts have been demonstrated as a source of fibroblast-like cells in liver fibrosis, renal fibrosis, and intestine fibrosis. **Cancer:** (i) Up to 40% of cancer-associated fibroblasts (CAFs) in pancreatic cancer or melanoma animal models were shown to be derived from EndMT. Tumor cells secrete abundant growth factors, including TGF-β, which stimulates endothelial cells to differentiate into CAFs. CAFs can promote cancer invasion and metastasis and immune evasion. (ii) Cancer cell-secreted growth factors (e.g., TGF-β) induce the EndMT of endothelial cells that line tumor blood vessels. EndMT-derived mesenchymal cells weaken the endothelial barrier permeability due to elongation of the cell shape and the loss of adhesion molecules such as claudins and VE-cadherin. These effects facilitate cancer cell intravasation and extravasation.

model) and PAH patient samples by the colocalization of vWF and α-SMA expression. More EndMT cells (vWF and α-SMA double-positive cells) were found in hypoxia/SU5416 mice sections and patient samples. Pulmonary artery ECs (PAECs) undergo EndMT following stimulation with the inflammatory cytokines IL-1β, TNFα, and TGF-β, and in turn secrete more proinflammatory cytokines that may further promote PAH progression (Good et al., 2015). Hopper et al. (2016) showed that dysfunctional BMPRII signaling in PAECs upregulated the expression of High Mobility Group AT-hook 1 (HMGA1), which might promote EndMT and contribute to PAH. Zhao et al. (2020) found that overexpression of miR-181b in the lung inhibited the monocrotaline-induced PAH-like phenotypic response in rats as demonstrated by a decreased right ventricular systolic pressure (RVSP), mean pulmonary artery pressure (mPAP), pulmonary vascular hypertrophy, and right ventricular remodeling. Mechanistically, overexpression of miR-181b in rat pulmonary arterial ECs (rPAECs) was found to inhibit TNF-α, TGF-β1, and IL-1β-induced EndMT by inhibiting the expression of TGF-βR1 and circulating proteoglycan endocan (Zhao et al., 2020).

Atherosclerosis

Atherosclerosis (AS) refers to the formation of atherosclerotic, calcified plaques. Although still asymptomatic, the vascular remodeling associated to this progressive condition is thought to begin after the first decade of life, due to the combined action of cytokines that induce the accumulation of SMCs, fibroblasts, and osteoblasts in the arterial wall, resembling the process of endochondral bone formation (Souilhol et al., 2018; Kovacic et al., 2019). The expansion and rupture of atherosclerotic plaques may disturb the blood flow and lead to

myocardial infarction, stroke, aneurysm, or pulmonary embolism (Alexopoulos and Raggi, 2009). Although many different groups (including ours) have identified ECs as a source of mesenchymal cells within the plaque, two groups have confirmed the presence of double-positive endothelial–mesenchymal cell populations using lineage tracing strategies (Chen et al., 2015; Evrard et al., 2016). As such, Evrard et al. (2016) made use of the tamoxifen-inducible endothelial-specific lineage tracing system end*SclCreERT;R26RstopYfp* in a pro-atherosclerotic $ApoE^{-/-}$ background to identify double-positive FSP-1/vWF or fibroblast activating protein (FAP)/CD31 cells in vulnerable atherosclerotic lesions. By using *in vitro* modeling, they found that both oxidative stress and hypoxia, which are hallmarks of AS, enhanced TGF-β-induced EndMT (Evrard et al., 2016). In an elegant study by Chen et al. (2015), using VE-cadherin-labeled reporter mice in combination with an $ApoE^{-/-}$ $Frs2a^{ECKO}$ atherogenic background, increased TGF-β signaling was observed to be related to EndMT in atherosclerotic plaques. Kim et al. (2013) showed that AS might be a severe side effect of radiation by inducing EndMT. Radiation can induce EndMT in heart aortic ECs (HAoECs), accompanied by the decreased expression of CD31 and VE-cadherin and increased expression of FSP-1 and α-SMA. They observed more atherosclerotic plaques in irradiated than in non-irradiated $ApoE^{-/-}$ mice. By immunofluorescence staining of aortic sinus sections for endothelial CD31 and mesenchymal α-SMA marker proteins, higher levels of cells undergoing EndMT were found in the irradiated $ApoE^{-/-}$ mice, which suggests that radiation-triggered EndMT might promote AS (Kim et al., 2013).

Organ Fibrosis

EndMT has also been implicated in the development of fibrosis in other organs, such as the lung, kidney, and liver (Piera-Velazquez et al., 2016). The origin of the fibroblasts in kidney fibrosis was studied by Zeisberg et al. (2008) using three different mouse chronic kidney disease models. In the kidney sections, up to 50% of fibroblasts showed the expression of both an endothelial marker (CD31) and fibroblast and myofibroblast markers (FSP-1 and α-SMA, respectively). Their results suggest the contribution of EndMT to the accumulation of fibroblasts in the kidney and related renal fibrosis diseases. Li et al. (2009) also provided evidence that EndMT occurs and promotes the early development of diabetic renal interstitial fibrosis. They used endothelial lineage tracing with Tie2-cre;LoxP-enhanced green fluorescent protein (EGFP) mice to distinguish endothelial-derived cells. A considerable number of ECs in the fibrotic kidneys of diabetic nephropathy mice were found to express α-SMA. α-SMA positive cells with an endothelial origin were also found in afferent/efferent arterioles in glomeruli, suggesting that the EndMT-derived myofibroblasts can promote glomerulosclerosis (Li et al., 2009). However, in the literature and at scientific meetings discussion remains about existence of EndMT (and EMT) in kidney fibrosis (Cruz-Solbes and Youker, 2017). EndMT has also been linked to liver fibrosis. The liver tissue sections from idiopathic portal hypertension (IPH) showed double-positive staining for CD34 and S100A4, which are EC and myofibroblast markers, respectively. Based on an increase in phosphorylated Smad2 levels, TGF-β signaling may be linked to EndMT in the portal vein endothelium and lead to eventual portal vein stenosis and obliteration in IPH (Kitao et al., 2009). A recent report showed that defective autophagy induced by suppression of *ATG5* expression resulted in EndMT in human microvascular ECs (HMVECs) mediated by an abnormal accumulation of IL-6. Feeding endothelial-specific *ATG5* knockout mice with high-fat diet (HFD) resulted in profound tubular damage and interstitial fibrosis in the kidney and stronger perivascular fibrosis in the heart compared to control animals. Increased EndMT was also found in *ATG5* deficient mice, which supported the notion that disruption of autophagy triggers EndMT can contribute to organ fibrosis *in vivo* (Takagaki et al., 2020).

EndMT in Cancer

ECs and angiogenesis are known to have critical function in tumor development and metastasis (Sobierajska et al., 2020). Emerging evidence has shown that EndMT not only plays roles in promoting cancer development and metastasis, but also influences the response to cancer therapy (Potenta et al., 2008; Platel et al., 2019). Tumor progression is facilitated by fibroblasts within the tumor. The origin of these CAFs has been investigated using Tie1Cre;R26R stop lacZ transgenic mice, and up to 40% of the CAFs in pancreatic cancer or melanoma models may have originated from EndMT (Zeisberg et al., 2007a). CAFs facilitate cancer progression by influencing the tumor microenvironment. CAFs secrete various cytokines and chemokines that influence the behavior of different cell types (Allen and Louise Jones, 2011; Polanska and Orimo, 2013). For example, VE growth factor (VEGF), which is secreted by CAFs, promotes vascular formation at tumor sites and may thereby provide more nutrition for tumor growth. CAFs secrete TGF-β to promote cancer invasion and metastasis (Xiao et al., 2015). Other CAF-derived factors, such as EGF, FGF, and matrix metalloproteinases (MMPs), have been identified as contributors of cancer progression that promote proliferation and invasion (Mendelsohn and Baselga, 2000; Katoh and Nakagama, 2014; Ciszewski et al., 2017). Interestingly, CAFs may also play a role in awakening dormant cells to induce metastasis (De Wever et al., 2014). In addition to supporting the fibroblast population, EndMT may contribute to weakening of the endothelial barrier due to the elongation of the cell shape and the loss of adhesion molecules such as claudins and VE-cadherin, supporting tumor metastasis (Anderberg et al., 2013; Gasparics et al., 2016). Krizbai et al. (2015) found that after inducing EndMT by treating ECs with cancer cell conditioned medium, the transendothelial electrical resistance was decreased indicative for loss of barrier function, and more melanoma cells were able to adhere to ECs and transmigrated through the endothelial layer. Therefore, EndMT might play a role during metastatic trans-endothelial migration.

Moreover, recent studies showed that the response of cancer cells to chemo- and targeted therapy can be influenced by EndMT. Kim et al. (2019) showed that HUVECs undergoing EndMT enhanced the resistance of tumor spheroids against EGFR inhibitor gefitinib and chemotherapy cisplatin. Furthermore, CAFs originated at tumor sites via EndMT

influence chemotherapy in several ways. CAFs secrete some factors, such as IL-6 and IL-8, and matricellular proteins to regulate chemoresistance (Shintani et al., 2016; Leask, 2019). At the same time, CAFs reduce the levels of therapeutic reagents in tumors by decreasing the expression of drug transporters and trapping active agents (Chen and Song, 2019). EndMT is also related to radiation therapy. Choi et al. (2018) showed that radiation could induce EndMT, which triggered tumor-associated macrophage (TAM) polarization toward an M2 phenotype and resulted in radiation resistance. Additionally, CAFs can support immune evasion and act as an immunosuppressive agent in cancer immunotherapy, by inducing the secretion of multiple chemokines and cytokines, such as TGF-β and IL-6/8/13, and thereby inhibit the antitumor immune response. Additionally, the ECM produced by CAFs at tumor sites enhances ECM stiffness and obstructs the infiltration of effector T cells into the tumor (Chakravarthy et al., 2018; Liu et al., 2019; Monteran and Erez, 2019). In conclusion, EndMT is a promising target for cancer therapy, although more investigation is needed.

EndMT in Cerebral Cavernous Malformation

EndMT has also been shown to contribute to the development of CCM, a disease that can result in brain hemorrhage, seizure, and paralysis (Bravi et al., 2015, 2016). Loss-of-function mutations in CCM1 is one of the causes of CCM. In endothelial-specific *Ccm1* (also known as KRIT1)-ablated mice, ECs in the vascular lesions of the brain underwent EndMT; N-cadherin was increased that promoted the formation of vascular malformations. The deletion of *Ccm1* in ECs upregulated the secretion of BMP6 and, in turn, increased the sensitivity of the response to TGF-β and activated BMP signaling to induce EndMT (Maddaluno et al., 2013). EndMT was shown to be critical in the onset and progression of CCM. In line with these results, Takada et al. (2017) found that ECs in cerebral and orbital CCM expressed both the endothelial marker CD31 and the mesenchymal markers α-SMA and CD44, also demonstrating the occurrence of EndMT.

EndMT IN TISSUE REGENERATION AND ENGINEERING

In addition to the pathological effects of (myo)fibroblast generation, the beneficial aspects of EndMT are gradually being discovered. EndMT has the potential to drive ECs to mesenchymal multipotent cells (MSCs), able to further differentiate into various different cell types that can be applied in tissue engineering and regeneration (Medici and Kalluri, 2012; **Figure 4**).

The ability of EndMT to generate various cell types has been described *in vivo* and *in vitro*. Fibrodysplasia ossificans progressiva (FOP) patients, which suffer from heterotopic bone formation, have a gain-of-function mutation in the BMP type I receptor ALK2 (Shore et al., 2006). Endothelial-like cells were identified as a source of heterotopic cartilage and bone formation in Tie2-GFP reporter mice injected with adenoviral particles expressing a constitutively active form of ALK2. Moreover, immunostaining performed in patient-derived tissue sections revealed the existence of double positive cells expressing either Tie2 or vWF and Osteocalcin (osteoblast marker) or SOX9 (chondrocyte marker). Overexpression of this mutated ALK2 in ECs induced EndMT, and the cells adapted the characteristics of MSCs, which have the ability to differentiate into osteoblasts, chondrocytes, or adipocytes. Similar results were found in TGF-β-treated cells, which verified the utilization of TGF-β-induced EndMT to generate MSCs (Medici et al., 2010). Although whether ECs contribute to ectopic bone formation in FOP patients remains controversial, we have recently demonstrated how circulating ECs isolated from FOP donors exhibit enhanced EndMT and osteogenic differentiation *in vitro*, which was used as a functional readout to identify novel small molecules targeting ALK2 (Sánchez-Duffhues et al., 2019b). This illustrates the potential of EndMT to establish surrogate models for research without the need to go through iPSCs. Osteoprogenitor cells formed after the EndMT process were also found in calcifications of the aortic tract (Yao et al., 2015; Boström et al., 2016), valves (Hjortnaes et al., 2015), and tumors (Dudley et al., 2008). Furthermore, via VE-cadherin lineage tracing in mice, EndMT was also shown to be involved in the transformation of ECs into white and brown fat cells (Tran et al., 2012). Recent manuscripts identified that microvascular ECs within adipose tissue in patients with obesity undergo EndMT, thereby modifying their secretome and enhancing systemic inflammation (Haynes et al., 2019). ECs isolated from tumor vessels can undergo EndMT to subsequently differentiate into adipocytes, pericytes, and SMCs (Huang et al., 2015), which suggests that artificially modified EndMT-derived cells may be useful to induce tissue repair in a paracrine manner. Furthermore, ECs were discovered to have the potential to form skeletal myocytes in muscle repair (Huang et al., 2014). ECs also contribute to cardiac renewal (Fioret et al., 2014). Evidence has also shown that a subset of valvular ECs behave as progenitor cells that can undergo EndMT and replenish valvular interstitial cells to repair valves (Bischoff and Aikawa, 2011).

The potential of ECs to generate different cell types via EndMT makes steering this process a potential tool in tissue regeneration. For example, EndMT-derived osteoblasts or chondrocytes could be used in skeletal conditions, such as osteoporosis, bone fracture healing, or osteoarthritis. In addition, EndMT-induced myogenesis may generate cardiomyocytes to alleviate myocardial infarction (Medici, 2016). EndMT-mediated chondrogenesis could be employed in osteoarthritis or temporal mandibular joint disorder (TMJD) therapies. Due to its ability to generate SMCs and pericytes, steering EndMT could be an option for vascular formation-related tissue engineering. EndMT might also have the potential to promote angiogenesis as Snail1 mediated EndMT was shown to play a role in regulating vessel formation (Sun et al., 2018). Zheng et al. (2007) showed that myoendothelial cells isolated from human skeletal muscle have the potential to differentiate into myogenic, osteogenic, and chondrogenic cells after culturing in special formulated media supplemented with cytokines. After injecting isolated human myoendothelial cells into damaged muscles in immune compromised mice, dystrophin and human-specific lamin A/C double positive myofibers were observed in mice muscle. This result suggests the

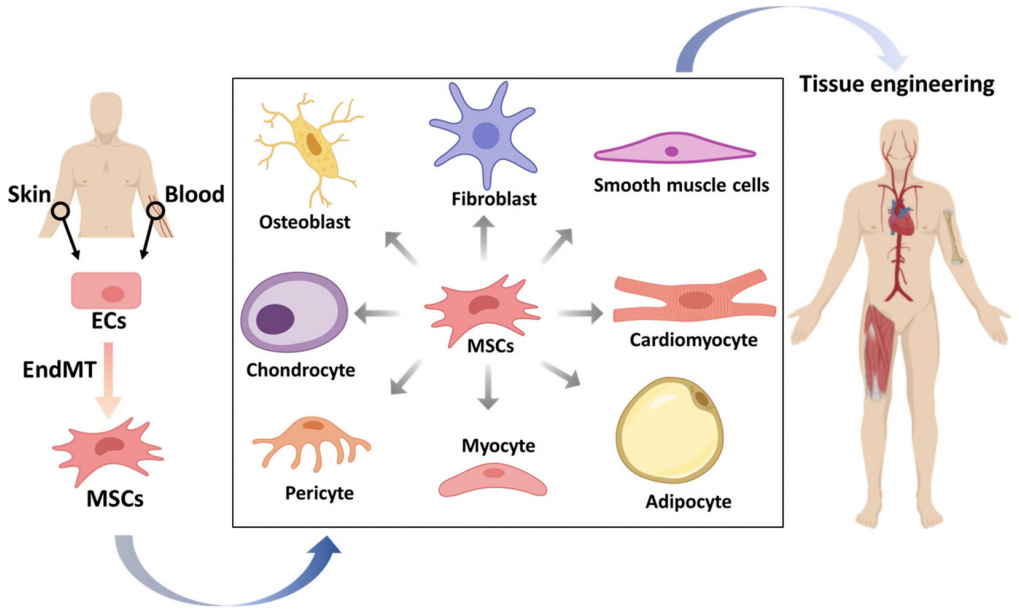

FIGURE 4 | The potential applications of mesenchymal stem cells (MSCs) that originate from endothelial cells (ECs) in tissue engineering. ECs from patients isolated from tissues, such as skin or blood, can be stimulated to undergo endothelial to mesenchymal transition (EndMT) to generate MSCs. These multipotent MSCs can be differentiated into various cell types, which may be used to form desired tissue types that can be transplanted into patients.

potential of regulating myoendothelial cells differentiation for the treatment of muscle related disease.

The potential of EndMT may also be considered in combination with the emerging use of organ-on-chips. ECs grown *in vitro* on chips can mimic the function of blood vessel networks, e.g., they contain a functional endothelial lumen sensitive to flow. Moya et al. (2013) set up a 3D dynamic perfused capillary network model *in vitro* using human endothelial colony forming cell-derived ECs (ECFC-ECs) isolated from cord blood. In addition, Mathur et al. (2019) explored the potential of blood outgrowth ECs (BOECs), which were isolated from venous circulation, to reconstitute vascular networks on vessel-chips. The authors used this 3D complex model constituted with swine BOECs to study the response of the endothelium in diabetes. Noteworthy, perfusion of 3D vessels with whole blood from diabetic pigs led to an enhanced formation of thrombi compared to control animals, such as lower proliferation, more intact lumen, reactive oxidative stress, and platelet adhesion, which also are expected in diabetic patients. This demonstrates the possibility of developing personalized vessel structures on a chip device (Mathur et al., 2019). Although EndMT was not the specific aim of the study, Kolesky et al. (2014) successfully developed a 3D chip resembling vascular calcification using a bio-printing approach with three different cell types (i.e., mesenchymal stem cells, fibroblasts, and ECs). This perfusable vascular tissue was useful to study vascular calcification and monitor osteocalcin expression and collagen deposition.

In vitro 3D organ cultures can be used to study EndMT-related diseases. For example, Wagner et al. (2020) established 3D vascularized cardiac tissue mimetics (CTMs) by co-culturing cardiomyocytes (CM) and fibroblasts (FB) in spheroids and then complementing them with HUVECs to investigate the heterocellular crosstalk in different culture conditions. In this system, TGF-β stimulation could induce EndMT as vimentin/SM22α was expressed in Isolectin B4 stained ECs, and more vascularization was observed in CTMs. In summary, although not so many mature applications have been established to date, the role of TGF-β-induced EndMT in tissue engineering and 3D *in vitro* modeling is emerging.

CONCLUSION

EndMT, a complex process in which ECs change their morphology into that of fibroblast-like mesenchymal cells, is accompanied by changes in cell function and endothelial and mesenchymal marker protein expression. TGF-β, a major inducer of EndMT, regulates the underlying mechanisms via the Smad/non-Smad signaling pathways and interacts with other signaling cascades to orchestrate this process. An in-depth understanding of the dynamic mechanisms of TGF-β signaling in the EndMT process would help to precisely regulate this transition. The EndMT process is a double-edged sword. EndMT is needed for proper development of the embryo and wound healing, but also contributes to some fatal diseases, such as tissue fibrosis and cancer. Inhibition of the EndMT process, e.g., by inhibiting TGF-β signaling, is being pursued for the treatment of diseases associated with/caused by EndMT. But, the discovery of EndMT-derived multipotent cells has inspired scientists to explore the therapeutic potential of TGF-β-induced EndMT in tissue regeneration and tissue engineering.

Since almost all tissues in the body are highly vascularized, the EndMT-derived multipotent cells in vascular engineering might be applied in other cell types to enable the regeneration of a well-contained vascular tissue. In addition, resident ECs within or near damaged tissues could be used in a similar way to enable tissue repair by reprogramming them into mesenchymal multipotent cells and thereafter stimulating the formation of differentiated derivatives. The potential of EndMT in tissue regeneration and engineering is promising.

AUTHOR CONTRIBUTIONS

JM wrote the initial draft of the manuscript. GS-D and M-JG provided feedback and comments on the manuscript. PD supervised and coordinated the writing. All authors have approved the manuscript for publication.

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REVIEW
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RIPK3: A New Player in Renal Fibrosis

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Chronic kidney disease (CKD) is the end result of a plethora of renal insults, including repeated episodes of acute or toxic kidney injury, glomerular, or diabetic kidney disease. It affects a large number of the population worldwide, resulting in significant personal morbidity and mortality and economic cost to the community. Hence it is appropriate to focus on treatment strategies that interrupt the development of kidney fibrosis, the end result of all forms of CKD, in addition to upstream factors that may be specific to certain diseases. However, the current clinical approach to prevent or manage renal fibrosis remains unsatisfactory. The rising importance of receptor-interacting serine/threonine-protein kinase (RIPK) 3 in the inflammatory response and TGF-β1 signaling is increasingly recognized. We discuss here the biological functions of RIPK3 and its role in the development of renal fibrosis.

Keywords: RIPK3, Receptor interacting serine/threonine-protein kinase 3, renal fibrosis, TGF – β1, necroptosis, dabrafenib

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INTRODUCTION

Chronic kidney disease (CKD) is defined as a loss of glomerular filtration and or proteinuria, persisting for at least 3 months or structural abnormalities in the kidney. In the majority of cases, CKD eventually leads to end-stage kidney disease (ESKD) requiring renal replacement therapy or death will ensue. CKD affects a large proportion of the population and considerably more than is widely appreciated by the general public. In 2016–2017, 1.8 million hospitalizations in Australia were associated with CKD, which accounts for 16% of all hospital admissions in Australia. Of those hospitalizations, 80% were for regular dialysis (AIHW, 2019). Having CKD increases the length of stay, cost, and complications of non-CKD related hospital admissions. In the United States, the overall prevalence of CKD in the general adult population was 14.8% in 2011–2014 (United States Renal Data System, 2018). Regardless of the cause of the initial renal injury, progressive renal fibrosis is common to all forms of CKD, characterized pathologically by extracellular matrix (ECM) accumulation, myofibroblast activation, and inflammatory cell infiltration (Lee and Kalluri, 2010; Carew et al., 2012).

To date, inhibition of the renin-angiotensin-aldosterone system (RAAS) is the crucial strategy utilized to slow deterioration of renal functional decline. However, this influences intrarenal and extrarenal hemodynamics, and only secondarily reduces the development of renal fibrosis. It is primarily beneficial in patients with proteinuric renal disease, and at best, it delays the time to ESKD, leading to renal replacement therapy or death, by a factor of months. More recently, sodium-glucose linked transport inhibitors have been shown to reduce the development of end-stage kidney disease in patients with diabetic kidney disease (Ingelfinger and Rosen, 2019), and a recent trial in both diabetic and non-diabetic CKD was prematurely terminated in light of positive results in favor

of the SGLT2 inhibitor (ClinicalTrials.gov, 2020). However, a treatment gap remains, and novel therapies directed toward reducing the ultimate fibrotic response in the kidney are urgently needed to arrest the progression of CKD and improve the outcome of patients.

TRANSFORMING GROWTH FACTOR BETA-1 (TGF-β1)

TGF-β is the prototype of a family of secreted polypeptide growth factors. Three isoforms of TGF-β have been identified in mammals, including TGF-β1, TGF-β2 and TGF-β3 (Yu et al., 2003). All TGF-βs are synthesized as homodimeric proproteins together with the latency-associated peptide (LAP), which binds to the TGF-β homodimer to promote the formation of the latent TGF-β binding protein (LTBP) (Robertson et al., 2015). The synthesized complex, consisting of TGF-β dimer, LAP dimer, and LTBP, remains inactive and stored in the ECM (Hinz, 2015). LTBP serves as a localizer to interact with the ECM (Annes et al., 2003). LAP inhibits TGF-β activity by preventing TGF-β binding to its receptors (Annes et al., 2003; Hinz, 2015). This mechanism controls free and, therefore, active TGFβ tissue levels. To cleave the TGF-β complex into the active component and release active TGF-β, one or more of a wide range of proteases, including plasmin, matrix metalloproteinase (MMP) 2, and MMP9, thrombospondin, integrins, and the cationic independent mannose 6 phosphate receptor, are needed (Annes et al., 2003).

It is well accepted that overexpression of active TGF-β1 induces a fibrotic response in multiple organs, including the kidney (Sanderson et al., 1995). TGF-β1 is a well-characterized key mediator in the pathogenesis of tubulointerstitial fibrosis, due to its direct and indirect effect on various cells types (Roberts, 1998; Wang et al., 2005; Bottinger, 2007). The direct action of TGF-β1 includes the transition of cells to a fibroblastic phenotype and synthesis of profibrotic proteins, such as collagens and fibronectin (Border et al., 1990; Haberstroh et al., 1993; Wilson et al., 1993). TGF-β1 also facilitates an indirect fibrotic response, via accelerating apoptosis of resident healthy cells and promoting resident and infiltrating cells to increase ECM deposition (Lebrin et al., 2005; Das et al., 2014; Mack and Yanagita, 2015). Inhibiting TGF-β1 in animal models of kidney disease attenuates fibroblast activation and ECM accumulation (Moon et al., 2006; Russo et al., 2007; Murphy et al., 2012; McGaraughty et al., 2017).

The central signal transduction in response to TGF-β1 is mediated by two specific receptors, TGF-β type II receptor (TGFβRII) and the TGF-β type I receptor (TGFβRI). TGF-β1 firstly binds with TGFβRII in an active form (homodimers) and recruits the low-affinity receptor (TGFβRI) by the ligand-bound high-affinity receptor (TGFβRII) (Groppe et al., 2008). The activation of TGFβRI initiates receptor signaling (Xu et al., 2012a) and phosphorylates the substrates, the Smad proteins. Specifically, TGF-β1 signaling stimulates receptor-regulated Smad (R-Smad) phosphorylation. This is followed by translocation of R-Smads and the common mediator Smad (Co-Smad) complexes in the nucleus to regulate gene transcription (Moustakas et al., 2001). By contrast, inhibitory Smads (I-Smads) antagonize the activity of the R-Smads by preventing phosphorylation of R-Smads (Hill, 1999).

TGF-β1-SMAD PATHWAY

Smads separate into different classes with regards to their functions: two TGF-β R-Smads (Smad2 and Smad3), three bone morphogenetic protein (BMP) R-Smads (Smad1, Smad5, and Smad8), one Co-Smad (Smad4) and two I-Smad (Smad6 and Smad7) (Hill, 1999; Heldin and Moustakas, 2012).

R-SMADS

Smad2 and Smad3 are extensively studied in the TGF-β1 facilitated fibrotic response using various animal models and in human kidney disease, including diabetic (Isono et al., 2002; Fujimoto et al., 2003; Li et al., 2004; Chung et al., 2010; Chen et al., 2011) and obstructive nephropathy (Terada et al., 2002; Lan et al., 2003; Sato et al., 2003; Huang et al., 2008a; Chung et al., 2009), remnant kidney disease (Hou et al., 2005; Yang et al., 2010), hypertensive nephropathy (Wang et al., 2006), drug-associated nephropathy, and immunologically mediated glomerulonephritis (Ka et al., 2007; Huang et al., 2008b).

TGF-β1/Smad3 signaling mediates transcription of multiple downstream genes, such as the collagen chains ColIa1, ColIa2, ColIIIa1, ColaVa2, ColVIa1, and ColVIa3, and tissue inhibitor of metalloproteinases (TIMP)-1 (Verrecchia et al., 2001). The deletion of Smad3 in mice suppresses fibrosis in rodent models of kidney disease (Fujimoto et al., 2003; Sato et al., 2003; Zhou et al., 2010).

Relative to Smad3, the function of Smad2 in renal fibrosis is not fully elucidated. Because of the unavailability of Smad2 knock out (KO) mice, conditional kidney tubular epithelial cells Smad2 KO mice were generated by crossing the Smad2 floxed mouse with the kidney-specific promoter (Cadherin 16)-driven Cre transgenic mouse (Shao et al., 2002). Unexpectedly, deletion of Smad2 in tubular cells significantly enhances fibrosis, with an associated elevated Smad3 signaling in the UUO mouse model (Meng et al., 2010). Similarly, Smad2-/- fibroblasts have an increased fibrotic response (Meng et al., 2010). Additional evidence has shown that Smad3, but not Smad2, mediates fibrotic process (Wang et al., 2006; Yang et al., 2009, 2010; Chung et al., 2010; Zhou et al., 2010). Hence Smad2 and Smad3 may have distinct roles in mediating the fibrosis upon exposure to TGF-β1.

Among the R-Smads, BMP R-Smads (1, 5, 8) mediate the development of kidney and renal cell cancer (Oxburgh and Robertson, 2002; Blank et al., 2008; Markic et al., 2010). The BMP-7-Smad1/5/8 pathway has been shown to accelerate ECM deposition in the kidneys of unilateral ureteral obstruction (UUO) rats (Cao et al., 2015). The activin receptor-like kinase (ALK)-1 /Smad1/5 pathway may influence ECM protein expression in several cell types, such as rat myoblasts, hepatocytes, and human chondrocytes (Munoz-Felix et al., 2013). However, the role of BMP R-Smads in fibrotic disorders remains largely unknown.

CO-SMAD (SMAD4)

Smad4 promotes TGF-β1 signaling by dimerizing with R-Smads and facilitating nuclear translocation (Massague and Wotton, 2000; Gomez-Puerto et al., 2019). Deleting Smad4 from renal tubular cells alleviates renal fibrosis in a mouse model of UUO by suppressing Smad3 function (Meng et al., 2012). In mesangial cells, the loss of Smad4 inhibits TGF-β1 induced ECM accumulation (Tsuchida et al., 2003).

I-SMADS

Smad 6 and Smad7 are inhibitory mediators in the TGF-β1 signaling pathway. They provide a negative feedback loop through multiple mechanisms, including competing with R-Smads in activating the receptors by associating directly with TGFβRI (Hanyu et al., 2001; Nakayama et al., 2001), indirectly affecting the activity of TGFβRI by cooperation with BMPs (Murakami et al., 2003; Yan et al., 2009), interference in the formation of R-Smad/Co-Smad complex (Hata et al., 1998; Yan et al., 2016) and abolishing transcription in the nucleus (Lin et al., 2003; Zhang et al., 2007).

The deletion of Smad7 accelerates fibrogenesis in a number of mouse models, including UUO (Chung et al., 2009), diabetic (Chen et al., 2011), and hypertensive nephropathy (Liu et al., 2013). However, the importance of Smad6 in renal fibrogenesis is unclear.

NON-SMAD PATHWAYS

TGF-β1 also independently and directly activates other pathways, such as Ras/Raf/extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways, c-Jun N-terminal kinase (JNK), p38 MAPK signaling and Rho-like GTPase signaling pathways (Loeffler and Wolf, 2014).

TGF-β1 increases phosphorylation of tyrosine residues on TGFRs (I and II) and recruits ERK through Ras, Raf, and their downstream MAPK cascades. Specifically, ERK regulates target gene transcription through its downstream transcription factors in conjunction with Smads to control epithelial-mesenchymal transition (EMT) (Lee et al., 2007). ERK also regulates the activity of R-Smads, including Smad1, Smad2, and Smad3 (Kretzschmar et al., 1997, 1999; Funaba et al., 2002; Matsuura et al., 2005). Moreover, ERK is involved in the autoinduction of TGF-β1 via distinct transcriptional and translational mechanisms in tubular epithelial cells (Zhang et al., 2006). These studies suggest a dominant role of ERK in the non-Smad mediated transduction of TGF-β1.

The Rho-like GTPases, including RhoA, Rac, and Cdc42, play crucial roles in controlling dynamic cytoskeletal organization, cell motility, and gene expression through a variety of effectors (Jaffe and Hall, 2005). In addition to MAPK pathways, RhoA is a vital regulator, which can be activated by TGF-β1 via either Smad-dependent or independent pathways to promote stress fiber formation during EMT (Bhowmick et al., 2001a; Edlund et al., 2002).

JNK and p38 MAPK pathways are the best characterized non-Smad pathways involved in renal fibrosis. TGF-β1 can rapidly activate JNK and p38 MAPK via MAPK kinase (MKK) 4 and MKK 3/6, respectively (Frey and Mulder, 1997; Engel et al., 1999; Hanafusa et al., 1999; Hocevar et al., 1999; Sano et al., 1999; Bhowmick et al., 2001b; Yu et al., 2002). The activated JNK/p38 conjuncts with Smad2/3 to regulate apoptosis and EMT by controlling the activities of downstream transcription factors (Shibuya et al., 1998; Liao et al., 2001; Bakin et al., 2002; Yu et al., 2002; Edlund et al., 2003; Yamashita et al., 2008; Zhang, 2009). The phosphorylated JNK also regulates Smad 3 activity directly (Zhang, 2009; Grynberg et al., 2017).

RECEPTOR-INTERACTING SERINE/THREONINE-PROTEIN KINASE (RIPK) 3

The RIP kinase family contains seven members, each of which possesses a homologous kinase domain. To date, the functions of RIPK4–7 are poorly understood (Zhang et al., 2010). RIPK2 is a mediator of mucosal immunity. Extensive studies have clarified the importance and physiological roles of RIPK1 and RIPK3 in inflammation and cell death (Christofferson et al., 2014; Newton, 2015).

The RIPK3 gene is located on chromosome 14 in both humans and mice (Kasof et al., 2000; Shlomovitz et al., 2017). This gene encodes a 518 amino acid (aa) protein with a molecular mass of 57 kDa in humans (Sun et al., 1999), whereas it encodes a 486 aa protein of 53 kDa in mice (Pazdernik et al., 1999). RIPK3 is a threonine/serine protein kinase that shares almost 30% identity and 60% with the other two RIPK members, RIPK1 and RIPK2 (Sun et al., 1999; Yu et al., 1999). Compared with RIPK2, RIPK3, and RIPK1 have a unique C-terminal RIP homotypic interaction motif (RHIM) (Sun et al., 1999), which enables homotypic protein interactions (Sun et al., 2002).

To date, several phosphorylation sites of RIPK3 have been identified. The human Ser227 site (Thr231/Ser232 for mouse RIPK3) and Ser199 site (Ser204 in mouse) are particularly crucial for the activation of its downstream substrate in the necroptosis pathway, mixed-lineage kinase domain-like (MLKL) (He et al., 2009; Sun et al., 2012; Chen et al., 2013; McQuade et al., 2013).

RIPK3 IN NECROPTOSIS

In response to physiological signals and pathological stimuli, cell death is crucial to maintaining homeostasis. To date, several types of cell death have been identified. Among them, necrosis is a type of cell death characterized by loss of intracellular contents and the triggering of subsequent inflammatory response. For many years, necrosis was considered to be accidental and, therefore, unregulated cell death (Proskuryakov et al., 2003; Festjens et al., 2006; Zong and Thompson, 2006; Vandenabeele et al., 2010). The recognition that necroptosis is programmed necrosis that has provided new insights into

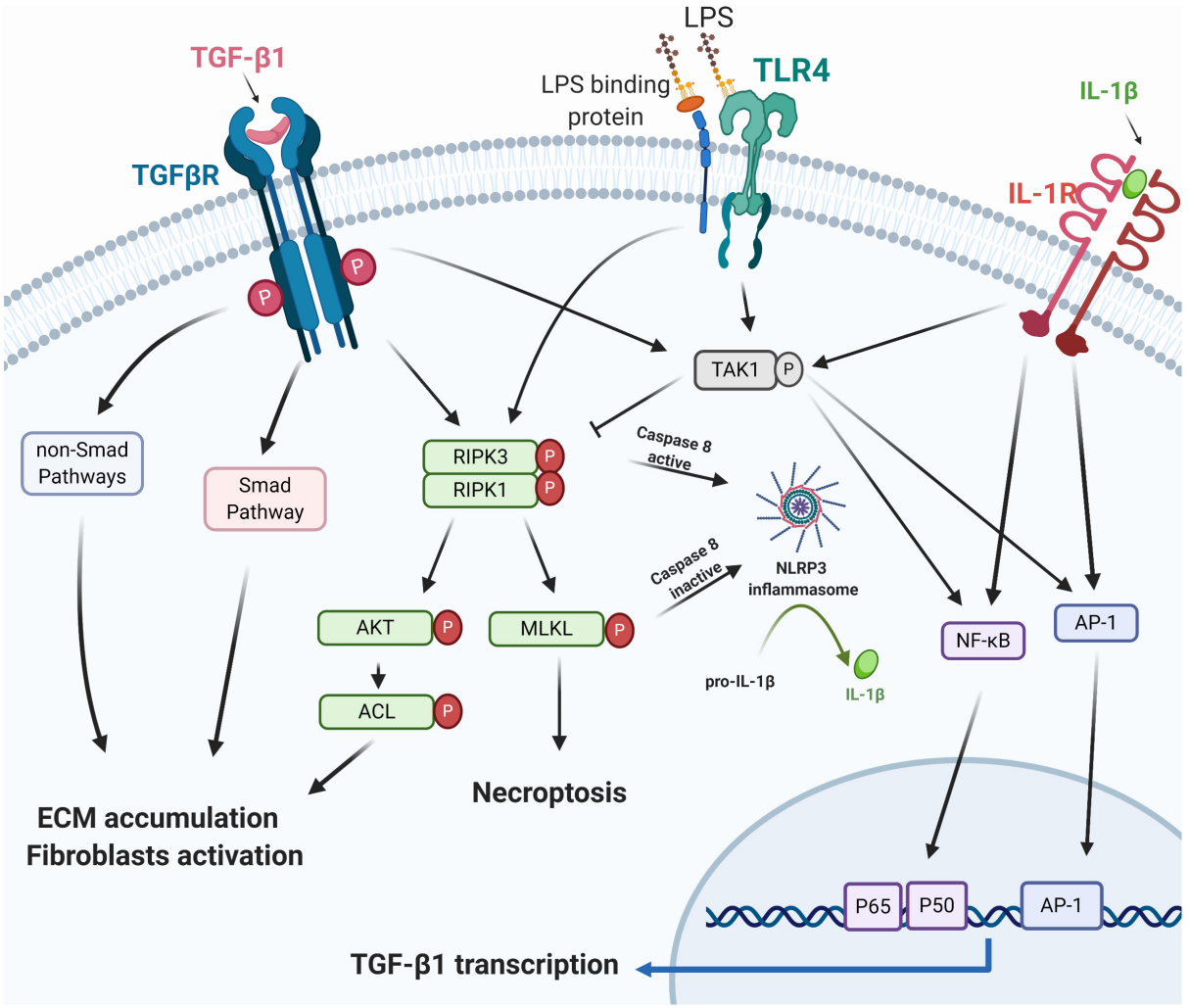

FIGURE 1 | RIPK3 and TGF-β1. TGF-β1 exhibits its biological function via the canonical Smad/non-Smad pathways or TAK1/necrosome/AKT/ACL signaling to mediate ECM accumulation and fibroblast activation. Necroptosis or RIPK3 facilitates NLRP3 inflammasome assembly, triggers mature IL-1β secretion, and promotes the TGF-β1 transcription via the IL-1β regulated AP-1 and NFκB pathway (Lee et al., 2006). IL-1β, TGF-β, and TLR signaling pathways all activate TAK1 and its regulated inflammatory mediators (Kim and Choi, 2012; Fechtner et al., 2017). RIPK3, Receptor-interacting serine/threonine-protein kinase 3; TGF-β1, transforming growth factor beta-1; TAK1, TGF-β-activated kinase 1; AKT, protein kinase B; ACL, ATP citrate lyase; ECM, extracellular matrix; TLR4, toll-like receptor 4; LPS, lipopolysaccharides; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; IL-1β, interleukin-1β; AP-1, activator protein 1; NFκB, nuclear factor-kappa B. Created with BioRender.com.

necrosis-initiated cell death. Necroptosis is mediated by the dimerization of RIPK1-RIPK3, which forms the necrosome associated with the downstream expression of MLKL (Li et al., 2012; Newton and Manning, 2016; Weinlich et al., 2017). RIPK1 and RIPK3 both possess RHIM domains, with bilateral interaction between RIPK1 and RIPK3 (Li et al., 2012; Mompean et al., 2018). Subsequently, the necrosome facilitates the aggregation of phosphorylated RIPK3 and phosphorylation of MLKL by RIPK3 (Li et al., 2012; Newton and Manning, 2016; Weinlich et al., 2017). The phosphorylated MLKL translocates to the cell membrane and thus promotes necroptosis (Li et al., 2012).

In contrast to the obligatory involvement of RIPK3, RIPK1 is not always required to cause necroptosis. The M45-mutant strain of murine cytomegalovirus (MCMV) infection causes RIPK3 activation in the absence of activation of RIPK1 (Upton et al., 2010, 2012). There is also evidence that the RHIM-containing protein (ICP) 6 protein of herpes simplex virus 1 recruits RIPK3 directly and independent of RIPK1 (Wang et al., 2014b). In addition, RIPK1 may have dual influences on cell death by both promoting necroptosis and protecting cells from death under certain conditions (Filliol et al., 2017).

RIPK3 IN KIDNEY FIBROSIS

To date, few studies have investigated the role of RIPK3 in kidney fibrosis, and most of them have not dissected the role of RIPK3

from necroptosis. The majority of these studies used acute injury models where there is known increased necroptosis.

The RIPK1 inhibitor necrostatin-1 reduces renal ischemia and reperfusion injury (Shen et al., 2019) and sepsis-associated acute kidney injury (Dong et al., 2018). Lacking RIPK3 protects kidney tubular injury in the sepsis-induced acute kidney injury (Sureshbabu et al., 2018). The deletion of either RIPK3 or MLKL prevents kidney damage in the oxalate crystal-induced AKI (Mulay et al., 2016) and kidney ischemia-reperfusion injury mouse models (Moerke et al., 2019). However, blockade of MLKL in folic acid-induced AKI (Martin-Sanchez et al., 2017) and 7-day unilateral ureteral obstruction (UUO) models (Imamura et al., 2018) failed to protect against kidney injury. Hence blockade of RIPK1, RIPK3, or MLKL may have differential benefits depending on the model under study.

Necrostatin-1 reduces interstitial fibrosis in a mouse model of UUO (Xiao et al., 2017) by inhibiting necroptosis, associated with lower protein and mRNA expression of RIPK, RIPK3, and MLKL and TGF-β1. In parallel, collagen accumulation and fibroblast activation (Xiao et al., 2017) were reduced. This study showed the integral relationship between necroptosis and TGF-β1 activation leading to renal fibrosis.

RIPK3 IN APOPTOSIS AND INFLAMMATION

Under certain conditions, RIPK3 also serves as a pro-apoptosis adaptor, which recruits RIPK1 and Fas-associated protein with death domain (FADD) to activate caspase 8 and thus apoptosis. This effect relies on the involvement of caspase 8 when RIPK3 is inactive, or MLKL is absent (Mandal et al., 2014; Newton et al., 2014). RIPK3 deficient animals develop normally, whereas mice expressing catalytically inactive RIPK3 ^{D161N} die around embryonic day 11.5 from increased RIPK1- and caspase-8-dependent apoptotic injury (Newton et al., 2014). Similar effects are observed in a study using compounds that selectively inhibit RIPK3 interaction with caspase 8 independent of pro-necrotic kinase activity and MLKL (Mandal et al., 2014).

Recent studies also identify that RIPK3 is an essential mediator in NOD-, LRR- and pyrin domain-containing protein (NLRP) 3 inflammasome formation (Wang et al., 2014a; Lawlor et al., 2015; Chen et al., 2018; Guo et al., 2019). In LPS-treated mouse bone marrow-derived dendritic cells, activation of the NLRP3 inflammasome was initiated by necroptosis (Kang et al., 2014). In a podocyte cell line, the RIPK3 specific inhibitor GSK'872 blocked both the necroptosis pathway and the NLRP3 inflammasome activation (Guo et al., 2019). These indicate the RIPK3 mediated NLRP3 inflammasome can be dependent of the necroptosis. Specifically, RIPK3–MLKL triggers NLRP3 activation when the activation of caspase 8 is reduced (Lawlor et al., 2015). In this setting, RIPK3 activity is required. RIPK3 can also promote NLRP3 inflammasome independent of the MLKL and RIPK3 kinase activity when caspase 8 is active (Lawlor et al., 2015). Collectively, RIPK3 mediated NLRP3 activation can be in both a necroptosis-independent and -dependent manner, depending on the levels of caspase-8 activity.

RIPK3 AND TGF-β1

Necrostatin-1 in a mouse UUO model attenuates IL-1β, TNF-α, and TGF-β1 levels (Xiao et al., 2017). In contrast, another study demonstrated that RIPK3 deficiency in the same UUO model prevents renal fibrosis without altering the mRNA expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and TGF-β1 (Imamura et al., 2018). These conflicting results may indicate that IL-1β, TNF-α, and TGF-β1 are "co-existing" as the downstream cytokines in the RIPK3 signaling. Mature IL-1β, the critical effector of the NLRP3 inflammasome (Jo et al., 2016; Kelley et al., 2019), has been demonstrated to increase TGF-β1 transcription (Lee et al., 2006). As described above, RIPK3 can regulate NLRP3 inflammasome (Lawlor et al., 2015). We, therefore, hypothesize that the "on/off switch" of RIPK3 in regulating TGF-β could be NLRP3 inflammasome activation (**Figure 1**). The trigger to promote NLRP3 inflammasome activation in RIPK3 signaling remains to be elucidated.

In vitro studies using NIH 3T3 fibroblasts, RIPK3 targeted siRNA, the RIPK3 inhibitor GSK'872 or necrostatin-1 abolished TGF-β dependent ECM and α- smooth muscle actin (α-SMA) expression (Imamura et al., 2018), suggesting that the necrosome RIPK1/RIPK3 is a downstream regulator of TGF-β in stimulating ECM deposition and fibroblast activation. The necrosome/RIPK3- Protein Kinase B (AKT)-dependent ATP citrate lyase (ACL) pathway has previously been identified as downstream of TGF-β (Imamura et al., 2018).

TGF-β-activated kinase 1 (TAK1), interacts with TGF-β1 and contributes to the development and progression of organ fibrosis through TGF-β1/TAK1/MKK3/p38MAPK, TGF-β1/TAK1/MKK4/JNK, and TGF-β1/TAK1/ NFκB pathways (Kim and Choi, 2012; Biesemann et al., 2015; Li et al., 2017; Wu et al., 2017; Zhou et al., 2018; Bao et al., 2019). Few studies of TAK1 on necroptosis have been reported, and these mostly report on RIPK1-dependent cell death. A recent study explored TAK1 regulated endothelial necroptosis in tumor progression and

TABLE 1 | The RIPK3 inhibitors (Martens et al., 2020).

Inhibitor types	*Inhibitors*
Type I	Dabrafenib
	GSK'843
Type II	Sorafenib
	Ponatinib
	HS-1371
	GSK'067
	GSK'074
	Inhibitor 9
	Inhibitor 18
Unclassified	DCC-2036
	GSK'840
	GSK'872
	ZINC71828321
	ZINC72474191
	ZINC72454060
	GW'39B

showed that TAK1 deficiency increases necroptosis and RIPK3 expression in endothelial cells in both *in vitro* and *in vivo* studies (Yang et al., 2019). Endothelial knockout of RIPK3 or MLKL abolishes the effects of TAK1-deficiency on the enhancement of necroptosis, suggesting an inhibitory role of TAK1 on necroptosis (Yang et al., 2019). TAK1 may, therefore, negatively regulate the necroptosis in the TGF-β1 signaling network (**Figure 1**).

IMPLICATIONS FOR ANTI-FIBROTIC THERAPY

TGF-β1-specific, humanized, neutralizing monoclonal antibody added to RAAS inhibitors failed to slow the progression of diabetic nephropathy (Voelker et al., 2017). Therefore, targeting the full spectrum of downstream TGF- β1 signaling to prevent renal disease is unlikely to be fruitful, and the development of blockers of more targeted downstream pathways, such as the RIPK3/necroptotic pathway may be more beneficial.

To date, several small-molecule compounds (Li et al., 2014; Fauster et al., 2015; Martens et al., 2017, 2020; Park et al., 2018; Pan et al., 2019; Hart et al., 2020) have been developed to inhibit RIPK3 activity, providing an impressive toolbox for the investigation of the role of RIPK3 in diverse tissues. These inhibitors of RIPK3 can be divided into three types: ATP mimetic inhibitors targeting the active ATP-binding site in the kinases located between two catalytic domain lobes (type I), targeting the inactive states (type II), and unclassified inhibitors (Muller et al., 2015; Martens et al., 2020; **Table 1**).

GSK'872 is the most widely used cell-permeable inhibitor of the RIPK3-selective kinase, with >1,000-fold selectivity over a vast majority of more than 300 other kinases (Kaiser et al., 2013). GSK'872 binds the kinase domain and inhibits kinase activity with high specificity, targeting a broad range of pro-necrotic stimuli (Mandal et al., 2014) and has been used to specifically inhibit RIPK3 (Lu et al., 2017; Chen et al., 2018; Imamura et al., 2018).

The serine/threonine kinase B-Raf ^{V600E} inhibitor dabrafenib is the only type I RIPK3 inhibitor approved for clinical use (Rheault et al., 2013; Li et al., 2014; Sugaya et al., 2019). Previous studies have reported that dabrafenib is a selective RIPK3 inhibitor in various models, including human hepatocytes (Li et al., 2014), mouse models of acetaminophen-caused liver injury (Li et al., 2014), and ischemic brain injury (Cruz et al., 2018). In addition, dabrafenib is a well-known inhibitor of B-Raf, which suppresses the downstream Ras/Raf/ERK/MAPK pathway (Spagnolo et al., 2014), which has been approved for clinical use for the treatment of non-small cell lung cancer expressing B-Raf ^{V600E} mutations and in melanoma (Odogwu et al., 2018). Inhibition of Raf kinase has found to attenuate renal fibrosis (Xu et al., 2012b; Chen et al., 2019).

Collectively, inhibition of RIPK3 is a promising anti-fibrotic strategy. RIPK3 facilitates necrosome and necroptosis. RIPK3 stimulates downstream activation of TGF-β1 cascades and regulates TGF-β1 transcription through NLRP3 inflammasome activation. Given inhibitors of RIPK3 are already approved for use in patients with non-small cell lung cancer and melanoma, an accelerated route to market in patients with CKD should be available if early phase clinical studies prove positive.

AUTHOR CONTRIBUTIONS

YS wrote the manuscript. YS, CP, XC, and CH provided the critical discussion of the manuscript. YS and CP revised the manuscript.

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Figure 1 was created with BioRender.com.

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REVIEW
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The Post-translational Modifications of Smurf2 in TGF-β Signaling

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Smad ubiquitin regulatory factor 2 (Smurf2), an essential negative regulator of TGF-β signaling, ubiquitinates TGF-β receptors (TβRs) and Smad proteins, inducing their proteasomal degradation. Smurf2 plays crucial roles in regulating TGF-β signaling and maintaining normal cellular functions and tissue homeostasis; dysfunction of Smurf2 triggers abnormal TGF-β signaling in pathological states. Smurf2 has been reported as a potentially strong candidate for targeting therapies for related diseases. Recent work has begun to focus on the regulation of Smurf2 itself, and emerging evidence indicates that Smurf2 is regulated by post-translational modifications (PTMs) mechanisms. These mechanisms predominantly regulate the expression level and E3 ligase activity of Smurf2, strongly suggesting that this protein contributes to complicated roles under multiple pathophysiological conditions. In this review, we cover some significant and novel mechanisms of the PTMs that potentially control Smurf2 participation in TGF-β signaling, including ubiquitylation, SUMOylation, neddylation, phosphorylation, and methylation in order to provide a broad view of the depth and sophistication of Smurf2 function in TGF-β regulation, as well as perspectives for future therapeutic directions for its associated diseases.

Keywords: post-translational modifications, Smurf2, SUMOylation, ubiquitylation, neddylation, phosphorylation, methylation

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INTRODUCTION

Signaling mediated by the transforming growth factor β (TGF-β) family controls many cellular responses and diverse biological processes, such as cell growth, differentiation, adhesion, migration, and apoptosis (Drabsch and ten Dijke, 2012). The TGF-β signaling transduction network entails a complex series of protein interactions, including the activation of serine/threonine kinase receptors, SMAD protein phosphorylation and mobilization to the nucleus, and subsequent regulation of transcription factors that modulate target gene expression. Furthermore, dysregulation of TGF-β signaling can lead to various pathologies such as fibrosis, cardiovascular pathology, and cancer (Eichhorn et al., 2012; Iyengar et al., 2015; Shu et al., 2016; He et al., 2017; Chanda et al., 2018). Smad ubiquitin regulatory factor 2 (Smurf2), a HECT (homologous to the E6-accessory protein C-terminus)-type E3 ubiquitin ligase located mainly in the nucleus, has been demonstrated to play pivotal roles in the negative regulation of TGF-β signaling (David et al., 2013). Typically, Smurf2 translocates out of the nucleus in response to the activation of TGF-β receptor and forms a complex with I-Smads to ubiquitinate TGF-β type I receptors (TβRI) and R-Smads, thereby leading to their proteasomal degradation, and subsequently attenuating the TGF-β signaling.

Other biological functions of Smurf2 have been reported in addition to the regulation of TGF-β signaling. Emerging evidence has demonstrated that Smurf2 contributes to genomic stability, cell polarity, tissue homeostasis, and tumorigenesis (Koganti et al., 2018). Smurf2 acts as both a tumor promoter and suppressor. Knock out of Smurf2 results in tumorigenesis in mice (Ramkumar et al., 2012). In contrast, functional Smurf2 inhibits cancer cell proliferation and tumorigenesis via ubiquitination and degradation of several critical cellular proteins, such as Sirtuins (Yu et al., 2020), SIRT1 (Yu et al., 2019), ChREBP (Li et al., 2019), and RNF20 (Manikoth Ayyathan et al., 2020). However, some studies have documented evidence that Smurf2 functions as a tumor promoter rather than a tumor suppressor under some specific circumstances (David et al., 2013). Additionally, high levels of Smurf2 expression have been found in association with several types of cancer (Jin et al., 2009; Klupp et al., 2019) and were correlated with poor prognosis (Fukuchi et al., 2002; Klupp et al., 2019). However, the mechanism underlying these dual roles for Smurf2 in cancer remain poorly understood. Most recently, Emanuelli et al. (2019) found that altered expression and localization may potentially diminish its tumor-suppressive activities. Elucidating the regulatory mechanisms Smurf2 activity and expression is imperative for understanding its role in multiple pathophysiological conditions.

An increasing number of studies have demonstrated that Smurf2 activity and expression are regulated by a series of post-translational modifications (PTMs), including ubiquitylation, phosphorylation, methylation, SUMOylation, and neddylation (Xu et al., 2012). Given that PTMs not only regulate the activity of Smurf2 to control TGF-β signaling but are also involved in the development of several diseases, it is an urgent priority to resolve the underlying mechanisms of how PTMs affect Smurf2 function for identification of potential therapeutic targets. Herein, we review the PTMs that have been thus far reported to affect Smurf2 activity and stability.

STRUCTURE AND FUNCTIONS OF SMURF2

First discovered in 2000, Smurf2 is a member of the Neural Precursor Cell-expressed Developmentally Down-regulated Protein 4 (NEDD4) subfamily (Kuratomi et al., 2005). The human *smurf2* gene, located on chromosome 17, and Smurf2 protein contain three regions: a C2 domain at the N-terminal, three WW domains containing two conserved tryptophan residues each, and a highly conserved HECT catalytic domain at the C-terminal (**Figure 1**; Lin et al., 2000). Moreover, the WW domain is responsible for substrate recognition through specific binding to a PPXY motif (Zhu et al., 1999). In the resting state, e C2 domain associates with the HECT domain on Smurf2 to prevent the WW domain from interacting with substrates. This mechanism potentially contributes to maintaining the stable expression of Smurf2 in cells. Furthermore, Smurf2 requires adaptor proteins to facilitate the induction of its active state to proceed with enzymatic interactions with its substrates (Wiesner et al., 2007). To date, many adaptors have been found to interact and promote the function of Smurf2. The first reported, canonical protein adaptor is Smad7. Smad7 binds to Smurf2, forming a complex, to initiate Smurf2 translocation out of the nucleus for targeting of the TGF-β receptor complex for degradation (Kavsak et al., 2000).

As a C2-WW-HECT type E3 ubiquitin ligase, Smurf2 was described as a negative regulator of TGF-β signaling, and a substantial number of reports subsequently demonstrated that Smurf2 primarily targets signaling components and downstream protein expression induced by TGF-β. For instance, Smurf2 not only associates with the I-Smads to down-regulate type I TGF-β receptor (TβRI) and R-Smads (Kavsak et al., 2000; Lin et al., 2000; Zhang et al., 2001) but also degrades SnoN by assembling a complex with Smad2 (Bonni et al., 2001). Moreover, TGF-β was shown to up-regulate the transcription level of Smurf2, thus generating a negative feedback loop for TGF-β signaling (Ohashi et al., 2005). Smurf2 and Smad7 are the strongest negative regulators of TGF-β (Wegner et al., 2012). Notably, the negative feedback loop can be disrupted by Ring finger protein 11 (RNF11) activity, which is overexpressed in cancer cells. RNF11 binds directly to Smurf2, preventing the formation of the Smad7-Smurf2 complex, resulting in constitutive induction of TGF-β signaling (Malonis et al., 2017). This mechanism has major implications for the role Smurf2 in related diseases, such as pancreatic and breast cancer (Seki et al., 1999; Subramaniam et al., 2003).

Previous studies have also demonstrated that Smurf2 is autoinhibited by its C2 domain. The C2 domain interacts with the HECT domain via the catalytic cysteine, thereby inhibiting the formation of the ubiquitin thioester between Smurf2 and its substrates. Notably, Smad7 has been shown to antagonize this process to activate Smurf2 (Wiesner et al., 2007). A mechanistic study also found that, in some circumstances, the Smurf2 WW1 domain associates with the C2-WW1 linker and strongly enhances the C2-HECT interaction, effectively down-regulating its E3 ligase activity. Intriguingly, the WW domain in Smurf1 does not exert this effect. To better understand the role of the WW1 domain in Smurf2, a customized Smurf1 with an additional Smurf2 WW1 domain and a recombinant Smurf2 lacking the WW1 domain were used to determine that Smurf1 carrying a Smurf2 WW1 domain exhibited auto-inhibition, while deletion of the WW1 domain led to Smurf2 activation. The results indicated that the WW1 domain in Smurf2 is

Abbreviations: TGF-β, Transforming growth factor β; PTM, Post-translational modification; Smurf2, Smad ubiquitin regulatory factor 2; TβR, TGF-β receptor; HECT, Homologous to the E6-accessory protein C-terminus; RNF, RING finger protein; ChREBP, Carbohydrate response element-binding protein; SIRT1, The NAD-dependent deacetylase sirtuin 1; FBXL15, F-box and LRR domain-containing protein 15; USP, Ubiquitin-specific protease; NEDD4, Neural Precursor Cell-expressed Developmentally Down-regulated Protein 4; RNF11, Ring finger protein 11; TRAF4, Tumor necrosis factor receptor-associated factor 4; TRB3, Tribbles homolog 3; TTC3, Tetratricopeptide repeat domain 3; SUMO, Small ubiquitin-like modifier; PIAS3, The protein inhibitors of activated STATs 3; Erk5, Extracellular signal-regulated kinase 5; HGF, Hepatocyte growth factor; PRMT1, Protein arginine methyltransferase 1; Nedd8, Neural precursor cell expressed developmentally downregulated protein 8; UBL, Ubiquitin-like protein; CRL, Cullin-RING E3 ligase.

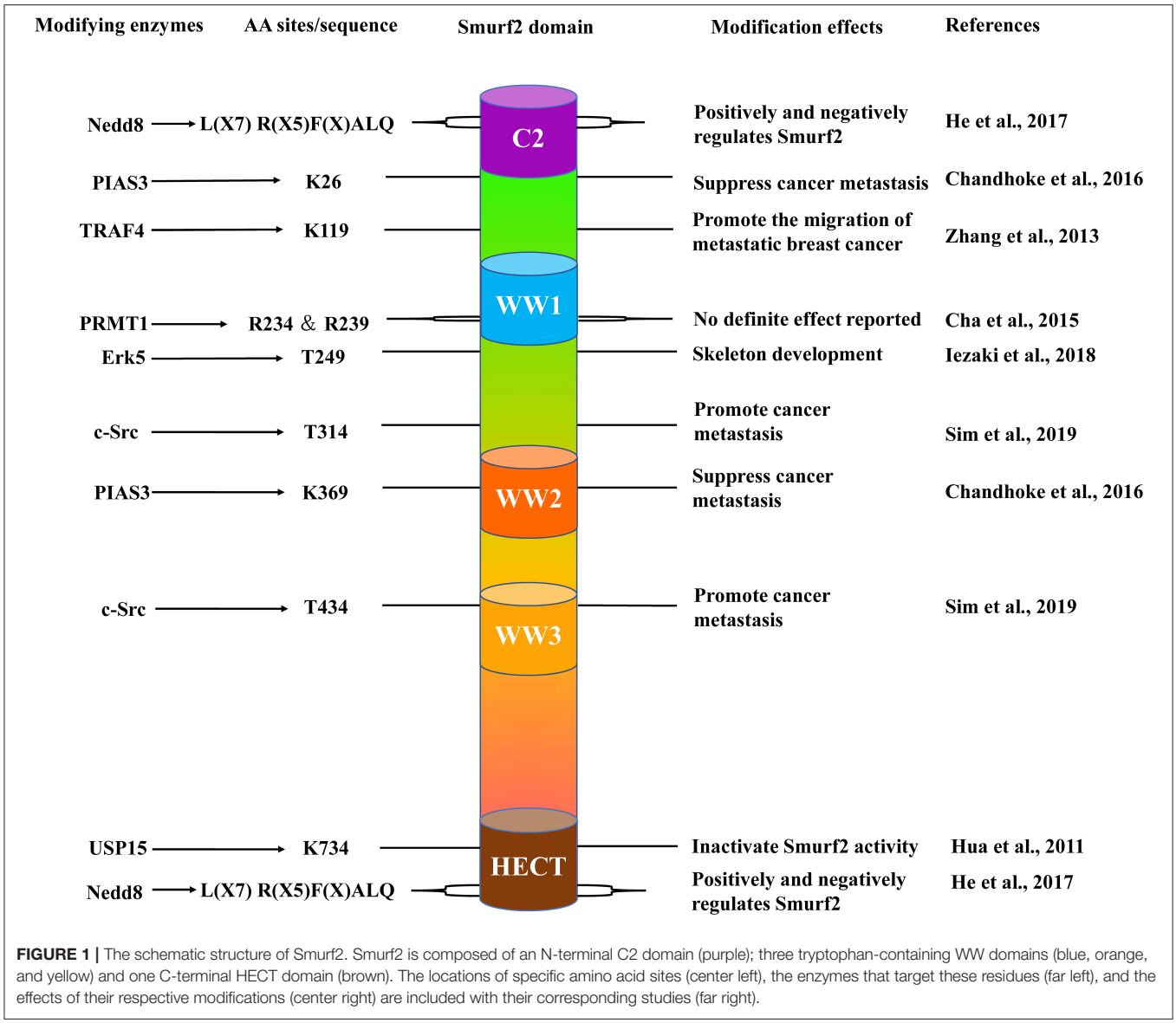

FIGURE 1 | The schematic structure of Smurf2. Smurf2 is composed of an N-terminal C2 domain (purple); three tryptophan-containing WW domains (blue, orange, and yellow) and one C-terminal HECT domain (brown). The locations of specific amino acid sites (center left), the enzymes that target these residues (far left), and the effects of their respective modifications (center right) are included with their corresponding studies (far right).

essential for its autoinhibition (Ruetalo et al., 2019). In agreement with this finding, in bladder cancer, the C2-HECT interaction between Smad7 and Smurf2 was prevented by an abnormal PTM, a phenomenon which we discuss in further detail below (Sim et al., 2019).

UBIQUITYLATION AND DEUBIQUITYLATION OF SMURF2

Ubiquitylation is highly conserved among animal organisms and is fundamental for the regulation of protein stability. As an E3 ligase, Smurf2 can polyubiquitinate TβRI as well as Smad2/3 to attenuate TGF-β signaling. Similarly, Smurf2 is also subjected to negative regulation by ubiquitylation via other E3 ligases, such as tumor necrosis factor receptor-associated factor 4 (TRAF4) (Zhang et al., 2013), Tribbles homolog 3 (TRB3) (Hua et al., 2011) and tetratricopeptide repeat domain 3 (TTC3) (Kim et al., 2019). A recent study revealed that Smurf2 is both the substrate and the direct target of TRAF4 through TRAF4 interactions with the Smurf2 C2 and WW domains. Overexpression of wild-type TRAF4 in HEK-293T cells was found to significantly enhance the polyubiquitylation of Smurf2, while TRAF4 deletion stabilized Smurf2, thus suggesting that TRAF4 contributes to polyubiquitylation and degradation of Smurf2 (Zhang et al., 2013).

Similar to TRAF4, the silencing of TRB3 led to the up-regulation of Smurf2 protein levels, but not its mRNA levels. In contrast, TRB3 overexpression decreased Smurf2 protein levels (Hua et al., 2011). Furthermore, immunoprecipitation assays confirmed that TRB3 promoted Smurf2 ubiquitylation and degradation. In addition, TTC3 was found to interact

with the catalytic domain of Smurf2, directly triggering Smurf2 ubiquitylation (Kim et al., 2019). Another report on Smurf2 degradation found that F-box and LRR domain-containing protein 15 (FBXL15) targeted Smurf2, leading to its ubiquitylation and degradation (Cui et al., 2011). Additionally, Smad7 was shown to induce Smurf2 E3 ligase activity as well as mediate Smurf2 autoubiquitylation and degradation via interaction with the HECT domain (Ogunjimi et al., 2005).

Deubiquitinating enzymes function in the reversal of the ubiquitylation process. For example, ubiquitin-specific protease 15 (USP15) can directly deubiquitinate Smurf2, thus causing the loss of Smurf2 catalytic activity (Iyengar et al., 2015). Further studies found that USP15 targets the essential catalytic site residue, Lysine 734, for deubiquitination (Iyengar et al., 2015). Notably, unlike USP15, USP11 appears to indirectly enhance the ubiquitylation of Smurf2, although the mechanism remains unclear (Iyengar et al., 2015).

Accumulating evidence indicates that ubiquitylation and degradation of Smurf2 promote the development of some diseases, such as fibrosis and cancer. A recent study in HepG2 cells showed that TRB3 promoted cancer cell migration and invasion through enhancement of Smurf2 ubiquitylation (Hua et al., 2011). In BEAS-2B cells and NHLFs cells, TTC3 was found to induce Smurf2 proteasomal degradation by ubiquitination in a Lys48-linked manner, hence contributing to TGF-β-induced epithelial-mesenchymal transition (EMT) and myofibroblast differentiation (Kim et al., 2019). Moreover, TRAF4 was found to promote the migration of metastatic breast cancer through Lys48-linked ubiquitylation of Smurf2 at Lys 119 (Zhang et al., 2013). These findings strongly suggest that targeting Smurf2 may be a viable strategy for the treatment of its related diseases.

SUMOYLATION OF SMURF2

The small ubiquitin-like modifier (SUMO) system is a post-translational modification system associated with ubiquitylation. SUMOylation of Smurf2 was first observed at K26 and K369 by SUMO E2 Ubc9 and E3 enzyme, the protein inhibitors of activated STATs 3 (PIAS3) in NMuMG epithelial cells (Chandhoke et al., 2016). Moreover, the SUMOylation of Smurf2 was found to be reversed by sentrin-specific proteases (SENP) 1 and SENP2 but not SENP3, suggesting that SENP1 and SENP2 might be deSUMOylases for Smurf2 (Chandhoke et al., 2016).

SUMOylation modification has been shown to enhance the Smurf2-mediated induction of TβR degradation (Chandhoke et al., 2016). However, a Smurf2 double mutant carrying arginine replacements of Lysine 26 (K26R) and 369 (K369R) (Smurf2KdR), which was still capable of binding activated TβR similar to wild-type Smurf2, lost its ability to attenuate TGF-β signaling and failed to inhibit EMT. This finding suggested that the SUMOylation of Smurf2 is essential for its suppression of TGF-β-induced EMT.

Notably, Smad7 binds to Smurf2 through association with NTD–HECT, and thus promotes the autoubiquitylation of Smurf2 by recruiting E2s (Wiesner et al., 2007). Since KdR mutation has little effect on the interaction of Smurf2 to Smad7, ostensibly SUMOylation does not affect its autoinhibition (Kavsak et al., 2000; Wiesner et al., 2007; Ruetalo et al., 2019). Further investigation revealed that PIAS3 potentially maintained a non-invasive phenotype through Smurf2 SUMOylation in human MDA-MB-231 breast cancer cells, indicating an anti-metastatic activity of SUMOylated Smurf2 (Chandhoke et al., 2017).

PHOSPHORYLATION OF SMURF2

Phosphorylation is a prevalent PTM that regulates protein function. Akt was the first kinase identified to phosphorylate Smurf2, which led to the down-regulation of its protein levels through ubiquitin/proteasome-mediated degradation. However, the use of an anti-phospho-Akt substrate motif (RXX*/T*) antibody to detect Smurf2 phosphorylation in that study prevented the identification of specific phosphorylation sites (Choi et al., 2014). Recently, extracellular signal-regulated kinase 5 (Erk5) was found to phosphorylate Smurf2 at Thr249, thereby enhancing its ability to target Smad 1, Smad2, and Smad3 for ubiquitylation and proteasome-mediated degradation. Moreover, a Smurf2 T249A mutant, defective for phosphorylation by ERK5, was not able to induce Smad protein ubiquitylation, whereas a T249E mutation, which mimicked phosphorylation by ERK5, caused extensive Smad ubiquitylation, irrespective of the presence or absence of ERK5. These findings implied that, under certain conditions, ERK5-mediated phosphorylation is a prerequisite for ubiquitin E3 ligase activity by Smurf2 (Iezaki et al., 2018).

Work by other groups has shown that c-Src phosphorylated Smurf2 at Tyr314/Tyr434. This activity inhibited Smad7 binding and maintained Smurf2 in a closed, inactive conformation by promoting its own C2-HECT domain interaction. A conversion mutation of Tyr314/Tyr434 to glutamines, which mimicked phosphorylation by c-Src, completely abrogated Smurf2-mediated TβRI degradation. In contrast, a phosphorylation-defective mutant generated by conversion of the Tyr314/Tyr434 residues to phenylalanine nullified the ability of c-Src to downregulate Smurf2 activity (Sim et al., 2019). Taken together, these findings demonstrated that distinct phosphorylation patterns induced by various protein kinases result in different outcomes for the regulation of Smurf2.

METHYLATION OF SMURF2

Smurf2 was also found to be methylated by protein arginine methyltransferase 1 (PRMT1) (Cha et al., 2015), with methylation sites identified within the amino acid region 224-298, including residues Arg232, Arg234, Arg237, and Arg239. Among these four sites, Arg234 and Arg 239 are specific to PRMT1. Moreover, PRMT1 knockdown led to the up-regulation of Smurf2 protein levels, which implied that methylation of Smurf2 by PRMT1 is involved in the maintenance of Smurf2 stability. However, wild-type PRMT1 overexpression or catalytic inactivation of PRMT1

exerted no detectable effects on the inhibitory role of Smurf2 in TGF-β signaling (Cha et al., 2015).

NEDDYLATION OF SMURF2

Protein neddylation is an essential biological process in which Nedd8 (neural precursor cell expressed developmentally downregulated protein 8), a ubiquitin-like protein, is activated by Nedd8 E1 and E2 enzymes and then conjugated to lysine residues in the target protein by Nedd8 E3 enzyme (Kamitani et al., 1997; Rabut and Peter, 2008; Zhou et al., 2018). In addition to the cullin-RING E3 ligase (CRL) family members, which are the most widely studied substrates known to be activated by neddylation, an increasing number of non-cullin proteins have been reported to be modified by Nedd8 (Xie et al., 2014; Enchev et al., 2015; Shu et al., 2016; He et al., 2017; Zhou et al., 2018).

Both Smurf1 and Smurf2 were found to be modified by the neddylation system (He et al., 2017). For Smurf1, covalent binding to Nedd8 results in a Nedd8-thioester intermediate, which consequently causes the neddylation of multiple lysine residues, notably in the C2 and HECT domains, as well as in the WW-HECT linker. However, Smurf2 is neddylated primarily at sites in the HECT region. Recently, a conserved non-covalent Nedd8 binding sequence, L(X7)R(X5)F(X)ALQ, was verified in the catalytic HECT domain of both Smurf1 and Smurf2. Moreover, the conversion of these conserved residues to alanine in both the N- and C-lobes of the Smurf2 HECT domain prevented its interaction with Nedd8 and attenuated its ability to induce Smad3 degradation. These results suggested that the non-covalent binding with Nedd8 is essential for Smurf2 regulation of BMP/TGF-β signaling. Intriguingly, neddylation promotes Smurf2 degradation while also enhancing its E3 ligase activity (Shu et al., 2016; He et al., 2017).

In addition, Nedd8 overexpression was shown to significantly increase the poly-ubiquitylation of Smurf2, thus enhancing its turnover by proteasomal degradation. In contrast, Smurf2 ubiquitylation was unaffected by overexpression of a Nedd8 ΔGG mutant, which lacked the ability to covalently conjugate Smurf2. Moreover, neddylation also promoted the ubiquitylation of a ligase-inactive mutant of Smurf2, which differed from Smurf1 due to its neddylation-augmented auto-ubiquitylation (Shu et al., 2016). Furthermore, *in vitro* experiments demonstrated that Smurf2 was effectively neddylated in the absence of any other Nedd8 E3 ligase, strongly suggesting that Smurf2 itself is a potential neddylation E3 ligase, and which supports its autoneddylation. However, *in vivo* neddylation assays with HA-Nedd8 and Myc-tagged-Smurf2 mutants carrying alanine conversions of each cysteine residue in the HECT domain, co-transfected into failed to identify the responsible, active site since all mutants were neddylated to the same extent as wild-type Smurf2 (Shu et al., 2016). Given that Smurf1 was demonstrated to function as a Nedd8 ligase and also to interact with Smurf2, it is possible that the above mentioned Myc-tagged-Smurf2 mutants were potentially neddylated by Smurf1, although this hypothesis requires further investigation.

CONCLUSION AND DISCUSSION

Smurf2 is a C2-WW-HECT-domain E3 ubiquitin ligase that contributes pivotal functions in a variety of physiological and pathological processes through the regulation of protein stability and TGF-β pathway signaling (Koganti et al., 2018). Multiple reports have demonstrated that Smurf2 undergoes extensive post-translational modifications that regulate its function and stability, and some of which have identified specific amino acid site targets for modification (**Figure 2**).

Based on the available evidence, the PTM system governing Smurf2 activity and accumulation involves a complex and sophisticated suite of interacting proteins. Together, several studies have demonstrated that TTC3, TRAF4, and TRB3 promote Smurf2 ubiquitylation and lead to its degradation. All three ubiquitylation enzymes reportedly promote tumor growth, invasiveness, or EMT. In light of results demonstrating that ubiquitylation is a reversible process, deubiquitylation enzymes have been proposed as a means of reducing ubiquitylation levels of Smurf2 for interference with tumor growth. However, it was also reported that USP11 increased the ubiquitination level of Smurf2 through an unknown mechanism, while the high expression of another deubiquitylation enzyme USP15 was correlated with high TGF-β activity (Iyengar et al., 2015).

Modification by PIAS3 results in Smurf2 SUMOylation, thereby enhancing its E3 ligase activity. In addition, Smurf2 was shown to be phosphorylated by Akt, Erk5, and c-Src, although activity by each of these proteins resulted in different regulatory outcomes. Specifically, Akt-mediated phosphorylation induced Smurf2 degradation, and ERK5-mediated phosphorylation increased its E3 ligase activity to degrade Smad proteins, while c-Src-mediated phosphorylation prevented Smurf2 activation by Smad7 and induced its proteasomal degradation. Furthermore, neddylation was revealed to promote both the ubiquitin ligase activity and the degradation of Smurf2, while Smurf2 methylation mediated by PRMT1 apparently exerted little effect on its function. These studies together present an intricate system of Smurf2 regulation by PTMs, and provide a strong basis for in-depth interrogation of the specific mechanisms by which Smurf2 dysregulation can lead to pathogenic outcomes. Given the complexity of PTM-mediated regulation of Smurf2 activity and stability, it is also unsurprising that there are endogenous mechanisms for reversal of these modifications, or that there is potentially substantial overlap or redundancy in protein interactions within this network that can lead to crosstalk or dysregulation.

Furthermore, multiple PTMs can positively or negatively influence each other's activity, i.e., through PTM crosstalk. Under certain conditions, PTM crosstalk may potentially function to maintain cellular proteostasis, that is, the capacity to adapt to stresses or stimuli while protecting the normal function of individual proteins (Frauke and Vertegaal, 2016). In the case of Smurf2, a body of work has shown that both phosphorylation and neddylation promote its ubiquitylation. However, it remains unclear if there are other mechanisms mediated by PTM crosstalk that can contribute to disease development and progression, and if so, by what underlying mechanisms they are controlled.

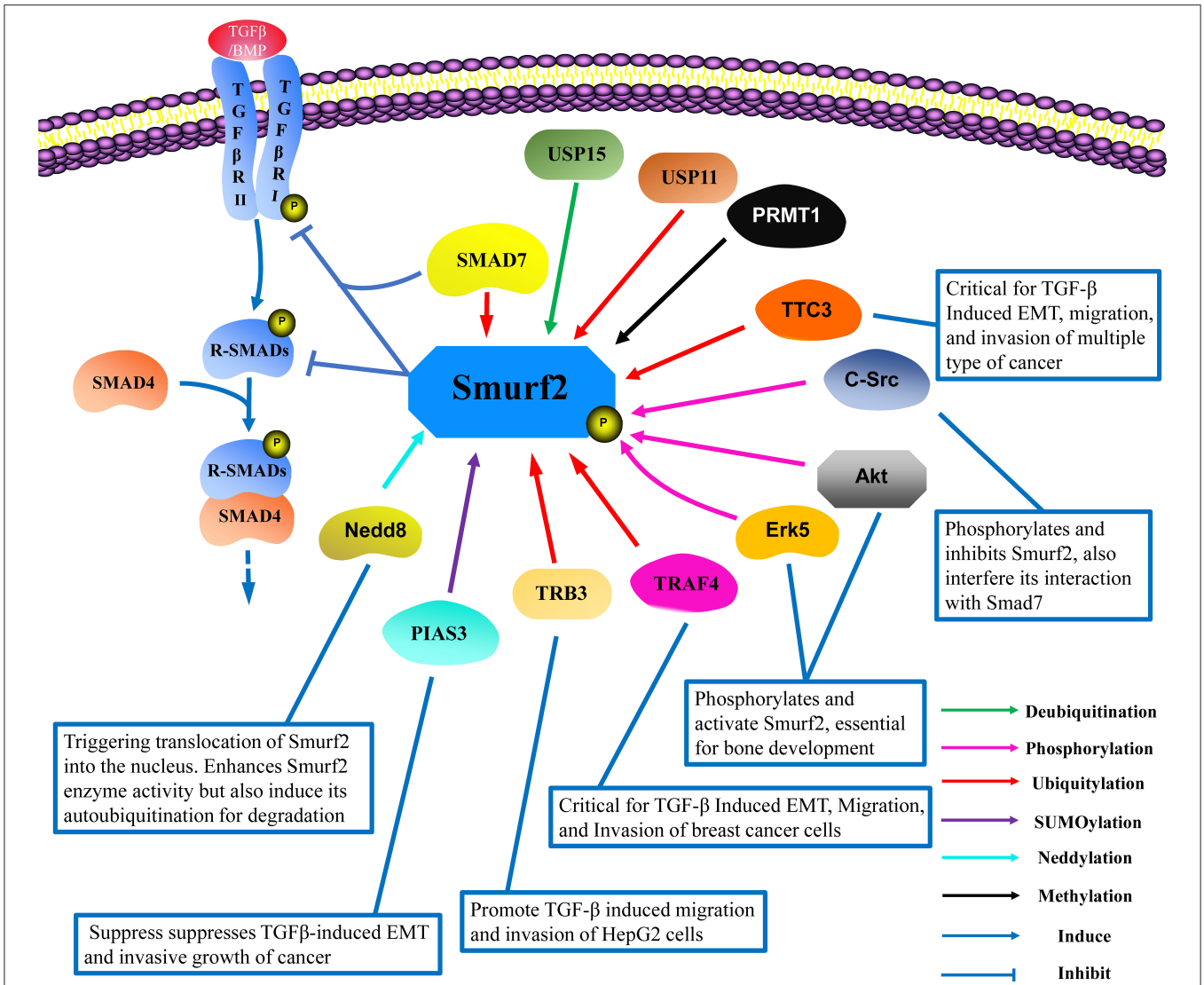

FIGURE 2 | The interacting protein network and post-translational modifications of Smurf2. Smurf2 works in conjunction with SMAD7 to degrade R-Smads and TβRI. To date, the reported post-translational modifications of Smurf2 include ubiquitylation (red lines), deubiquitylation (green lines), SUMOylation (purple lines), neddylation (light blue lines), phosphorylation (pink lines), and methylation (black lines). TRAF4, TRB3, and TTC3 induce Smurf2 degradation in a ubiquitin-dependent manner. USP15 can deubiquitinate Smurf2, while USP11 can increase the ubiquitylation level of Smurf2, although the mechanism remains unknown. Neddylation by Nedd8 enhances Smurf2 function, but also induces Smurf2 degradation. PIAS3 mediates the SUMOylation of Smurf2, which promotes Smurf2 attenuation of TGF-β signaling. Methylation by PRMT1 exerts no clear effect on Smurf2 functions. Phosphorylation by Erk5 and Akt enhance Smurf2-mediated interference with TGF-β signaling, which is essential for bone development. Additionally, phosphorylation by c-Src inhibits the activation of Smurf2 in cancer development, thus inducing epithelial mesenchymal transition (EMT). USP, Ubiquitin-specific protease; TRAF4, Tumor necrosis factor receptor-associated factor 4; TRB3, Tribbles homolog 3; TTC3, Tetratricopeptide repeat domain 3; PIAS3, The protein inhibitors of activated STATs 3; Erk5, Extracellular signal-regulated kinase 5; PRMT1, Protein arginine methyltransferase 1; Nedd8, Neural precursor cell expressed developmentally downregulated protein 8.

Additionally, future work will also identify whether other modifications, such as acetylation and glycosylation, play a role in modulating Smurf2 activities.

In conclusion, PTMs play central roles in the regulation of the many functions of Smurf2. A thorough and comprehensive understanding of these roles and the mechanisms by which these PTMs control Smurf2 is critical for understanding the biological and pathological networks in which Smurf2 participates. Moreover, these PTMs will likely prove invaluable for the identification of novel therapeutic targets for diseases caused by dysregulation of TGF-β signaling, such as cancer and fibrosis.

AUTHOR CONTRIBUTIONS

YB contributed to manuscript preparation and editing. YY contributed to literature research, revise, and final approval of

the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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On-Target Anti-TGF-β Therapies Are Not Succeeding in Clinical Cancer Treatments: What Are Remaining Challenges?

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Metastasis is the leading cause of death for cancer patients. During cancer progression, the initial detachment of cells from the primary tumor and the later colonization of a secondary organ are characterized as limiting steps for metastasis. Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are opposite dynamic multistep processes that enable these critical events in metastasis by altering the phenotype of cancer cells and improving their ability to migrate, invade and seed at distant organs. Among the molecular pathways that promote tumorigenesis in late-stage cancers, transforming growth factor-β (TGF-β) is described as an EMT master inducer by controlling different genes and proteins related to cytoskeleton assembly, cell-cell attachment and extracellular matrix remodeling. Still, despite the successful outcomes of different TGF-β pharmacological inhibitors in cell culture (*in vitro*) and animal models (*in vivo*), results in cancer clinical trials are poor or inconsistent at least, highlighting the existence of crucial components in human cancers that have not been properly explored. Here we review most recent findings to provide perspectives bridging the gap between on-target anti-TGF-β therapies *in vitro* and in pre-clinical models and the poor clinical outcomes in treating cancer patients. Specifically, we focus on (i) the dual roles of TGF-β signaling in cancer metastasis; (ii) dynamic signaling; (iii) functional differences of TGF-β free in solution vs. in exosomes; (iv) the regulatory effects of tumor microenvironment (TME) – particularly by cancer-associated fibroblasts – on TGF-β signaling pathway. Clearly identifying and establishing those missing links may provide strategies to revitalize and clinically improve the efficacy of TGF-β targeted therapies.

Keywords: cancer therapy, epithelial to mesenchymal transition, exosome, metastasis, signaling, TGF-β, tumor microenvironment

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Abbreviations: ASO, antisense oligonucleotide; BMP, bone morphogenetic protein; CAF, cancer-associated fibroblast; Co-SMAD, common SMAD; CTC, circulating tumor cell; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; GREM1, gremlin 1; I-SMAD, inhibitor SMAD; LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β binding protein; MET, mesenchymal-epithelial transition; R-SMAD, receptor SMAD; sRII/III, soluble TβRII/III; TGF-β, transforming growth factor-beta; TME, tumor microenvironment; TβRI/II/III, TGF-β receptor type I/II/II.

INTRODUCTION

Affecting human populations in the whole world, cancer is a disease that can virtually compromise all biological human tissues. More than 18 million new cases of cancer were expected for 2018 and more than 9 million patients died in the same year (Bray et al., 2018; Ferlay et al., 2019). Other than different factors distinguishing particular cancer types, metastasis is considered to be the most important cause of death related to this disease and patients affected by metastasis at diagnosis can present a reduced survival rate of 60–90% (Hanahan and Weinberg, 2000; Australian Institute of Health and Welfare [AIHW], 2019).

Tumor metastasis is a multistep process through which cancer cells leave their primary site to colonize distant organs (Zhou et al., 2014; Ren et al., 2019; **Figure 1**). In order to migrate and invade, epithelial cancer cells undergo phenotypic alterations to detach from surrounding cells, degrade the basement membrane and remodel the extracellular matrix (ECM) in a process known as epithelial-mesenchymal transition (EMT) (Nieto et al., 2016). These cancer cells will reach blood or lymph vessels and then proceed to vasculature intravasation. Some of the circulating tumor cells (CTCs) which survive into blood or lymph will adhere to vessel walls and escape from the vessel lumen by vasculature extravasation (Kim et al., 2009; **Figure 1**). Still, while mesenchymal cells present enhanced ability to invade different tissues and proceed to vasculature intravasation/extravasation during metastasis, this phenotype impairs their establishment in a secondary site, limiting the growth of macrometastasis (**Figure 1**). Thus, after reaching a new organ, neoplastic cells reverse their phenotype through the mesenchymal-epithelial transition (MET), improving their interaction with the microenvironment and increasing their proliferation rate and chance of survival (Chaffer et al., 2006; Biswas et al., 2014). Therefore, the two opposite processes of EMT and MET in metastasis early and late-stages, respectively, are considered to be critical steps in cancer metastasis.

Different molecular pathways are associated with the phenotypic changes observed in metastatic cells, including those mediated by transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), hepatocyte growth factor (HFG), platelet derived growth factor (PDGF), notch, and wnt (Nieto et al., 2016). Among these important pathways, TGF-β signaling is considered to act as a master inducer of EMT, invasion and metastasis by controlling different genes and proteins related to cytoskeleton assembly (Gladilin et al., 2019), cell-cell attachment (Kim et al., 2019) and ECM remodeling (Mori et al., 2015). TGF-β is a secreted dimeric polypeptide that elicits cellular effects via cell surface TGF-β type I and type II receptors (TβRI and TβRII). They have intrinsic serine/threonine kinase activity and activate intracellular (non)SMAD signaling pathways (Hao et al., 2019). Each step in the TGF-β signaling pathway is tightly regulated, and subject to crosstalk with other signaling pathways (Liu et al., 2018). TGF-β signaling pathway is well-characterized and many strategies have been used to interfere with its activity (Colak and Ten Dijke, 2017). Nevertheless, even if the selective inhibition of TGF-β bioavailability, TGF-β/TGF-β receptor interaction or TGF-β receptor kinase activity is efficacious *in vitro* and *in vivo*, outcomes observed for anti-TGF-β therapies in clinical settings are often unsatisfactory. In the next sections, we provide a brief overview of TGF-β signaling pathways (section "TGF-β as a Critical Driver in Cancer Progression"); describe and compare different TGF-β signaling inhibitors used *in vitro*, *in vivo*, and in human patients (section "Anti-TGF-β Therapies and Their Poor Outcomes in Cancer Clinical Trials"); and discuss critical issues in preclinical experiments that so far have been largely ignored/overlooked that could explain the poor outcomes observed in cancer clinical trials (sections "Controlling Metastasis Critical Steps: The Dual Role of TGF-β," "TGF-β Dynamic Signaling," "Tumor Microenvironment Regulates TGF-β Signaling," and "Exosomes as a Mechanism of TGF-β Secretion and Signaling Amplification"). When used in particular studies, TGF-β isoforms are indicated during this discussion, otherwise they are referred as TGF-β if this specificity is not relevant.

TGF-β AS A CRITICAL DRIVER IN CANCER PROGRESSION

Until early 1980s, thanks to studies exploring the role of infectious agents on cancer development, the acquisition of a malignant phenotype was greatly associated with a virus-induced reprogramming of normal cells (Stehelin et al., 1976; Levinson et al., 1978). Products of avian, murine and feline tumor viruses' genomes were shown to drive the malignant transformation of normal cells by the hyperactivation of signaling pathways (Todaro et al., 1976; Levinson et al., 1978; Hackett et al., 1981). In this scenario, the elevated secretion of growth factors was described as an important mechanism able to cause normal fibroblasts transformation, as observed by an increased anchorage-independent growth potential *in vitro* that was intimately associated with cancer cells behavior *in vivo* (de Larco and Todaro, 1978). These molecules later named as transforming growth factors (TGF) were later purified and assigned as TGF-α and TGF-β, being the later characterized as a critical component in the process of malignant transformation (Roberts et al., 1980, 1981; Anzano et al., 1982). Since then, many other related molecules were studied and nowadays TGF-β is part of a protein family of growth factors and cytokines.

Based on similarity in sequence and function, TGF-β family is divided in two subgroups: TGF-βs, activins, and nodals forming one group and bone morphogenetic proteins (BMP)s and anti-muellerian hormone the other. The cellular responses to TGF-β and BMP are highly context-dependent, and have been attributed both anti- and pro-tumorigenic roles in different cancer types and/or stages of cancer progression (Biswas et al., 2008; Zhong et al., 2010; Luwor et al., 2015; Sachdeva et al., 2019; Vollaire et al., 2019). The biphasic role of TGF-β family pathways in cancer were already reviewed in details by others (Lebrun, 2012; Seoane and Gomis, 2017). Among all TGF-β family members, the targeting of TGF-β pathway has been explored most for therapeutic gain in the treatment of cancer patients (Colak and Ten Dijke, 2017; Hao et al., 2019). In this review, therefore, we focus on the TGF-β signaling pathway and selective intervention strategies as

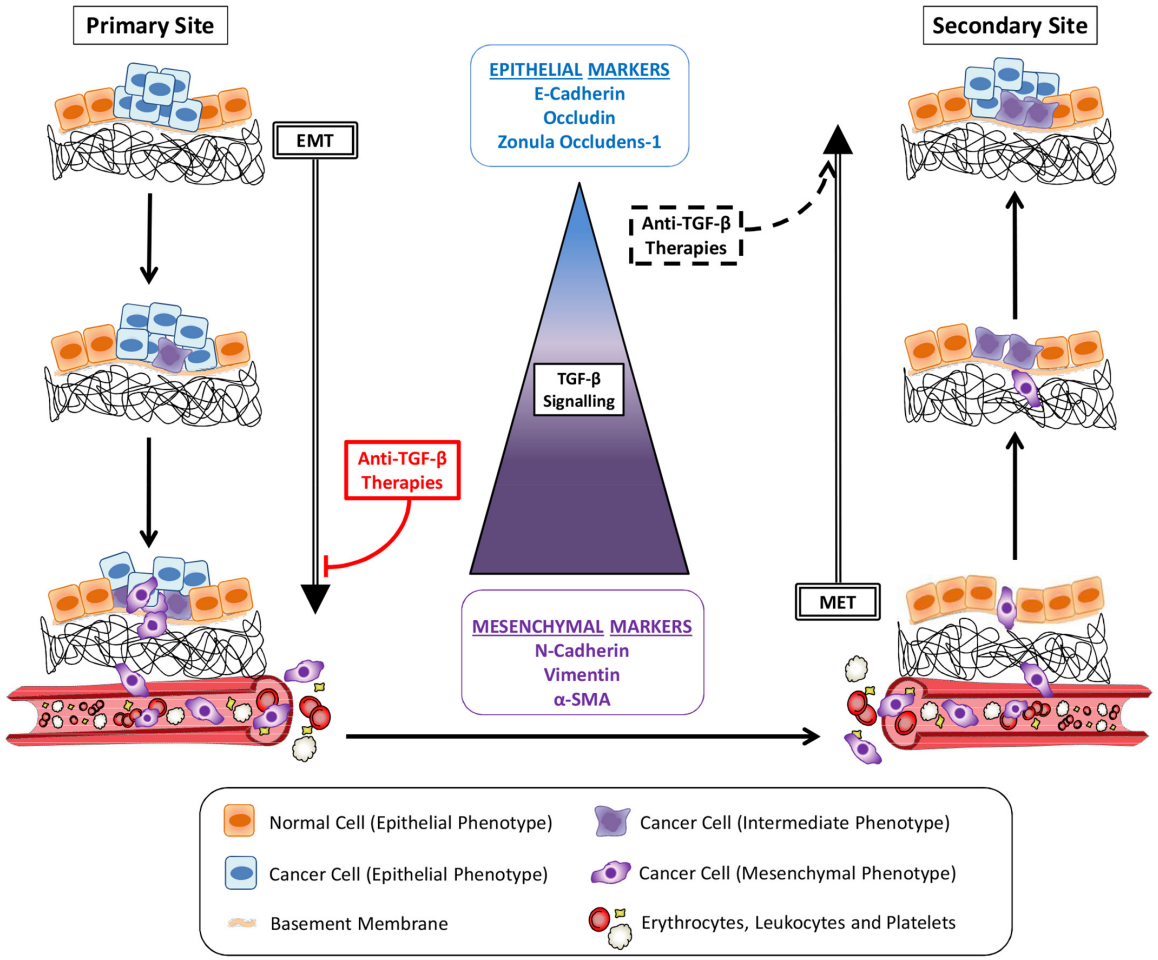

FIGURE 1 | Cancer metastasis and TGF-β signaling. Cancer cells alter their morphology through epithelial-mesenchymal transition (EMT) induced by TGF-β signaling pathway activity, increasing their migratory potential. Invading the basement membrane and the extracellular matrix, tumor cells reach the vasculature (blood or lymph vessels) and become circulating tumor cells (CTCs) after intravasation. Gradually, the magnitudes of TGF-β signaling increase dramatically to enable the EMT-invasion processes. Cancer cells reach a secondary site after extravasation. Following TGF-β signaling reduction and consequent mesenchymal-epithelial transition (MET), cancer cells colonization proceeds to the growth of a metastatic lesion. Anti-TGF-β therapies administered in early stage cancers, before initial invasion, would inhibit metastasis by avoiding EMT. The same strategies used to treat late-stage cancers would also induce MET and seeding of secondary tumors.

a background to discuss problems related to pharmacological inhibitors for TGF-β family members used in preclinical and clinical cancer studies.

TGF-β Secretion and Activation

The expression TGF-β isoforms (TGF-β1-3) is coordinated in tissues according to physiopathological conditions (Stenvers et al., 2003; Cooley et al., 2014; Denney et al., 2015; Hachim et al., 2018). Importantly, TGF-β is secreted in an inactive form in which the N-terminal sequence (also termed latency-associated peptide, LAP), and a C-terminal sequence (active cytokine) are non-covalently linked (Walton et al., 2010). Dimers of TGF-β:LAP associate with the latent TGF-β binding protein (LTBP) to form the large latent complex (LLC) (Taipale et al., 1994; Walton et al., 2010). While LAP prevents TGF-β activation, LTBP promotes secretion and can mediate the TGF-β association with proteins in ECM. Besides enzymatic cleavage, a non-enzymatic mechanism of TGF-β activation is also reported and relies on the interaction of LLC with integrins. In cells with enhanced contractility, the tension created by cytoskeleton exerts physical forces that unfold LAP and release active TGF-β (Taipale et al., 1994; Shi et al., 2011).

TGF-β Receptor Signaling Pathways

After secretion and activation, TGF-β ligands bind to heteromeric complexes of type I and type II serine/threonine kinase receptors (i.e., TβRI and TβRII). TβRII is a constitutive active kinase that phosphorylates TβRI upon ligand binding, thereby enabling the transduction of extracellular signal into the cell (Zhu and Sizeland, 1999). The activated TβRI initiates intracellular signaling by phosphorylation of downstream effector molecules. Besides TβRI and TβRII, TGF-β can interact with more abundant auxiliary receptors, e.g., TGF-β type III receptor (TβRIII), that lack an enzymatic intracellular motif (Andres et al., 1992;

Stenvers et al., 2003). These co-receptors can enable presentation of TGF-β to TβRI and TβRII and thereby regulate cellular responsiveness (López-Casillas et al., 1993; Stenvers et al., 2003). Moreover, as TGF-β isoforms bind with different affinity to co-receptors, they contribute to isoform specific responsiveness to different cell types (Andres et al., 1992; Itoh et al., 2003).

SMADs act as specific effectors downstream of activated TGF-β family type receptors. In the canonical TGF-β-SMAD signaling pathway (**Figure 2**), TβRI kinase induces the phosphorylation of a Sma- and Mad- related (SMAD) 2 and 3. BMP type I receptors mediate the phosphorylation of distinct set of R-SMADs, i.e., SMAD1, 5, and 8. Common SMAD (Co-SMAD), i.e., SMAD4 binds to phosphorylated R-SMADs to form heteromeric complexes that accumulate in the nucleus and control target gene expression. Another set of SMADs are the inhibitory SMADS (I-SMADs), i.e., SMAD6 and 7. I-SMADs antagonize signal transducing SMADs via multiple mechanisms, including direct competition with R-SMADs for SMAD4, and recruitment of ubiquitin ligases that drive type I receptor polyubiquitination and degradation. Besides canonical SMAD signaling, TGF-β family type I receptors can also initiate so-called non-SMAD signaling pathways that follow intracellular downstream routes, controlling the stability, activity and expression of genes and proteins (Nakao et al., 1997; Shi et al., 1997; Itoh et al., 2003; Zhang et al., 2007; Fleming et al., 2013). Different studies have demonstrated for example the TβRI-induced activation of mitogen-activated protein kinase (MAPK) (Tang et al., 2019), and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (PKB/AKT) pathways (Kattla et al., 2008).

By activating its canonical and non-canonical pathways, TGF-β controls multiple processes in cell homeostasis. In non-malignant cells and in early stage cancers, TGF-β exerts a tumor-suppressive role inducing cell cycle arrest and apoptosis. In fact, inactivating mutations in TGF-β receptors and SMADs are frequently observed in cancers (e.g., colorectal, pancreas, and lung cancers) (Hahn et al., 1996; de Jonge et al., 1997; Shi et al., 1997; Zhang et al., 2003; Biswas et al., 2008; Fleming et al., 2013). Nonetheless, many other cancer types, such as brain, breast and skin, bypassing TGF-β cytostatic or pro-apoptotic effects through mutations in different pathways (e.g., PI3K/AKT), become invasive by subverting TGF-β activity to their own benefit (Biswas et al., 2014; Yang et al., 2016). In this scenario, TGF-β tumor-promoter role contributes directly and indirectly with metastatic potential of cancer cells. Directly, TGF-β induces EMT to support migration and invasion of cancer cells as previously mentioned. Indirectly, TGF-β acts on distinct elements of tumor microenvironment, suppressing immune surveillance, promoting angiogenesis and activating cancer-associated fibroblasts that will further contribute to metastasis (Itoh et al., 2009; Liu et al., 2016a,b; Stockis et al., 2017).

The accumulated evidences about critical steps in TGF-β signaling activation combined to the relevance of TGF-β in cancer progression led to the development of multiple strategies to abrogate its activity. Anti-TGF-β therapies have been extensively investigated, but despite their very well-established efficacies and ability to act on target, clinical trials are still unable to reproduce these outstanding results obtained *in vitro* and *in vivo* (Ahmadi et al., 2019). On the next section we present different mechanisms to block the TGF-β signaling pathway and a brief compilation of preclinical and clinical data obtained from studies using TGF-β inhibitors in order to contextualize the missing points when these therapies are translated from bench-to-bedside.

ANTI-TGF-β THERAPIES AND THEIR POOR OUTCOMES IN CANCER CLINICAL TRIALS

Multiple strategies have been developed to target the TGF-β signaling pathway, including interference with activation of latent TGF-β, ligand-receptor interactions, and receptor kinase inhibitors. While *in vitro* and preclinical models have been clearly successful, so far the outcomes from clinical trials to treat different types of cancers have frequently shown (at best) only a minor survival benefit and even sometimes adverse effects. One reason for poor clinical translation may well be that the preclinical data may suffer from publication bias for positive results, and that the animal models used in these studies poorly reflect the cancers developed in patient. In addition, with TGF-β being a multifunctional cytokine of key importance to the maintenance of tissue homeostasis, targeting of TGF-β signaling has been associated with on-target cardiovascular toxic side effects and formation of benign tumors (Colak and Ten Dijke, 2017). Inappropriate patient selection in clinical trials may also contribute to the inability to demonstrate favorable survival benefit. Moreover, as targeting TGF-β will not kill the cancer cell, but is aimed at inhibiting invasion and metastasis, it will have to be used with other agents that do kill cancer cells (Bhola et al., 2013; Zhu et al., 2018). Furthermore, targeting TGF-β signaling has been frequently aimed at inhibiting cancer cell invasion and metastasis, but inhibition of immune evasion by blocking the potent immune suppressive function of TGF-β might actually be more important for anti-cancer activity of TGF-β targeting agents (Ghiringhelli et al., 2005; Yang et al., 2008; Rong et al., 2016; Xia et al., 2017; Biswas et al., 2019). Thus, drugs used so far do not recapitulate preclinical data and the outcomes reported for these tests are inconsistent among patients as discussed in the following sections. In order to understand the mechanisms of action on which these strategies are based and the possible reasons for their failure in clinical tests, four categories of anti-TGF-β therapies will be further discussed: (i) antisense oligonucleotides (ASOs), (ii) anti-integrins, (iii) ligand traps, and (iv) kinase inhibitors (**Figure 2**).

Antisense Oligonucleotides

ASOs are designed to bind to and prevent TGF-β mRNA translation, consequently decreasing its expression. Tests in mesothelioma and prostate cancer cells lines, for example, demonstrated its effectiveness in dramatically reducing TGF-β protein expression and inhibiting anchorage-independent growth (Fitzpatrick et al., 1994; Matthews et al., 2000). Further experiments *in vivo* showed reduced tumor growth in animals subjected to ASOs treatments and these results were associated

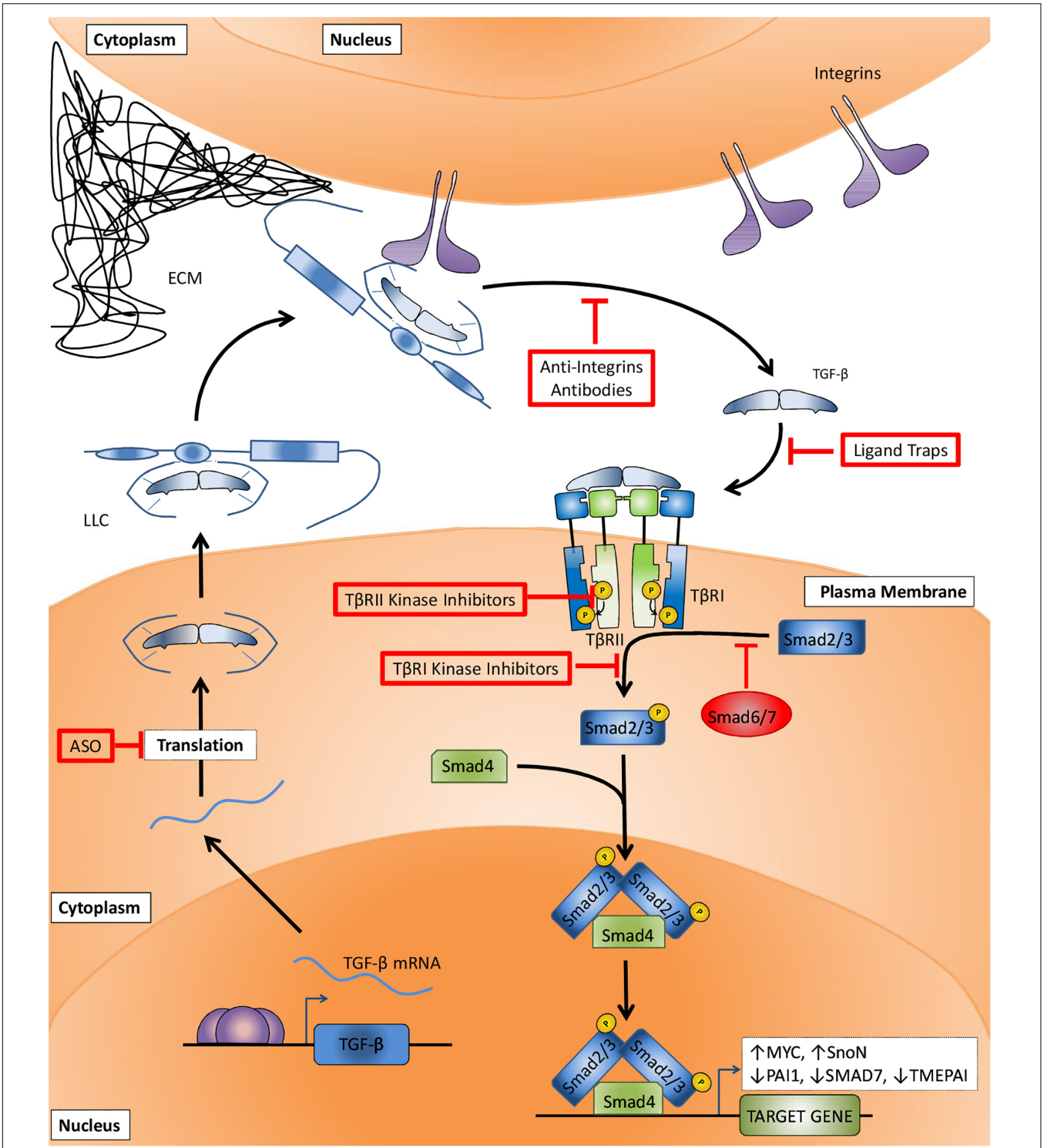

FIGURE 2 | Canonical TGF-β signaling pathway and TGF-β signaling targeting therapies. After TGF-β mRNA translation (step I) and secretion, the large latent complex (LLC) composed of TGF-β, latency associated peptide (LAP), and latent TGF-β binding protein (LTBP) is deposited to the extracellular matrix (ECM). The interaction between LTBP and integrins increases TGF-β:LAP dissociation and TGF-β activation (step II). TGF-β binding to surface receptors (step III) is followed by TβRII-mediated TβRI transphosphorylation (step IV). The signaling is then transduced to cytosol by TβRI-induced phosphorylation of SMAD2 and 3 (step V), followed by their association with SMAD4, accumulation in the nucleus and regulation of target genes transcription. Anti-TGF-β therapies target critical steps in order to impair TGF-β signaling. Antisense oligonucleotides (ASOs) prevent the translation of TGF-β mRNA (step I). Anti-integrins prevent TGF-β activation (step II). Ligand traps avoid cytokine binding to its receptors (step III). TβRII and TβRI kinase inhibitors block type II-mediated type I receptor phosphorylation (step IV) and type I-mediated SMAD2 and 3 phosphorylation (step V), respectively.

with impairment of TGF-β-mediated immune suppression (Fitzpatrick et al., 1994; Matthews et al., 2000).

Based on its proven specificity observed in preclinical models, ASOs have progressed to clinical trials. AP 12009 (or Trabedersen), an ASO targeting TGF-β2 mRNA, was used to treat multiple cancer types. The safety of Trabedersen was demonstrated in phase I trials in patients with pancreas, colon, and skin cancers (NCT00844064). In a phase II trial, Trabedersen was administered to patients with glioblastoma and anaplastic astrocytoma (NCT00431561), achieving a particularly interesting outcome: compared to patients treated with standard chemotherapy (i.e., Temozolomide or Procarbazine/Lomustine/Vincristine), patients submitted to this ASO appeared to exhibit an improvement in cognitive functions. Nevertheless, the same study failed to demonstrate increased antitumor responses in patients treated with Trabedersen compared to patients treated with standard chemotherapy. Finally, the only phase III clinical trial using Trabedersen, also to treat brain cancer patients (NCT00761280), has been terminated by its inability to recruit the projected number of patients and only descriptive analyses are available. **Table 1** summarizes main results obtained in cancer clinical trials using ASOs.

Anti-integrins

TGF-β activation by dissociation from LAP is a crucial step that precedes its binding to TβRI/II. As mentioned previously, different mechanisms work toward TGF-β activation, binding of LTBP to integrins is considered one of them to greatly improve the activation process. In fact, integrins expression is associated with elevated availability of activated TGF-β and consequent increase of EMT, migration and invasion *in vitro* for many cancer cell lines (Roth et al., 2013; Moore et al., 2014; Dutta et al., 2015; Takasaka et al., 2018). Furthermore, the activation of TGF-β signaling pathway is shown to induce integrins expression leading to a positive feedback (Mori et al., 2015; Liu and Shang, 2020; van Caam et al., 2020). Consequently, many strategies targeting TGF-β signaling by blocking integrin-mediated TGF-β activation were developed and tested in preclinical models. For instance, antibodies blocking integrins (e.g., 10D5 and 264RAD) efficiently impair the growth of primary and secondary tumors in models of breast and prostate cancers, though the effects exerted by these therapies could also be related to reduced TGF-β-mediated immunosuppression and angiogenesis (Moore et al., 2014; Dutta et al., 2015).

Seven cancer clinical trials exploring the effects of integrins inhibitors were conducted so far, but two were terminated (NCT01122888 and NCT02337309) before prematurely conclusion and three others do not present results publicly available (NCT00721669, NCT00284817, NCT00635193). The two remaining studies evaluated the use of EMD 121974 (or Cilengitide), an antibody targeting integrins αvβ3 and αvβ5, to treat patients with head and neck squamous cell carcinoma (phases I and II, NCT00705016) and glioblastoma (phase III, NCT00689221). Unfortunately, both trials report that administration of Cilengitide did not result in improved antitumor activity or increased overall survival compared with standard chemotherapies. **Table 2** shows an overview of clinical studies that evaluated integrin inhibitors to treat cancer patients.

Interfering With Ligand-Receptor Interactions

TGF-β signals when the active cytokine binds to surface receptors that will further transduce the signal to cytoplasm and two main strategies were developed so far as ligand trap to prevent this step: (i) administration of antibodies against ligand or its receptors, and (ii) the use of soluble TGF-β receptors (sRII or sRIII) or receptors fused to immunoglobulins (TβRII:Fc) as ligand sequesters. Many molecules designed as ligand traps have been characterized *in vitro* and *in vivo*. Their ability to reduce the availability of the active cytokine, diminish SMAD2/3 phosphorylation and decrease the expression of TGF-β target genes, support their on-target activity (Ganapathy et al., 2010). For instance, treatments with 1D11 or 2G7 (monoclonal anti-TGF-β antibodies) were shown to reduce the metastatic burden and angiogenesis in breast cancer models and further experiments associated these results to an increased cytotoxicity exhibited by natural killer (NK) cells (Arteaga et al., 1993; Ganapathy et al., 2010; Biswas et al., 2011). Similar results were obtained by employing antibodies raised against the extracellular domain of TGF-β receptors (particularly against TβRII), reducing the growth of primary and secondary tumors as well as increasing the numbers of NK and cytotoxic T cells (Zhong et al., 2010). Also, mice models treated with the sRIII (Bandyopadhyay et al., 1999) or TβRII:Fc (Muraoka et al., 2002; Yang et al., 2002) showed a reduced number of metastases in different organs analyzed (i.e., lung, liver, and pancreas). This approach has recently been expanded by fusing the extracellular domain of TβRII with an anti-programmed cell death ligand-1 (PD-L1) antibody to obtain a bifunctional therapy and circumvent the immunosuppression commonly observed in solid tumors. *In vitro*, this bifunctional therapy (M7824) was demonstrated to increase the lysis of urothelial carcinoma cells by T cells compared to effects of anti-PD-L1, a result that was associated to the upregulation of molecules involved in immunogenic modulation (i.e., intercellular adhesion molecule 1/ICAM-1, carcinoembryonic antigen/CEA, and Fas cell surface death receptor/FAS) (Grenga et al., 2018). A similar pattern has also been demonstrated for this strategy *in vivo*, in which the administration of an anti-PD-L1-TβRII reduced tumor burden and promoted activation of $CD8^+$ T lymphocytes and NK cells in breast and colorectal cancer models (Ravi et al., 2018).

Multiple observations in preclinical models led ligand traps to cancer clinical trials, but different from animal models, results in humans have been inconsistent. The TGF-β sequester GC1008 (also known as Fresolimumab), one of the best characterized monoclonal anti-TGF-β1-3 antibodies was used in patients with renal cell carcinoma (phase I, NCT00923169), melanoma (phases I and II, NCT00923169), glioma (phase II, NCT01472731), mesothelioma (phase II, NCT01112293), and breast cancer (phase II, NCT01401062). Even though a relationship between safety and antitumor activity was shown, it was also observed a decreased expression of activating surface proteins in NK cells

TABLE 1 | Overview of anti-TGF-β therapies based on antisense oligonucleotides used in cancer clinical trials.

Drug (Target)	Clinical trial (Phase)	Status	Cancer type	Patients enrolled	Arms	Outcomes
AP 12009 (TGF-β2)	NCT00431561 (Phase II)	Completed	Glioblastoma and anaplastic astrocytoma	141	AP 12009 (10 μM) AP 12009 (80 μM) Temozolomide or procarbazine, lomustine, and vincristine	Improved PFS Improved OS (Results for responders regardless drug concentration administered)
AP 12009 (TGF-β2)	NCT00761280 (Phase III)	Terminated	Glioblastoma and anaplastic astrocytoma	27	AP 12009 (10 μM) Temozolomide or carmustine or lomustine	NA
AP 12009 (TGF-β2)	NCT00844064 (Phase I)	Completed	Melanoma, pancreatic and colorectal neoplasms	62	Single-arm: AP 12009 (dose escalation)	NA

PFS, progression-free survival; OS, overall survival. NA, not available.

TABLE 2 | Overview of anti-TGF-β therapies based on integrin inhibitors used in cancer clinical trials.

Drug (Target)	Clinical trial (Phase)	Status	Cancer type	Patients enrolled	Arms	Outcomes
EMD 121974 (Integrins αvβ3 and αvβ5)	NCT01122888 (Phase I)	Terminated	Adult giant cell glioblastoma, adult glioblastoma, adult gliosarcoma, adult solid neoplasms and recurrent adult brain neoplasms	41	Sunitinib + EMD 121974 Sunitinib	NA
EMD 121974 (Integrins αvβ3 and αvβ5)	NCT00705016 (Phases I/II)	Completed	Head and NeckSquamous Cell Carcinoma	184	Cilengitide (2000 mg) once weekly + cetuximab + 5-FU + cisplatin Cilengitide (2000 mg) twice weekly + cetuximab + 5-FU + cisplatin Cetuximab + 5-FU + Cisplatin	No improvement in PFS No improvement in OS
EMD 121974 (Integrins αvβ3 and αvβ5)	NCT00689221 (Phase III)	Completed	Glioblastoma	545	Cilengitide + temozolomide + radiotherapy Temozolomide + radiotherapy	No improvement in PFS No improvement in OS
SF1126 (Integrin-targeted PI3 kinase)	NCT02337309 (Phase I)	Terminated	Neuroblastoma	4	Single-arm: SF1126	NA
IMGN388 (Integrins αv)	NCT00721669 (Phase I)	Completed	Melanoma, breast carcinomas, lung carcinomas and ovary carcinomas	60	Single-arm: IMGN388	NA
MEDI-522 (Integrin αvβ3)	NCT00284817 (Phases I/II)	Completed	Colorectal cancer	17	MEDI-522 (D0: 4 mg/kg; W1–W51: 1 mg/kg) MEDI-522 (D0: 4 mg/kg; W1–W51: 2 mg/kg) MEDI-522 (D0: 6 mg/kg; W1–W51: 2 mg/kg) MEDI-522 (D0: 6 mg/kg; W1–W51: 3 mg/kg)	NA
M200 (Integrin α5β1)	NCT00635193 (Phases I/II)	Completed	Ovarian cancer and primary peritoneal cancer	138	Liposomal doxorubicin (40 mg/m^2) + M200 (7.5 mg/kg) Liposomal doxorubicin (40 mg/m^2) + M200 (15.0 mg/kg) Liposomal doxorubicin (40 mg/m^2)	NA

5-FU, 5-fluoracil; D0, First day of treatment; W1–W51, weeks 1–51 of treatment; PFS, progression-free survival; OS, overall survival; NA, not available.

(i.e., CD226 and CD244) which could impair therapy effects and partially explain why most patients treated in these studies did not present improved overall survival. Currently two other clinical trials using anti-TGF-β antibodies are recruiting: a phase I/Ib trial (NCT02947165) using NIS793 (anti-TGF-β) to evaluate its safety and tolerability as a single agent or in combination with PDR001 (anti-programmed cell death-1, or PD-1), and another phase I study (NCT03192345) using SAR439459 (anti-TGF-β) to evaluate safety, pharmacokinetics, pharmacodynamics and antitumor activity as a monotherapy or in combination with Cemiplimab (anti-PD-1) in multiple cancers. Similar to antibodies targeting the ligand, many problems were also observed when using anti-TGF-β receptors antibodies. For example, a study evaluating the safety of LY3022859 (anti-TβRII) to treat solid tumors (NCT01646203) failed in establishing its maximum tolerated dose, restricting its usage in other phases. Finally, the only clinical trial (phase I) proposed so far to treat cancer patients by blocking TGF-β signaling pathway by the use of soluble TGF-β receptors is still recruiting patients. This study (NCT03834662) will evaluate AVID200 safety, tolerability, and dose-limiting toxicities in advanced or metastatic solid cancers. Results obtained in clinical trials evaluating the interference between ligand-receptor interactions in the treatment of cancer patients are summarized in **Table 3**.

Kinase Inhibitors

Kinase inhibitors block the binding of ATP to TGF-β receptors, reducing their kinase activity and limiting downstream signaling transduction. Similar to ligand traps, their ability to specifically target and impair TGF-β signaling pathway activation has been demonstrated by using cancer cells derived from different tumors (e.g., brain, breast, pancreas, and mesothelium). Acting exclusively on TβRI or interfering with type I and II TGF-β receptors, these inhibitors were shown to reduce tumor growth, metastasis, recurrence and angiogenesis in mouse models (Gaspar et al., 2007; Suzuki et al., 2007; Rausch et al., 2009; Zhang et al., 2011).

Given the outstanding results achieved *in vitro* and *in vivo*, kinase inhibitors were also investigated in clinical studies. LY2157299 (or Galunisertib) had its safety demonstrated in a phase I trial with glioblastoma patients (NCT01220271), but the antitumor response was only achieved in 3 of 28 patients. Still, 10 other studies are currently in development or recruiting patients with different types of cancer in advanced stage. LY3200882 is the most recent TGF-β inhibitor in this class and clinical studies (phases I and II) intend to recruit patients to evaluate safety and antitumor activity as single agent or in combination with other chemotherapies (NCT02937272, NCT04031872). **Table 4** presents an overview of cancer clinical trials using kinase inhibitors.

As described above, positive results in clinical tests using anti-TGF-β therapies were observed, but they are not common to all patients. Even when interesting outcomes were achieved, they are not satisfactorily distinct from those results reported for current therapies, as would be expected by data obtained *in vitro* and *in vivo*. This highlights a major problem: a gap in the current comprehension about TGF-β activity during cancer progression in human patients. Based on the most recent findings, we argue in next section that important points about TGF-β signaling have or are not being properly considered in preclinical studies. Specifically, we address (i) the dual role of TGF-β signaling in EMT and MET; (ii) TGF-β dynamic signaling; (iii) the functional difference of TGF-β secreted by exosomes; and (iv) the regulatory effects of tumor microenvironment (TME) – particularly by cancer-associated fibroblasts – on TGF-β signaling activities and its functions.

CONTROLLING METASTASIS CRITICAL STEPS: THE DUAL ROLE OF TGF-β

The critical role of TGF-β on EMT, increasing cancer cells migration and invasion *in vitro* (Hao et al., 2019) have been comprehensively established. By using pharmacological inhibitors studies have also demonstrated convincingly that blocking TGF-β signaling represents an effective strategy to impair metastasis *in vivo* (Matthews et al., 2000; Zhong et al., 2010; Biswas et al., 2011; Dutta et al., 2015). Still, few studies consider that TGF-β can exert an important anti-MET activity, avoiding a critical late-stage step in metastasis (**Figure 1**). Also, cancer patients and animal models differ in a very important point that could be critical to classify TGF-β as friend or foe: the timing at which the treatment is administered.

It is usual to start anti-TGF-β treatment in animal models as soon as the cancer reaches a palpable volume or even earlier. By treating cancer at such an early stage, researchers avoid that malignant cells invade surrounding tissues and progress to vasculature intravasation, inhibiting metastasis and reinforcing the dangerous role of TGF-β in metastasis. Nevertheless, cancers in humans are not always diagnosed at early stages because it takes time until the initial symptoms appear, not mentioning that the TGF-β targeting treatments were often for very late stage cancer patients. Thus, when diagnosis occurs, many tumor cells have already spread and are found in the blood and/or lymph. These CTCs and possible undetectable micrometastases underwent EMT before treatment has started. Therefore, administering anti-TGF-β therapies by this time could block one of the most important molecular pathways that sustain cancer cells mesenchymal phenotype, inducing MET and facilitating the growth of secondary tumors (**Figure 1**).

Based on their enhanced invasive potential, cancer cells with mesenchymal phenotype usually result in more metastasis than counterparts with epithelial phenotype when implanted in solid tissues. Nevertheless, to represent the CTCs usually observed in cancer patients, these cells should be evaluated after they reach the bloodstream. Indeed, the reduced ability of mesenchymal cells to colonize secondary organs and establish distant metastasis has been described for many cancer types in animal models. In a striking report Biswas et al. (2014) describe that blocking TGF-β activity resulted in different outcomes when employing cancer cells with distinct initial phenotypes. In this study, researchers used a TβRI kinase inhibitor to treat breast cancer cells carrying mesenchymal – or epithelial-like phenotypes. After intracardiac inoculation,

TABLE 3 | Overview of anti-TGF-β therapies based on the interference between ligand-receptor interactions used in cancer clinical trials.

Drug (target)	Clinical trial (phase)	Status	Cancer type	Patients enrolled	Arms	Outcomes
GC1008 (TGF-β1 and TGF-β2)	NCT00923169 (Phase I)	Completed	Renal cell carcinoma and melanoma	22	GC1008 (10 mg/kg) GC1008 (15 mg/kg)	Highest safe dose: 15 mg/kg
GC1008 (TGF-β1 and TGF-β2)	NCT01472731 (Phase II)	Completed	Glioma	12	Bioimaging with 89Zr-GC1008 (37 MBq total) Treatment with GC1008 (5 mg/kg)	NA
GC1008 (TGF-β1 and TGF-β2)	NCT01112293 (Phase II)	Completed	Mesothelioma	14	Single-arm:GC1008 (3 cycles)	NA
GC1008 (TGF-β1 and TGF-β2)	NCT01401062 (Phase II)	Completed	Metastatic breast cancer	23	GC1008 (1 mg/kg) + radiotherapy GC1008 (10 mg/kg) + radiotherapy	No improvement in abscopal effect Improved OS in arm II
NIS793 (TGF-β)	NCT02947165 (Phase I)	Recruiting	Breast, lung, hepatocellular, colorectal, pancreatic and renal cancers	220	NIS793 NIS793 + PDR001	NA
SAR439459 (TGF-β1, TGF-β2 and TGF-β3)	NCT03192345 (Phase I)	Recruiting	Advanced solid tumors	225	SAR439459 (dose escalation) SAR439459 (dose expansion) SAR439459 (dose escalation) + cemiplimab SAR439459 (dose expansion) + cemiplimab	NA
LY3022859 (TβRII)	NCT01646203 (Phase I)	Completed	Advanced solid tumors	14	IMC-TR1 (1.25 mg/kg) IMC-TR1 (dose escalation – 12.5 to 1600 mg) IMC-TR1 (dose escalation – 800 to 1600 mg)	DLT reported TEAE reported SAE reported
AVID200 (TGF-β1 and TGF-β3)	NCT03834662 (Phase I)	Recruiting	Malignant solid tumors	36	AVID200 (180 mg/m^2) AVID200 (550 mg/m^2) AVID200 (1100 mg/m^2)	NA

OS, overall survival; DLT, dose-limiting toxicity; TEAE, treatment emergent adverse events; SAE, serious adverse event; NA, not available.

TABLE 4 | Overview of anti-TGF-β therapies based on kinase inhibitors used in cancer clinical trials.

Drug (target)	Clinical trial (phase)	Status	Cancer type	Patients enrolled	Arms	Outcomes
LY2157299 (TβRI)	NCT01220271 (Phases I/II)	Completed	Glioma	75	Phase I LY2157299 (160 mg) + radiotherapy + temozolamide LY2157299 (300 mg) + radiotherapy + temozolamide Phase II LY2157299 (established dose) + radiotherapy + temozolamide Radiotherapy + temozolamide	NA
LY3200882 (TβRI)	NCT02937272 (Phase I)	Active, not recruiting	Solid tumors	223	LY3200882 LY3200882 + LY3300054 LY3200882 + gemcitabine + nab-paclitaxel LY3200882 + cisplatin + radiation	NA
LY3200882 (TβRI)	NCT04031872 (Phases I/II)	Active, not recruiting	Colorectal metastatic cancer	31	Single-arm: LY3200882 + capecitabine	NA

Cmax, maximum concentration; ORR, objective response rate; PFS, progression-free survival; CBR, clinical benefit rate; CR, complete response; PR, partial response; AUC, area under the curve; SAE, serious adverse event; OS, overall survival; NA, not available.

epithelial-like cancer cells treated or not with anti-TGF-β metastasized at the same rate, while mesenchymal-like cancer cells responded to TGF-β signaling pathway block by slight increasing the number of lung metastases. By disrupting TGF-β signaling, researchers probably induced MET in these cancer cells, given them more benefits than disadvantages in secondary organ colonization.

Thus, the generally not considered role of TGF-β in promoting anti-MET could actually make this cytokine an interesting friend to block cancer metastasis in advanced tumors that already started to spread. In addition, this potential TGF-β function highlights the relevance of clearly determining which patients should be submitted to TGF-β inhibitors and considering probable poor outcomes for post-operative patients or patients with cancer in advanced stage (**Figure 1**). A thorough understanding of detailed and exacting roles played by TGF-β at specific cellular stages of cancer metastasis is in urgent need in order to devise an effective, precise anti-TGF-β treatment regimen.

TGF-β DYNAMIC SIGNALING

How treatment schemes for anti-TGF-β therapies are defined? Do these protocols consider natural fluctuations in TGF-β signaling? Similar to observations in many other molecular pathways, TGF-β signaling is also controlled by negative feedbacks, being SMAD7 its best characterized feedback inhibitor. In a simplified model, high levels of stimulus (TGF-β activity) result in increased *SMAD7* expression and TGF-β/SMAD signaling inhibition (SMAD7 activity) that in turn disrupt the initial stimulus (Nakao et al., 1997; Jenkins et al., 2005; Zhang et al., 2007; Khatibi et al., 2017a,b). Therefore, careless administration of TGF-β inhibitors should be expected to result in rapid decrease of TGF-β activity, followed by its pronounced increase when TGF-β receptors are thereafter activated.

Other than proteins, miRNA and lncRNA targeting TGF-β, its receptors or downstream effectors (Hao et al., 2019), TGF-β signaling is also opposed by BMP signaling – commonly associated to a MET-promoter effect – in many metastatic cancers (Gao et al., 2012; Karagiannis et al., 2013; Vollaire et al., 2019). Considering this antagonism, TGF-β pharmacological targeting should increase BMP activity, preventing cancer cell invasion and reducing the risk of metastasis. Nevertheless, two different problems could arise from that strategy. First, as discussed in the previous section, by promoting MET in cancer cells after intravasation/extravasation, BMP signaling pathway would actually contribute to metastatic development. Second, it is not unusual to detect alterations in BMP pathway, being these effects imposed by malignant cells and other elements at the TME. Gremlin 1 (GREM1) is a BMP antagonist that binds to the ligand, preventing its interaction with membrane receptors and activation of downstream signaling pathway. As recently demonstrated by Ren et al. (2019) elevated levels of GREM1 correlate with a poor prognostic for breast cancer patients. Also, the same study showed that GREM1 promotes EMT and invasion of breast cancer cells *in vitro* and it is correlated with higher levels of intravasation and extravasation in a zebrafish model.

Therefore, considering the existence of TGF-β regulatory components, oscillations in TGF-β signaling pathway were mathematically modeled *in silico* and tested *in vitro*. Many groups demonstrated that dynamic changes occur, including fluctuations in levels of SMADs phosphorylation and activity (Zi et al., 2011; Wegner et al., 2012), differential nuclear-cytoplasmic shuttling (Giampieri et al., 2009; Warmflash et al., 2012) and irregular regulatory effects on gene transcription (Giampieri et al., 2009; Zi et al., 2011; Wegner et al., 2012). These alterations are natural results of intracellular homeostasis, but these effects can also be induced by exposure to different ligand concentrations over time (Schneider et al., 2012; Warmflash et al., 2012; Wegner et al., 2012; Wang et al., 2014). It is reasonable to assume that metastatic cells traveling through different tissues on their way to distant organs are not submitted to a homogeneous environment. Otherwise, malignant cells are likely to be subjected to other cell types with heterogeneous TGF-β secretion potentials, what is not commonly reproduced *in vitro* and could result in different states of cancer cell activation. Sorre et al. (2014) studying the influence of this cytokine on the development of *Xenopus* embryos showed that a pulsed stimulus is more effective than a constant elevation in TGF-β concentrations to promote signaling activation. Moreover, Nicolás and Hill (2003) analyzing the tumor suppressive role of TGF-β reported that the resistance to TGF-β-induced growth arrest exhibited by some pancreatic cancer cell lines derive from their ability to rapidly export R-SMADs to cytoplasm, while counterparts sensitive to TGF-β retain nuclear SMADs for longer periods.

However, it should not be expected that all cells in a population (i.e., malignant tumor) present similar responsiveness to TGF-β – or at least not in a synchronized pattern. Evaluating cancer cell migration, Luwor et al. (2015) demonstrated an interesting difference exerted by the dynamics of TGF-β among cells populations *in vitro*. Even though SMAD3 activity was enhanced in migratory cells compared to non-migratory cells, the behavior of migratory cells was uneven. Three subpopulations were classified among these migratory cells, but surprisingly, cells with higher SMAD3 activity moved smaller distances than migratory cells with low or medium SMAD3 activity. Interestingly, this heterogeneous pattern of TGF-β activity in different cells from the same population was also described *in vivo* by Giampieri et al. (2009). Importantly, they demonstrated that TGF-β signaling activity is not sustained during all metastatic steps, and while SMAD2 nuclear localization and SMAD3 activity were detectable in migrating cells, these results were not present in cancer cells in lymph node metastases.

Overall, these results demonstrate that TGF-β signaling is dynamic. Two main mechanisms explain this observation: (i) this molecular pathway is transiently regulated by a negative feedback involving molecules (proteins or RNAs) that present direct interaction with TGF-β or its effectors; and (ii) opposite signaling pathways can inhibit TGF-β signaling for prolonged times and avoid its control on cell phenotype. Understanding

how these processes occur in different contexts and reproduce this balance in pre-clinical models will help to establish a better treatment scheme in clinical trials at which anti-TGF-β therapies are carefully administered to prevent amplified TGF-β activation (**Figure 3**), as such normalizing its signaling.

TUMOR MICROENVIRONMENT REGULATES TGF-β SIGNALING

The immunosuppressive role of TGF-β has been explored in many diseases including cancer and the combination of TGF-β inhibitors and immunotherapies is suggested as an alternative to improve the antitumor effect of immune cells. Still, this scenario considers components of TME – especially immune cells – as targets rather than sources of TGF-β secretion. It has been demonstrated, for instance, that cells such as myeloid-derived suppressor cells (MDSCs) secrete TGF-β and contribute to cancer progression (Yang et al., 2008; Biswas et al., 2019). Still, other cell types (e.g., endothelial cells and pericytes) already described as important during metastasis could also be involved in this process, but their contribution to carcinogenesis based on TGF-β secretion still not properly explored (Flaumenhaft et al., 1993; Ma et al., 2007; Colak and Ten Dijke, 2017). Therefore, despite cancer cells, little is known about TGF-β secretion by TME cells.

Considering the interference of cancer-associated fibroblasts (CAFs) in multiple processes during carcinogenesis (e.g., ECM remodeling, angiogenesis, bioenergetics, cancer cells stemness, response to therapies and immune surveillance), their ability to promote TGF-β-mediated EMT, invasion and metastasis started to be evaluated. Yu et al. (2014) demonstrated that primary breast CAFs-conditioned medium induce EMT markers (e.g., vimentin upregulation and E-cadherin downregulation) in breast cancer cell lines, promoting migration and invasion *in vitro* that could be partially blocked by using an anti-TGF-β antibody or a TβRI kinase inhibitor. Similar results were obtained by Nagura et al. (2015) working with uterine cervical squamous cell carcinoma cells. Surprisingly, they also showed that an exclusive detection of phosphorylated SMAD3 at tumor boundaries were preferentially detected in samples from uterine cancer patients diagnosed with lymph node metastasis, suggesting that the activation of TGF-β signaling pathway in malignant cells is induced by tumor stroma. Liu et al. (2016a,b) using primary fibroblasts isolated from hepatocellular carcinoma patients confirmed the relevance of TME cells signaling on EMT, migration and invasion *in vitro* and metastasis *in vivo* as a result of TGF-β signaling activation.

As discussed before, TGF-β and BMP signaling pathways are commonly describe as opposite molecular pathways, but the consequent effects are not limited to cancer cells. Gao et al. (2012) showed that lung stromal cells restrict the growth of metastasis by secreting high levels of active BMP. Interestingly, the ability of breast cancer cells to overcome this anti metastatic mechanism was related to secretion of COCO (DAND5) and consequent inhibition of BMP signaling. Ren et al. (2019), in the same study mentioned before, described that TGF-β-induced CAFs activation results in GREM1 (BMP inhibitor) secretion *in vitro*. This suggestive feed forward loop between CAFs and malignant cells was further reinforced by results showing that GREM1 expression is restricted to tumor stroma in breast cancer patients.

Overall, these data highlight the importance of the microenvironment surrounding cancer cells – especially CAFs – secreting or controlling the secretion of TGF-β. Nevertheless, the presence of CAFs in animal models exploring the response to anti-TGF-β therapies is not commonly observed. Thus, together with the points discussed in the previous sections, ignoring the crosstalk between cancer cells and TME components – especially CAFs – could hamper the translation of preclinical results to clinical trials using TGF-β signaling pathway inhibitors, explaining the poor and inconsistent outcomes observed in cancer patients (**Figure 4**), highlighting the need for developing mouse tumor models containing TME or at least CAFs.

EXOSOMES AS A MECHANISM OF TGF-β SECRETION AND SIGNALING AMPLIFICATION

Exosomes are nanosized extracellular vesicles with a diameter ranging from 30 to 100 nm. The role of exosomes on cell communication relies on their ability to transport different types of cargo allowing cell-cell interaction and autocrine or paracrine signaling. Nucleic acids, lipids and many proteins were already described among exosomes cargo, but their specific mechanism of sorting into endosomes still poorly understood (Escrevente et al., 2011; Christianson et al., 2013; Li et al., 2017). Interestingly, even though TGF-β receptors traditionally localize at the plasma membrane, they present sequences used by cell machinery as signals for internalization (Di Guglielmo et al., 2003; Clement et al., 2013). Indeed, it has been shown that these receptors can be directed to early endosomes, activating the SMAD-dependent pathway before being recycled back to the cell surface (Di Guglielmo et al., 2003; Clement et al., 2013). Thus, considering the role of endosomes as precursors of exosomes, the secretion of TGF-β by these extracellular vesicles became a suggestive possibility.

In fact, some studies have showed the secretion of exosomal TGF-β by cancer cells and their interaction with other TME components. For instance, Rong et al. (2016) demonstrated *in vitro* that T cells treated with exosomes derived from breast cancer cell lines exhibit reduced proliferation through a TGF-β-dependent mechanism that could be only partially reverted by treatment with anti-TGF-β antibodies. Furthermore, after show that stage III-IV renal carcinoma patients present higher levels of exosomal TGF-β than patients in stages I-II, Xia et al. (2017) treated NK cells with tumor-derived exosomes and reported a decrease in their cytotoxicity. Also, using 786-O renal adenocarcinoma cells as a model, the same study demonstrated that TGF-β secreted in exosomes is more efficient to reduce NK cytotoxicity than its free-ligand form. In gastric cancer, Yen et al. (2017) showed that exosomal TGF-β isolated from peripheral blood is elevated in patients with lymph node metastasis and positively correlates with increased levels of T regulatory lymphocytes.

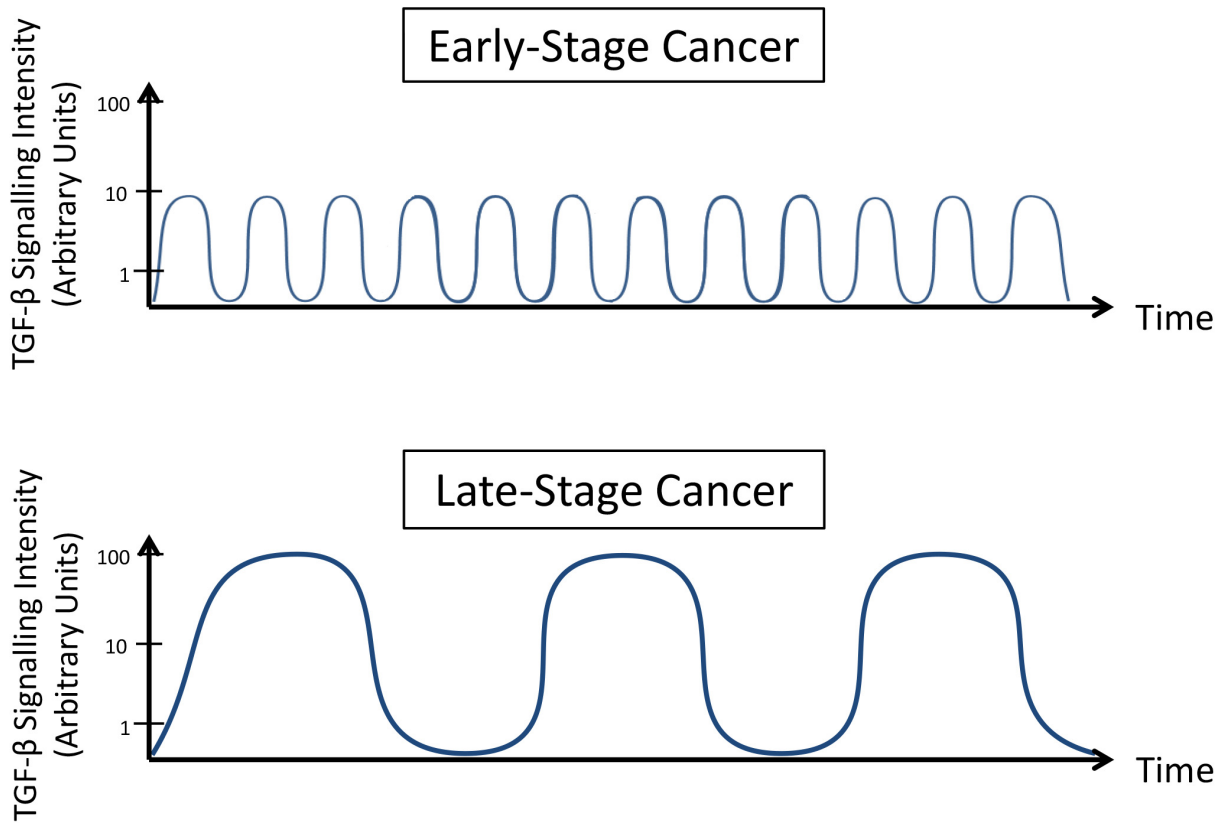

FIGURE 3 | TGF-β signaling dynamics change during cancer progression. Late-stage cancer cells present increased intensity and reduced frequency oscillation in TGF-β signaling pathway activity compared to early stage cancer cells.

Reinforcing the role of exosomes in paracrine signaling, Webber et al. (2010) reported that primary fibroblasts are activated after treatment with exosomal TGF-β secreted by cancer cells. Interestingly, although most TGF-β in these exosomes was present in the latent form, cells stimulated with exosomal TGF-β and TGF-β in its free-ligand form exhibited similar levels of SMAD3 activity. Still, in the opposite direction of this crosstalk, Li et al. (2017) demonstrated that CAFs can also secrete exosomal TGF-β. Even more, exosomal TGF-β was show to promote SMAD2/3 phosphorylation in ovarian cancer cells, decreasing their levels of E-cadherin and increasing vimentin expression. As a result of exposure to exosomal TGF-β secreted by CAFs, cancer cells presented enhanced migration and invasion *in vitro* and increased tumor growth *in vivo*.

While studies exploring the mechanisms of carcinogenesis have mostly focused on exosomes as the main type of extracellular vesicles mediating TGF-β transport as cargo, TGF-β secretion in microvesicles/ectosomes have also been reported in other contexts such as in immunology and infectious diseases (Cestari et al., 2012; Sadallah et al., 2014, 2016). For instance, Cestari et al. (2012) showed that *Trypanosoma cruzi* infection induces the release of microvesicles (MVs) enriched in TGF-β from blood cells. These MVs associate with the parasite surface, increasing *T. cruzi* invasion into host cells and escape from complement system. Other than that, two studies from Sadallah and collaborators demonstrated that platelet-derived MVs enriched in TGF-β promote immunosuppression by both promoting $CD4^+$ T cells differentiation toward a Treg phenotype (Sadallah et al., 2014) and decreasing NK cells activity (Sadallah et al., 2014).

Thus, the extracellular vesicles could work as an alternative mechanism to secrete TGF-β, and particularly in the context of cancer progression, promoting the crosstalk between TME cells and even amplifying TGF-β signaling activation. Furthermore, it is possible that extracellular vesicles prevent the interaction between TGF-β and ligand traps in a mechanism similar to what was shown for exosomes transporting PD-L1 and impairing anti-PD-L1 antibodies activity (Poggio et al., 2019). Also, the uptaking of extracellular vesicles enriched in TGF-β could activate TGF-β signaling from the cytoplasm. As such, the dynamics of extracellular vesicles trafficking in different cell types likely to influence the extent of downstream effects. As different mechanisms to block exosomes secretion and uptake have been tested *in vitro* (Ostrowski et al., 2010; Christianson et al., 2013), a combinatory approach targeting exosomes and TGF-β signaling simultaneously should be evaluated in order to block both TGF-β forms of secretion – as a free-ligand and as extracellular vesicles cargo.

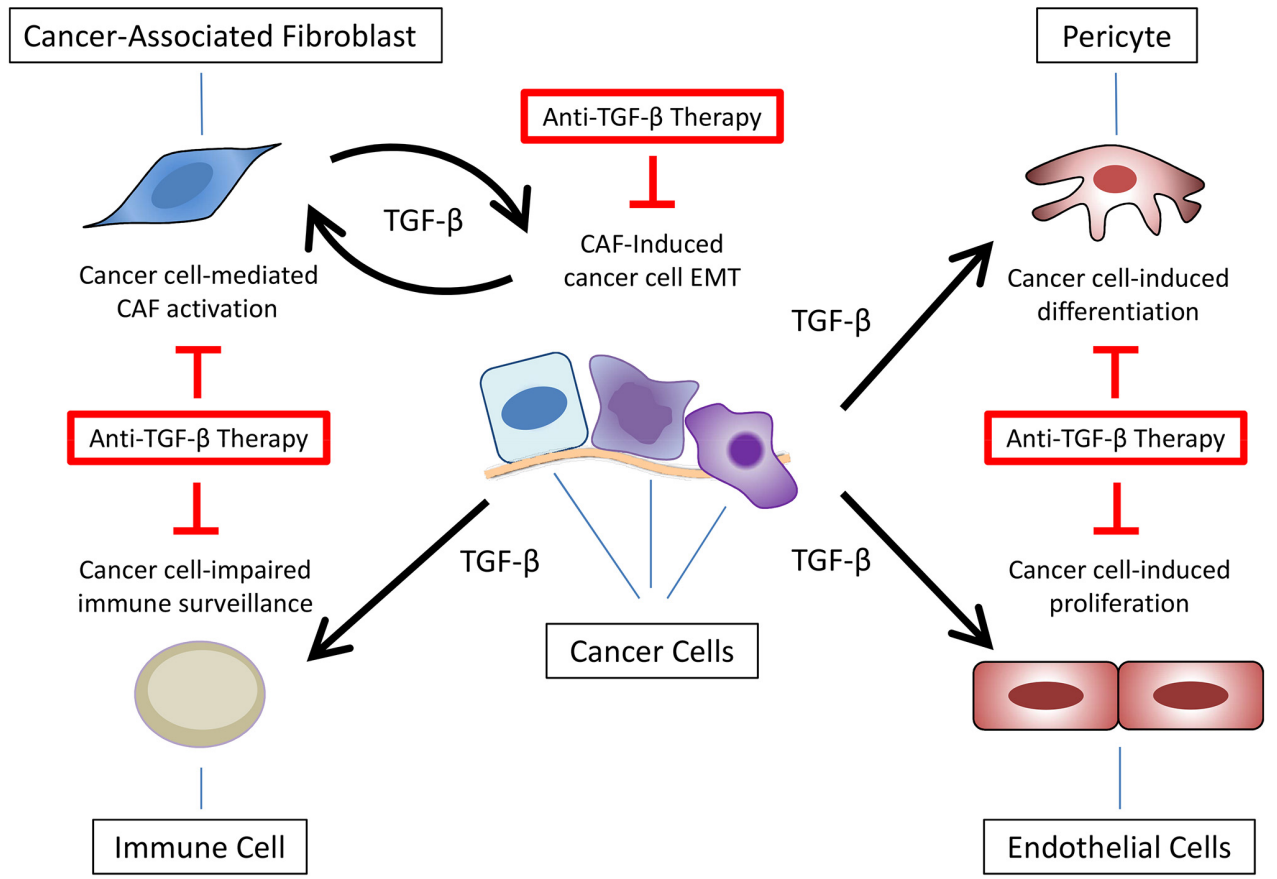

FIGURE 4 | Anti-TGF-β therapies targeting multiple components of tumor microenvironment. Besides epithelial-mesenchymal transition (EMT) in cancer cells, TGF-β secretion at the tumor microenvironment promotes cancer-associated fibroblast (CAF) activation, impaired immune surveillance, pericytes differentiation, and endothelial cell proliferation.

OTHER PERSPECTIVES

As discussed in the previous sections, anti-TGF-β therapies that successfully inhibit cancer cells EMT, migration, invasion and metastasis in pre-clinical models have faced multiple problems when used to treat cancer patients in clinical trials. However, the combination of these same drugs with immune checkpoint inhibitors has recently emerged as a highly promising approach that can lead to a prolonged anti-cancer response.

The immunosuppressive activity played by TGF-β critically impacts the activity of different immune cell types (Yang et al., 2010). More specifically, TGF-β is shown to reduce the proliferation (Rong et al., 2016) and activity (Thomas and Massagué, 2005) of cytotoxic T cells while induces the differentiation of $CD4^+$ cells toward a Treg phenotype (Chen et al., 2003; Ghiringhelli et al., 2005). Based on this premise, for instance, Dodagatta-Marri et al. (2019) have shown that outcomes obtained with an anti-PD-1 antibody can be improved by combining it with an anti-TGF-β neutralizing antibody treatment in xenograft models of skin cancer. In this study, while the outcome obtained with the anti-PD-1 therapy was limited to a partial regression and correlated with an increased $CD4^+$ Treg/$CD4^+$ Th cells ratio, a complete tumor regression was achieved by the synergistic response when anti-PD-1 and anti-TGF-β antibodies were combined. Results from Sow et al. (2019) have corroborated this pattern by demonstrating that the combination of an anti-PD-L1 antibody with a TβRI kinase inhibitor lead to increased survival in the highly immunogenic mouse MC38 colon adenocarcinoma model. Still, because the same combinatorial approach did not lead to similar outcomes in the poorly immunogenic mouse KPC1 pancreatic cancer model, these researches also suggested that combining anti-TGF-β therapies with immune checkpoint inhibitors may be beneficial only for certain types of cancer, highlighting the relevance of an appropriate selection of patients to undergo this therapeutic strategy. In addition, it is noteworthy that the role played by TGF-β in blocking the infiltration of cytotoxic T cells into the cancer mass may not necessarily be induced by cancer cells. In fact, Mariathasan et al. (2018) and Tauriello et al. (2018) demonstrated that the TGF-β-mediated immune exclusion is a response triggered during cancer progression by non-cancer cells from the tumor stroma, particularly by CAFs.

Overall, considering the studies highlighted in this section and the evidences from the use of bifunctional antibodies that

simultaneously target TGF-β and PD-1/PD-L1 (as presented in section "Interfering With Ligand-Receptor Interactions." Interfering with ligand-receptor interactions), it is suggestive that the poor outcomes obtained with anti-TGF-β therapies in clinical trials may be improved by their combination with other therapies, particularly with immune checkpoint inhibitors. In this context, experimental studies regarding the immunosuppressive activity played by TGF-β should be expanded and their results compared between "cold tumors" and "hot tumors" in order to obtain a better understanding about the use of TGF-β pharmacological inhibitors to overcome the immune exclusion that is common to different types of cancer. While the use of immune checkpoint inhibitors alone may favor the cytolytic activity of immune cells that are present within the cancer mass, their combination with TGF-β pharmacological inhibitors may increase the infiltration of these cells in "cold tumors." More details about the synergism between TGF-β pharmacological inhibitors and immune checkpoint inhibitors has been reviewed and discussed by others regarding its use in pre-clinical models and clinical trials (Ganesh and Massagué, 2018; Bai et al., 2019; Groeneveldt et al., 2020; Lind et al., 2020).

Given the occasional but serious side effect of anti-PD1/PD-L1 therapies on heart (Bajwa et al., 2019) and TGF-β's important role played in heart development and homeostasis (Dickson et al., 1995; Stenvers et al., 2003; Anderton et al., 2011), the challenges for the combined or bifunctional antibody therapies are to minimize the potential fatal side effect.

CONCLUDING REMARKS

In the past decade(s) many studies have been devoted to delineate the dynamic role of TGF-β signaling in the multistep process of metastasis. While substantial insights were obtained, new layers of complexity and regulation continue to be discovered. Its potent pro-oncogenic activities have been targeted using a scale of selective pharmacological inhibitors. Reported results in cancer models have been very promising. Yet, outcomes observed in more than 20 cancer clinical trials using anti-TGF-β therapies lack consistency and fail to recapitulate the preclinical data, raising questions about what is missing when translating these strategies from bench-to-bedside.

TGF-β function in cancer cell invasion and metastasis is pleiotropic and dynamically controlled. These critical aspects are somewhat overlooked when TGF-β targeting agents are applied to cancer patients. The administration of these drugs to post-operative patients or patients with advanced cancers could increase the seeding of CTCs and the growth of metastatic lesions by blocking its anti-MET activity and its tumor-suppressor function. Also, affected by the activity of antagonist molecular pathways, induced by surrounding elements in the TME and other cancer cells, the dynamics of TGF-β signaling might change from normal to malignant cells (and even from early to late-stage cancer cells) thereby influencing how different patients (or different cells in the same patient) respond to the therapy. Finally, the presence of TGF-β in exosomes could make it inaccessible from antibodies and ligand traps, limiting the effectiveness of these intervention strategies to the freely soluble ligand form of this cytokine. New *in vitro* and *in vivo* models exploiting these points will deepen our knowledge about the real complexity of TGF-β signaling in carcinogenesis. To do so, sensitive technologies such as single cell signaling and live signaling *in vitro* and *in vivo* are to be employed (Zhou et al., 2014; Luwor et al., 2015; Chen et al., 2018) and further developed. Moreover, clinical trials combining anti-TGF-β therapies to other target therapies such as immune checkpoint inhibitors, or strategies allowing a more spatio-temporal controlled intervention using nanocarriers, may allow for an improved treatment and perhaps even cure of cancer patients.

AUTHOR CONTRIBUTIONS

H-JZ: concept formation. AT: writing and original draft preparation. PD and H-JZ: editing and revision. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Roles of TGF-β Signaling in Cerebrovascular Diseases

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Cerebrovascular diseases are one of the leading causes of death worldwide, however, little progress has been made in preventing or treating these diseases to date. The transforming growth factor-β (TGF-β) signaling pathway plays crucial and highly complicated roles in cerebrovascular development and homeostasis, and dysregulated TGF-β signaling contributes to cerebrovascular diseases. In this review, we provide an updated overview of the functional role of TGF-β signaling in the cerebrovascular system under physiological and pathological conditions. We discuss the current understanding of TGF-β signaling in cerebral angiogenesis and the maintenance of brain vessel homeostasis. We also review the mechanisms by which disruption of TGF-β signaling triggers or promotes the progression of cerebrovascular diseases. Finally, we briefly discuss the potential of targeting TGF-β signaling to treat cerebrovascular diseases.

Keywords: cerebral cavernous malformation, hereditary hemorrhagic telangiectasia, cerebrovascular disease, endothelial-to-mesenchymal transition, cerebral angiogenesis, TGF-β signaling

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INTRODUCTION

According to the latest Global Burden of Disease Study, cerebrovascular disease is the second leading cause of mortality worldwide (Naghavi et al., 2017). Emerging clinical research data show that cerebrovascular disease is also the cause of many central nervous system diseases (Turner et al., 2016; Iadecola and Gottesman, 2018; Kummer et al., 2019). However, due to the lack of techniques to study cerebrovascular development and its regulatory mechanisms at the whole-animal level, our understanding of cerebrovascular diseases is still very limited. Emerging studies have begun to uncover the molecular mechanisms of cerebrovascular development and homeostasis, providing new basis and treatment strategies for the prevention and treatment of cerebrovascular and central nervous system diseases (Vanlandewijck et al., 2018; Munji et al., 2019).

Blood vessels of the brain form a highly specialized vascular network, which have complex interactions with the central nervous system, and has important physiological functions in the development and maintenance of the central nervous system (Zhao et al., 2015; Iadecola, 2017; Paredes et al., 2018). The development of cerebrovasculature begins with the angiogenic sprouting of perineural vascular plexus (PNVP) blood vessels, which forms a delicate hierarchical vascular structure through continued sprouting and remodeling (Tata et al., 2015). Blood vessels of the brain are mainly composed of highly specialized vascular endothelial cells (ECs), which have an arteriovenous differentiation pattern similar to that of peripheral vascular ECs. Brain ECs have obvious heterogeneity, complex tight junctions, more pericyte coverage, and form the

neurovascular unit (NVU) together with pericytes, smooth muscle cells (SMCs), astrocytes, and neurons. The brain ECs, pericytes and the endfeet of astrocytes together form the unique blood–brain barrier (BBB) to restrict potentially harmful substances and molecules from entering the brain. The nutrients, energy metabolites, metabolic waste and other essential molecules cross the brain endothelium via various substrate-specific transporters to ensure physiological functioning of the brain. The primitive BBB is formed at embryonic day 15 (E15) in mice and varies in other species (Zhao et al., 2015). BBB continues to mature under the influence of neural environment over a brief period after birth.

Cerebrovascular development is a highly conserved and complex process involving multiple signaling pathways. Using various model organisms, researchers have successively identified many genes and signaling pathways that regulate the formation and homeostasis of blood vessels of the brain, including vascular endothelial growth factor (VEGF), sonic hedgehog/Patched (Shh/PTC-1), platelet-derived growth factor B/platelet-derived growth factor receptor β (PDGFB/PDGFRβ), Wnt/β-catenin, orphan G protein coupled receptor 124 (GPR124), as well as transforming growth factor β/SMAD (TGF-β/SMAD) signaling (Stenman et al., 2008; Ferrari et al., 2009; James et al., 2009; Alvarez et al., 2011; Cullen et al., 2011; Posokhova et al., 2015; Sweeney et al., 2016). As one of the most important and complex signaling pathways in vascular development, TGF-β/SMAD signaling plays diverse functions during the development and homeostasis of the brain vessel, and dysfunction in this signaling pathway has been linked to various cerebrovascular diseases (Park et al., 2009; Nguyen et al., 2011; Maddaluno et al., 2013). In this review, we discuss the latest research progress on the physiological function of TGF-β signaling in cerebrovascular development, and the mechanisms by which disruption of TGF-β signaling causes cerebrovascular diseases.

TGF-β SIGNALING IN THE DEVELOPMENT AND HOMEOSTASIS OF CEREBROVASCULATURE

The TGF-β Signaling Pathway

The TGF-β signaling pathway is highly conserved in evolution, and plays multiple and complex physiological functions in the regulation of embryonic development and tissue homeostasis in a highly context-dependent manner (Morikawa et al., 2016; David and Massague, 2018; Zinski et al., 2018).

The TGF-β signaling pathway comprises of more than 30 kinds of ligands, mainly divided into subfamilies such as TGF-βs, bone morphogenetic proteins (BMPs), activins, inhibin, Nodal, anti-Müllerian hormone, and growth and differentiation factors (GDFs) (**Figure 1**). Most TGF-β ligands function as paracrine factors on adjacent cells. The TGF-β ligands are expressed in latent forms with latency-associated peptide (LAP) shadowing the active domains of TGF-βs in the latent complex, and mature TGF-β ligands are activated through cleavage by extracellular protease from the LAP or physical tension by integrins. Several milieu molecules interact specifically with latent TGF-β and are essential for the bioavailability of TGF-β ligands. It is widely accepted that αVβ6 and αVβ8 integrins convert the cytoskeletal tension into a mechanical force to dissociate LAP from the TGF-β active domain, thereby releasing the activated TGF-β molecule and initiating the signaling cascade (Aluwihare et al., 2009). Very recently, researchers used cryo-electron microscopy to analyze the intermediate conformation of the interaction between αVβ8 integrin and latent TGF-β, and found that latent TGF-β binding with αVβ8 can expose the active domain and directly activate the TGF-β signaling pathway without release of the mature conformation (Campbell et al., 2020).

Activated TGF-β ligands, which are usually disulfide-linked homodimers, directly bind to the serine/threonine protein kinase type II receptors on the cell membrane surface, sometimes with the assistance of co-receptors such as endoglin and β-glycan (Goumans and Ten Dijke, 2018). Various proteins including noggin, chordin, follistatin, gremlin, coco, and cerberus act as ligand-traps to prevent TGF-β ligands from binding to receptors (David and Massague, 2018). Regulatory molecules such as FKBP12 and BAMBI inhibit the signaling pathway by docking at the cytoplasmic domain of TGF-β type I receptors (Wang et al., 1996; Onichtchouk et al., 1999).

The type II receptors phosphorylate the type I receptors to form a receptor complex, which then phosphorylates the receptor regulated SMADs (R-SMADs) intracellularly. Type I receptors for the TGFβ subfamily (ALK4, ALK5, and ALK7) mainly phosphorylate SMAD2 and SMAD3, whereas type I receptors for the BMP subfamily (ALK1, ALK2, ALK3, and ALK6) mainly phosphorylate SMAD1, SMAD5, and SMAD8. The activated R-SMADs form a complex with the central mediator SMAD4 and translocate into the nucleus, where it binds to specific gene loci under the guidance of signal-driven transcription factors (SDTFs) and lineage-determined transcription factors (LDTFs) as well as tripartite motif 33 (TRIM33) to regulate chromatin accessibility and gene transcription (Xi et al., 2011; David and Massague, 2018). A negative feedback loop of TGF-β signaling is mediated by the inhibitory SMADs: SMAD6 and SMAD7. SMAD7 can recruit E3 ubiquitin protein ligase SMURF2 to degrade the TGF-β receptor (Kavsak et al., 2000). SMAD6 not only interferes with the activation of SMAD2 phosphorylation by the receptor, but binds to R-SMAD and inhibits its binding to SMAD4 (Imamura et al., 1997; Hata et al., 1998). The TGF-β ligands can also signal through SMAD-independent pathways including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (Derynck and Zhang, 2003; **Figure 1**).

It is intriguing that this seemingly simple "two-step" signal transduction of the TGF-β pathway has various and even opposite biological effects on a wide range of physiological processes, thereby reflecting the high spatiotemporal specificity of TGF-β signaling. The complexity of TGF-β signaling is manifested in the abundance and different combinations of its ligands, receptors and intracellular co-factors collaborating with SMADs. A single ligand can trigger multiple receptors [one of the 5 type II receptors (TGFBR2, BMPR2, ACVR2, ACVR2B, and AMHR2) in combination with one of the 7 type I receptors

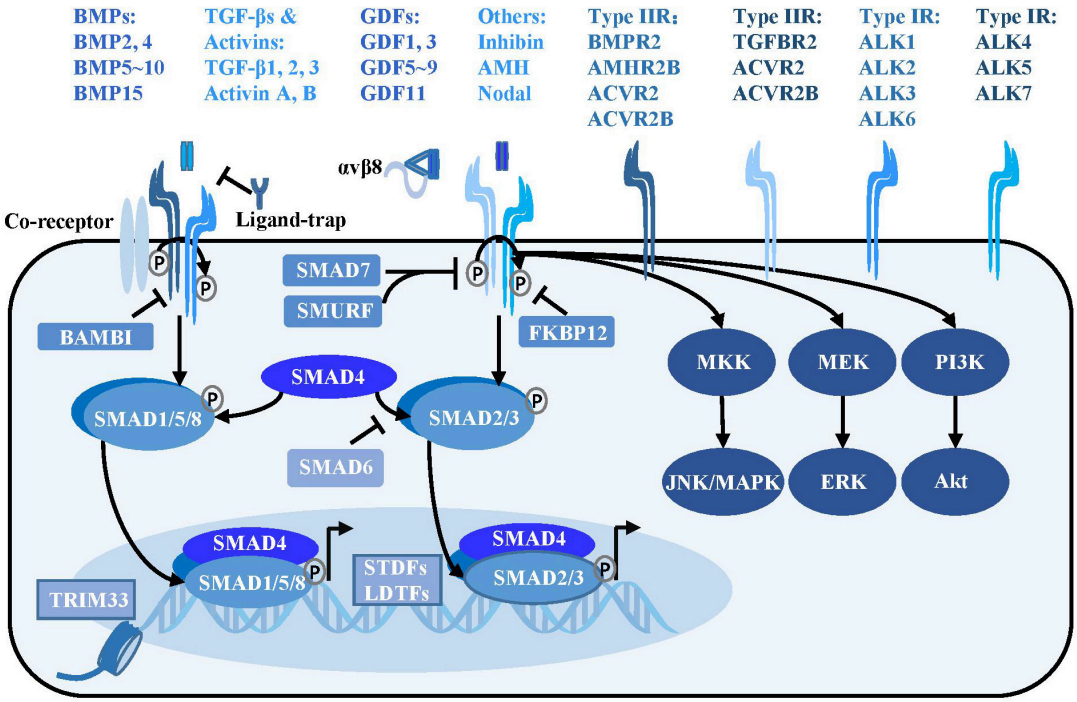

FIGURE 1 | TGF-β signaling pathway. Activated TGF-β ligands with or without αVβ8 integrins bind at serine/threonine protein kinase type II receptors, sometimes with the assistance of the co-receptors such as endoglin. The type II receptors subsequently phosphorylate type I receptors to form a tetrameric receptor complex, which subsequently phosphorylates the SMAD2, SMAD3 or SMAD1, SMAD5, and SMAD8 to form a trimeric complex with SMAD4 in cytoplasm. The SMAD complex then translocates into nucleus and binds at special loci under the guidance of the SDTFs and LDTFs to initiate the transcriptional response. Besides, TRIM33, a modulator of TGF-β signaling, is able to regulate chromatin accessibility and remodeling. In addition to the canonical TGF-β signaling, there are SMAD-independent pathways, such as PI3K/Akt, MEK/ERK and MKK/JNK/MAPK downstream of the TGF-β receptors. TGF-β signaling is negatively regulated at multiple levels. Various ligand traps (noggin, chordin, follistatin, gremlin, coco, and cerberus) can prevent TGF-β ligands from binding to receptors, while FKBP12 and BAMBI can dock at cytoplasmic domain of TGF-β type I receptors to inhibit TGF-β signaling. In addition, inhibitory SMADs including SMAD6 and SMAD7 play a critical function in suppressing the SMAD-mediated signaling.

(ALK1-7)], and single receptor can interact with different ligands as well. The diversity of TGF-β ligand-receptor combinations leads to superimposed, synergistic or antagonistic effects on cells harboring different transcription factors and co-factors interacting with SMADs, resulting in complex biological effects (Massague, 2012).

TGF-β Signaling in Cerebral Angiogenesis

The cerebrovascular network is developed via sprouting angiogenesis. The primary vessels of PNVP penetrate the CNS parenchyma and undergo remodeling to form a hierarchical vascular system composing of branched arteries and veins as well as capillaries, which is regulated by various signaling pathways including TGF-β signaling (Paredes et al., 2018).

Endothelial TGF-β-ALK5 Signaling in Sprouting Angiogenesis

Endothelial TGF-β signaling has been shown to be essential for cerebral angiogenesis, since Tgfbr2 or Alk5 gene knockout blood vessels fail to invade into the neuroepithelial layers and exhibit intracerebral hemorrhage (Nguyen et al., 2011). Genetic disruption of Smad4 in brain ECs leads to increased EC proliferation, impaired endothelial-pericyte interaction and intracerebral hemorrhage, providing a strong evidence that brain endothelial canonical TGF-β signaling plays essential roles in regulating brain angiogenesis and maintaining cerebrovascular integrity (Li et al., 2011).

A previous study has revealed the anti-angiogenic effect of TGF-β signaling in CNS vascular development (Arnold et al., 2014). Activated TGF-β signaling, by αVβ8 integrin, distributes as highest concentration in ventral brain regions and decreases in a gradient toward the dorsal brain regions, which is accompanied with stabilized vessels in ventral brain regions and greater vascular density, branch points and filopodia in dorsal brain region, suggesting that TGF-β signaling may play an anti-angiogenic role in cerebral angiogenesis. Consistently, loss of β8 integrin (Itgb8) or TGF-β1 or knockout of Alk5 or Tgfbr2 in ECs causes excessive vascular sprouting, branching and proliferation, which eventually leads to vascular dysplasia and cerebral hemorrhage (Arnold et al., 2014; Hirota et al., 2015). It has been further verified that neuroepithelial Itgb8 and endothelial neuropilin 1 (Nrp1) cooperatively promote cerebral angiogenesis by balancing TGF-β signaling. Endothelial Nrp1 inhibits β8 integrin activated TGF-β signaling to promote brain sprouting angiogenesis, and EC specific ablation of Nrp1 leads

to increased levels of phosphorylated SMADs and embryonic lethality associated with defective sprouting angiogenesis and cerebral hemorrhage (Hirota et al., 2015).

Transforming growth factor-β signaling has also been shown to promote angiogenesis. TGF-β1 derived from radial glial cells promotes murine microcapillary brain EC migration and tube formation in vitro and stimulates cerebrovascular branching angiogenesis in the cerebral cortex, and this effect may be mediated by the balanced expression of pro-angiogenic gene GPR124 or anti-angiogenic gene, brain-specific angiogenesis inhibitor-1 (BAI-1) (Siqueira et al., 2018).

Endothelial BMP-ALK1 Signaling in the Stabilization of Brain Vessels

Bone morphogenetic protein-ALK1 signaling has been shown to limit EC number and maintain the quiescence of nascent vessels. BMP9 and BMP10 are physiological ligands of ALK1 during vascular development (Chen et al., 2013). In zebrafish, ALK1 functions in transducing hemodynamic forces into a biochemical signal which limits nascent vessel caliber (Corti et al., 2011). In mouse, ALK1 has been shown to mediate fluid shear stress by inducing BMP9 to inhibit endothelial proliferation and promote the recruitment of mural cells, thus maintaining vascular quiescence (Baeyens et al., 2016). Circulating BMP10 acts through endothelial ALK1 to activate pSMAD1/5/8 which decreases pro-angiogenic chemokine receptor cxcr4a expression and induces vasoconstrictive peptide endothelin 1 (Edn1), thereby limiting EC number and stabilizing nascent arterial caliber (Laux et al., 2013).

The mechanisms by which BMP-ALK1 regulates cerebrovascular development are quite limited; therefore, certain studies on the developmental mechanisms of mouse retinal vasculature can help us understand the related processes. The study using heterozygous Acvrl1+/- mice revealed that BMP9-ALK1 signaling inhibits EC proliferation and migration by activating PTEN to inhibit PI3K/Akt and MEK/ERK cascades, thereby maintaining retinal vascular quiescence (Alsina-Sanchis et al., 2018). Consistently, simultaneously silencing Bmp10 and Bmp9 in developing mice increases the retinal vascular density by promoting angiogenesis (Ricard et al., 2012).

TGF-β Signaling in the Formation and Maturation of BBB

Transforming growth factor-β signaling has been implicated in BBB formation and permeability by regulating tight and adherens junctions. The BBB is mainly composed of ECs which are characterized by the presence of tight and adherens junctions, and pericytes play an important role in the formation and maintenance of the BBB (Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010; Li et al., 2011). Endothelial TGF-β/SMAD4 signaling upregulates the adhesion molecule N-cadherin to facilitate the EC-pericyte interaction and BBB formation, in collaboration with Notch signal transduction (Li et al., 2011). Knockout of Smad4 in the brain ECs causes decreased expression of N-cadherin and pericyte detachment, leading to intraventricular hemorrhage and BBB breakdown during the perinatal period (Li et al., 2011). Besides, TGF-β1 derived from pericytes upregulates the expression of claudin-5 and promotes BBB maturation via decreasing endothelial CD146 expression (Chen et al., 2017).

Bone morphogenetic protein signaling has also been demonstrated to participate in the maintenance of BBB function. In zebrafish, BMP3 has been shown to regulate BBB integrity by promoting pericyte coverage (Lei et al., 2017). In rat cerebral vessel, BMP9/ALK1 signaling increases expression of endothelial transporters such as organic anion transporting polypeptide 1a4 at the BBB (Abdullahi et al., 2017). And BMP9/Alk1 is required for BBB stability, since ALK1 haploinsufficiency worsens the vascular leakage in diabetic mice. Mechanistically, ALK1 signaling inhibits VEGF-induced VE-cadherin phosphorylation and induces occludin expression, thereby enhancing the BBB function (Akla et al., 2018).

Non-endothelial TGF-β Signaling in Cerebral Angiogenesis

Cerebral angiogenesis is not only programmed in ECs, but also orchestrated by dynamic TGF-β signaling in other cell types within or outside the NVU, including pericytes, astrocytes, oligodendrocyte precursor cells, neural progenitors, preosteoblasts and periosteal dura cells.

Brain pericytes have been shown to induce and upregulate the functions of BBB through continuous TGF-β production (Dohgu et al., 2005). Pericyte ALK5 upregulates tissue inhibitor of matrix metalloproteinase 3 (TIMP3) to control endothelial morphogenesis in the germinal matrix. Specific knockout of Alk5 in embryonic mouse pericytes causes degradation of the basement membrane by upregulated matrix metalloproteinases (MMPs), resulting in severe germinal matrix hemorrhage-intraventricular hemorrhage (GMH-IVH) (Dave et al., 2018).

Astrocytes, whose endfeet interact with ECs of the neural capillaries, play a critical role in cerebral angiogenesis and BBB formation though BMP signaling. Targeted disruption of BMP type IA receptor (BMPR1A) in telencephalic neural stem cells leads to upregulated expression of VEGF in mutant astrocytes, impaired EC-astrocyte interaction, and cerebrovascular malformation, demonstrating that BMP signaling in astrocytes is essential for a functional BBB (Araya et al., 2008). A very recent study showed that BBB breakdown in aging humans and rodents is associated with hyperactivation of TGF-β signaling in astrocytes. Conditional genetic knockdown of astrocytic TGF-β receptor-coding genes or pharmacological inhibition of TGF-β signaling rescues the phenotypes in aged mice (Senatorov et al., 2019).

Oligodendrocyte precursor cells have also been shown to maintain BBB integrity through TGF-β signaling. TGF-β1 derived from oligodendrocyte progenitor cells can activate the MEK/ERK signaling pathway in ECs to promote tight junction protein expression and improve BBB integrity, and knockout of Tgfbr1 in oligodendrocyte progenitor cells leads to cerebral hemorrhage and disruptive BBB in mice (Seo et al., 2014).

Several neural progenitors have also been shown to play important roles in brain region-specific angiogenesis via TGF-β signaling. Tgfbr2 silencing in forebrain-derived neural progenitors and neural cells impedes EC migration and

sprouting, decreases vessel density and branching via altered secretion of pro- and anti-angiogenic factors, thereby leading to intracerebral hemorrhage in the telencephalon (Hellbach et al., 2014). Neural progenitor S1P signaling regulates integrin β8 gene expression, thereby activating local TGF-β signaling that promotes germinal matrix vasculature development. Disruption of S1P signaling in neural progenitors results in defective angiogenesis and hemorrhage, as well as phenotypes mimicking the germinal matrix hemorrhage in humans (Ma et al., 2017).

In addition, BMP2 and BMP4 derived from preosteoblasts and periosteal dura are essential for dural cerebral vein formation. Loss of Twist1 or BMP2/4 signaling in skull progenitor cells and dura leads to cerebral vein malformations, similar to that in humans with craniosynostosis (Tischfield et al., 2017).

TGF-β Signaling in Endothelial-to-Mesenchymal Transition (EndMT)

Endothelial-to-Mesenchymal Transition is a complex biological process and mainly refers to the trans-differentiation of ECs into mesenchymal stem cells, fibroblasts, SMCs or pericytes (Dejana and Lampugnani, 2018). During the process of EndMT, ECs lose the expression of endothelial markers (such as CD31 and VE-cadherin), and exhibit increased expression of mesenchymal transcription factors and molecular markers [such as Snail1, Slug (Snail2), Twist, ZEBs, vimentin, α-SMA, fibroblast-specific protein-1 (FSP-1; also known as S100A4 protein), fibroblast activating protein (FAP), and fibrillary collagens type I and type III] to obtain a mesenchymal morphology. Mesenchymal cells derived from EndMT gain enhanced ability of cell migration and invasion via disturbing the paracellular connection and polarity of ECs.

Activation of the TGF-β signaling pathway is the most important onset of EndMT (Ma et al., 2020). All three TGF-βs (TGF-β1, TGF-β2, and TGF-β3) have been shown to induce EndMT, while TGF-β2 seems to be more effective than TGF-β1 or TGF-β3 (Sabbineni et al., 2018). TGF-β ligands activate the TGFBR2 and ALK2 or ALK5 in ECs, and induce the pSMAD2/3/4 complex to translocate into the nucleus, where they interact with other transcription factors required for EndMT including Snail1, Snail2, Zeb1, Zeb2, KLF4, TCF3, and Twist and subsequently trigger the expression of mesenchymal transcription factors and molecular markers. TGF-β ligands also trigger EndMT through the non-canonical TGF-β pathways including MAPK, PI3K, and RhoA pathways (Piera-Velazquez and Jimenez, 2019).

Emerging studies have revealed that BMP signaling serves as a gatekeeper by antagonizing TGF-β-induced EndMT in ECs. BMP7 has been shown to inhibit hypoxia-induced EndMT and gremlin-1-mediated EndMT (Zhang et al., 2018, 2020). Loss of Bmpr2 in ECs leads to EndMT characterized by conversion of VE-cadherin to junctional N-cadherin, Slug and Twist upregulation, as well as increased expression of extracellular matrix (ECM) proteins (Hiepen et al., 2019). BMPR2-JNK signaling axis has also been shown to antagonize inflammation-induced EndMT (Sanchez-Duffhues et al., 2019).

Physiologically, EndMT plays essential roles during cardiovascular development, such as angiogenic sprouting and cardiac valve formation (Kruithof et al., 2012; Welch-Reardon et al., 2014). Dysregulation of EndMT has been associated with pathological situations, such as malignant diseases, fibrotic disorders and vascular diseases (Piera-Velazquez and Jimenez, 2019).

Emerging evidence indicates that dysregulated EndMT contributes to certain cerebrovascular diseases (Piera-Velazquez and Jimenez, 2019). The first evidence that EndMT is involved in the pathological process of cerebrovascular diseases was from the study of cerebral cavernous malformation (CCM). TGF-β signaling mediated EndMT is a direct cellular mechanism leading to CCMs in either mouse models or human patients (Maddaluno et al., 2013; Cuttano et al., 2016). Shortly after, another study reported that a meningeal pathogen Group B Streptococcus infection induces Snail1 expression and endothelial dedifferentiation, leading to BBB disruption, suggesting that EndMT might also contribute to BBB deficiency (Kim et al., 2015). Very recently, several studies have revealed that EndMT occurs in multiple sclerosis (MS), ischemic stroke, as well as brain arteriovenous malformations (AVMs) in humans (Derada Troletti et al., 2019; Chen et al., 2020; Shoemaker et al., 2020). All these results indicate that dysregulated EndMT might be an important pathological process involved in a variety of cerebrovascular disorders. However, the causal link between EndMT and various cerebrovascular diseases needs to be further established.

DYSREGULATION OF TGF-β SIGNALING IN CEREBROVASCULAR DISEASES

Recent studies have shown that defects in TGF-β signaling are associated with human cerebrovascular diseases. Pathogenic mutations in TGF-β signaling, such as ENG, ALK1 gene mutations, are associated with type 1 and type 2 hereditary hemorrhagic telangiectasia (HHT), as well as Loeys-dietz syndrome with cerebrovascular events (McAllister et al., 1994; Cunha et al., 2017; Laterza et al., 2019). Some genome-wide association studies (GWAS) or whole exome trio sequencing have uncovered various pathogenic gene variants in the TGF-β pathway, which are associated with small vessel ischemic strokes, intracerebral hemorrhages and sporadic brain AVMs (Weinsheimer et al., 2016; Yilmaz et al., 2017; Wang et al., 2018; Chung et al., 2019). Increased expression of TGF-β1 has been found in the brain tissue after ischemic stroke, as well as in hereditary cerebral hemorrhage with amyloidosis-Dutch type (Krupinski et al., 1996; Grand Moursel et al., 2018), while a recent transcriptome-wide RNA sequencing study revealed that TGF-β signaling was downregulated in patients with brain AVMs (Hauer et al., 2020). All these evidences suggest that dysregulation of TGF-β signaling may contribute to the onset and progression of cerebrovascular diseases. While there are not many studies on the mechanisms of cerebrovascular diseases related to TGF-β dysfunction, we discuss the three most studied cerebrovascular diseases caused by dysregulation of TGF-β signaling.

Cerebral Cavernous Malformation (CCM)

Cerebral cavernous malformation is a cerebrovascular disease causing recurrent cerebral hemorrhage, headaches, seizures and stroke, which is histologically characterized by clusters of dilated vascular sacs with ECs lacking tight junctions and mural cell coverage (Goldstein and Solomon, 2017; Stapleton and Barker, 2018). Genetically, CCMs can be categorized into familial and sporadic types. Approximately, 20% of all CCMs are Familial CCMs which present autosomal dominant inheritance with loss-of-function germline mutations in any one of the following three genes: *CCM1/KRIT1*, *CCM2/malcavernin*, or *CCM3/PDCD10* (Zafar et al., 2019). The sporadic CCMs are non-hereditary and are probably caused due to somatic mutations of CCM genes (McDonald et al., 2014).

In both familial and sporadic CCM patients, TGF-β signaling is activated during pathological progression, as indicated by nuclear accumulation of endothelial pSMAD3 accompanied by expression of EndMT markers in lesions of familial and sporadic cavernomas (Maddaluno et al., 2013; Bravi et al., 2016). Besides, Kruppel-like factor 2 (KLF2) and KLF4, the activators of the BMP signaling, are significantly upregulated in ECs of familial and sporadic CCM lesions (Cuttano et al., 2016; Zhou et al., 2016). Activated TGF-β/BMP signaling has also been observed in cultured cells wherein all three Ccm genes were knocked down in ECs (Maddaluno et al., 2013; Cuttano et al., 2016), especially under low fluid shear stress conditions (Li et al., 2019).

The activation of TGF-β/BMP signaling has been confirmed in endothelial specific Ccm1 and Ccm3 knockout mice. Ablation of Ccm1 in ECs activates the expression of endogenous Bmp6 which induces the upregulation of pSMAD1 and pSMAD3 and triggers EndMT resulting in cerebral vascular malformations (Maddaluno et al., 2013). The upregulation of Bmp6 caused by mutant Ccm1 could be mediated by KLF4 which directly binds to the promoters of Bmp6 and some EndMT markers to induce their expression (Cuttano et al., 2016; **Figure 2**). Moreover, increased levels of pSMAD1 and pSMAD3 were observed in ECs of endothelial Ccm3 knockout mice (Bravi et al., 2015). Small-molecule inhibitors of TGFBR, pSMAD or BMP signaling could prevent EndMT and reduce the size and number of cerebral malformations, demonstrating that dysregulation of TGF-β/BMP signaling directly contributes to the onset and pathological process of CCMs (Maddaluno et al., 2013).

Some studies have uncovered the causal function of mitogen-activated protein kinase kinase Kinase 3 (MEKK3) and KLF2/4 in CCM pathogenesis, which is independent of TGF-β/SMAD signaling (Zhou et al., 2016). Endothelial-specific disruption of Mekk3, Klf2 or Klf4 significantly suppresses CCM and rescues the lethal phenotype in Ccm2 mutant mice. Consistently, the levels of KLF2 and KLF4 are increased in ECs of lesions in familial and sporadic CCM patients (Cuttano et al., 2016; Zhou et al., 2016). Supportively, ponatinib, a small-molecule compound inhibits MEKK3 activity to increase expression of the downstream Klf gene, suppresses CCM in neonatal Ccm1 deficient mouse models (Choi et al., 2018). In addition, activation of TLR4 by Gram-negative bacteria and lipopolysaccharide injection could increase the expression of Klf2/4 and promote CCM formation in Ccm1 and Ccm2 knockout mice (Tang et al., 2017; **Figure 2**). These inconsistencies with respect to the role of TGF-β signaling in the development and progression of CCM might be largely due to the different genetic backgrounds of the mouse models used, and the different stages of CCM pathogenesis analyzed in different experiments. Additional genetic rescue experiments might be helpful to further demonstrate the causal link between dysregulation of TGF-β signaling and the development and progression of CCM.

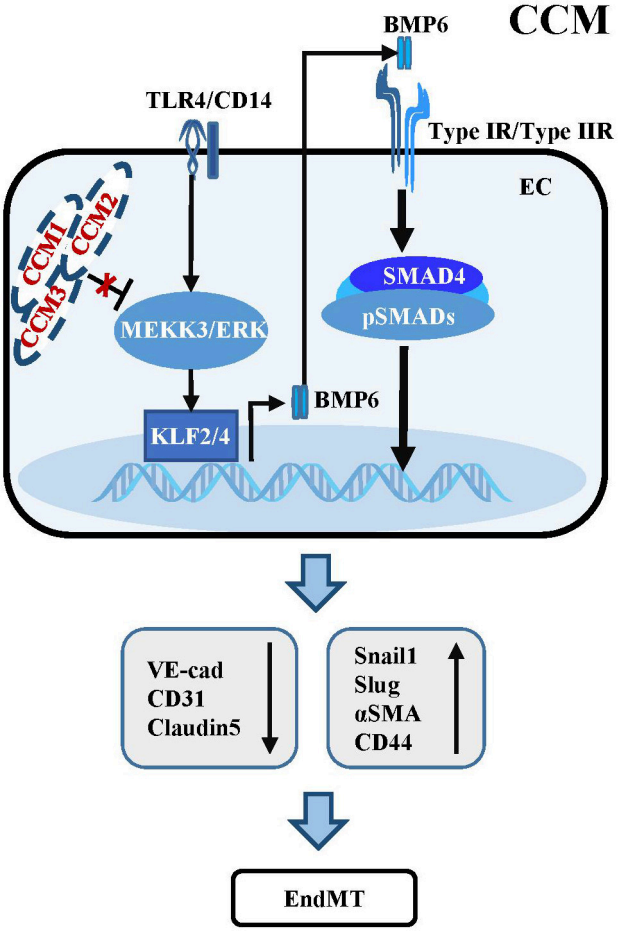

FIGURE 2 | Dysregulation of TGF-β/BMP signaling in CCM. Mutant CCM release the inhibition of MEEK3/ERK pathway, which trigger the expression of KLF2/4. KLF2/4 subsequently suppress the expression of endothelial markers and induce the expression of EndMT-related molecules. KLF4 could also transcriptionally upregulate BMP6 to active TGF-β/BMP cascades in ECs. Besides, activation of TLR4 associated with CD14 by Gram-negative bacteria and lipopolysaccharide injection could increase the expression of Klf2/4 and promote CCM formation.

Hereditary Hemorrhagic Telangiectasia (HHT)

Hereditary hemorrhagic telangiectasia, also known as Osler-Weber-Rendu syndrome, is an autosomal dominant genetic disorder characterized as telangiectasia and AVMs affecting

vessels in multiple organs and tissues including the brain (Brinjikji et al., 2015; Kritharis et al., 2018). Five types of HHT have been described, and HHT1 and HHT2 contribute to the disease in more than 80% of patients with definite HHT (Brinjikji et al., 2015). Some HHT patients display brain AVMs, often accompanied by cerebral hemorrhage, seizure, headache, or focal neurologic symptoms (Brinjikji et al., 2017a,b). Genetic screening of HHT patients has identified four mutated genetic loci, all of which are involved in the TGF-β signaling pathway, including BMP9 ligand encoding gene *GDF2* (HHT5 or HHT like), type I receptor ALK1 encoding gene *ACVRL1* (HHT2), co-receptor endoglin encoding gene *ENG* (HHT1) and intracellular mediator SMAD4 encoding gene *MADH4* (JP-HHT) (McAllister et al., 1994; Johnson et al., 1996; Gallione et al., 2004; Wooderchak-Donahue et al., 2013; **Figure 3**).

BMP9/10-ALK1 signaling suppresses HHTs through SMAD-dependent or SMAD-independent pathways. Endothelial-specific knockout of Alk1 triggers cerebral AVMs mimicking the pathologic characteristics of HHT (Park et al., 2009). In adult mouse, combined with VEGF stimulation, knockout of Alk1 could alter cerebral arteriovenous molecule specificity and induce AVMs (Walker et al., 2011). Zebrafish harboring mutations in Bmp9 and duplicate Bmp10 paralogs, Bmp10 and Bmp10-like exhibit cranial AVMs mimicking Acvrl1 mutants (Capasso et al., 2020). In postnatal retina, BMP9/10 ligand blockade and endothelial-specific homozygous ALK1 inactivation induces excessive angiogenesis via activating VEGF and PI3K/Akt signaling (Ola et al., 2016; Ruiz et al., 2016; Alsina-Sanchis et al., 2018). Pharmacological or genetic inhibition of PI3K rather than VEGFR could abolish ALK1-induced vascular hyperplasia in vivo, confirming that PI3K/Akt is the core mechanism downstream of BMP9/10-ALK1 signaling in maintaining vascular quiescence (Alsina-Sanchis et al., 2018; Ola et al., 2018; Iriarte et al., 2019; **Figure 3**).

Mice with homozygous or heterozygous deletion of Eng with VEGF treatment exhibit brain AVMs (Choi et al., 2012, 2014), and endothelial-specific Eng knockout mice spontaneously develop AVMs in the retina or brain (Mahmoud et al., 2010; Choi et al., 2014). In cerebral and retinal vessels, the Eng-null ECs cannot migrate against blood flow toward the arteries, leading to the accumulation and proliferation of ECs thereby triggering AVMs. Increased VEGFA expression which activates PI3K/Akt signaling through VEGFR2 may be responsible for stimulating sprouting angiogenesis and promoting venous differentiation in Eng mutant mice (Jin et al., 2017). Consistently, a recent study showed that ECs lacking Eng exhibit increased VEGF sensitivity and abnormal proliferation resulting in the formation of peripheral AVM (Tual-Chalot et al., 2020).

The essential role of endothelial SMAD4 in the maintenance of cerebrovascular integrity has been demonstrated by the study using a brain endothelial specific Smad4 knockout mouse, which develops phenotypes partially simulating HHT patients, such as dilated vessels, increased EC proliferation, intracranial hemorrhage and BBB breakdown (Li et al., 2011). Postnatally inducible endothelial Smad4 knockout results in AVM in neonatal and adult mice, which is comparable with the phenotypes observed in inducible endothelial Alk1 and Eng knockout mice (Crist et al., 2018; Kim et al., 2018; Ola et al., 2018). Mechanistically, SMAD4-mediated BMP9/10-ALK1 signaling inhibits the transcription of casein kinase 2 (CK2) which limits PTEN phosphorylation and PI3K/Akt activation, thereby preventing AVMs in the brain, retina, and gastrointestinal tract (Ola et al., 2018). In addition, Smad4 knockout leads to increased angiopoietin-2 (Angpt2) expression in ECs, which might cause AVM by changing the size and shape of ECs in the retina of Smad4 mutant mice (Lan et al., 2007; Crist et al., 2019; **Figure 3**).

These studies based on mouse models that mimic human HHT patients have provided the causal link between dysregulated TGF-β signaling and the pathogenesis of HHT. Blood flow stimulates BMP9-ALK1-ENG-SMAD4 signaling to maintain EC quiescence by suppressing EC proliferation and inducing pericyte

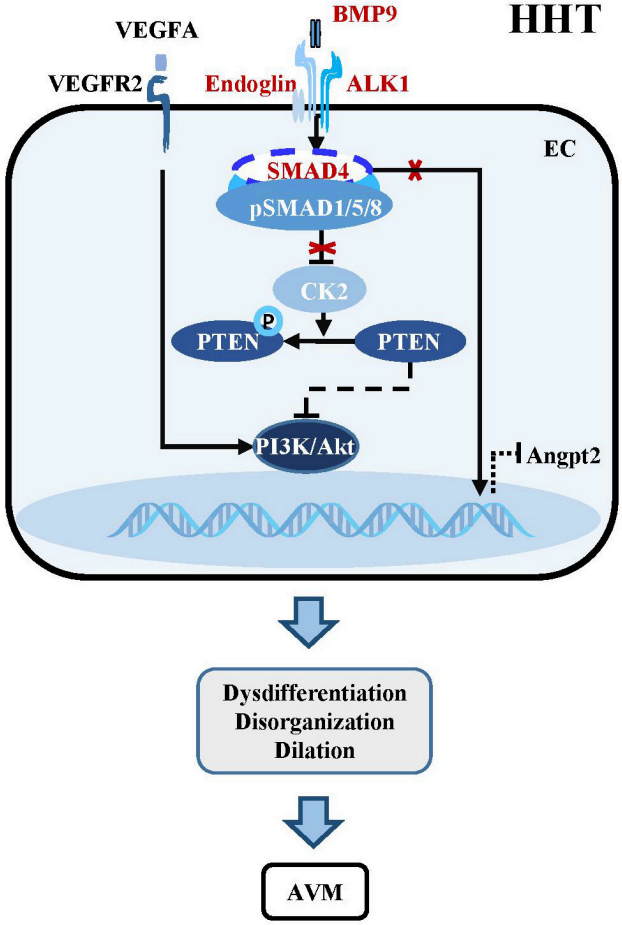

FIGURE 3 | Dysregulation of BMP-ALK1-Smad4 signaling in HHTs. The mutant components within the BMP-ALK1 pathway including BMP9, ALK1, Endoglin, and SMAD4 are unable to suppress the transcription of CK2. Therefore, PTEN is phosphorylated by CK2 and loss the capacity to inhibit the PI3K/Akt pathway, which result in dysdifferentiation, disorganization, dilation and subsequent AVMs in cerebrovasculatures. Besides, AVMs-caused hypoxia increases the level of VEGFA, which subsequently binds VEGFR2 to active downstream PI3K/Akt signaling to aggravate the pathological process of AVMs. In addition, mutant SMAD4 increases the expression of angiopoietin-2 (Angpt2), leading to AVMs.

recruitment (Baeyens et al., 2016), which involves PI3K/Akt signaling, Angpt2 signaling and possibly other factors (Alsina-Sanchis et al., 2018; Ola et al., 2018; Crist et al., 2019). It is worth noting that AVMs develop due to a combination of gene mutations in TGF-β signaling with angiogenic induction (via VEGF stimulation or wounding) (Park et al., 2009; Garrido-Martin et al., 2014), supporting the "Two hit mechanism" in HHT. Consistently, the tissues that are most vulnerable to AVMs or telangiectasia are those-susceptible to repeated damage and repair, such as the face, lips, and fingers in HHT patients (Brinjikji et al., 2015). Further investigation is required to elucidate whether dysregulation of other signaling pathways which cross talk with the TGF-β signaling pathway could serve as the second hits in the pathogenesis of HHT.

Cerebral Autosomal Recessive Arteriopathy With Subcortical Infarcts and Leukoencephalopathy (CARASIL)

Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy is a rare autosomal recessive cerebrovascular disease that mainly occurs in cerebral white matter and basal ganglia, causing early adult-onset dementia, gait disturbance, alopecia, and low back pain (Nozaki et al., 2014; Tikka et al., 2014). Histologically, CARASIL displays cerebral arteriopathy showing fibrous proliferation of intima, loss of vascular SMCs and thickening of meningeal and parenchymal arteries. Fibrous hyperplasia in arteries results in the impaired contraction, leading to subcortical lacunar infarcts and subsequent vascular dementia (Oide et al., 2008; Ito et al., 2016). Genetically, mutations in high-temperature requirement serine peptidase A1 (*HTRA1*) gene have been identified to be associated with CARASIL (Hara et al., 2009). In patients, TGF-β1-pSMAD2 activation was observed in cerebral small arteries (Hara et al., 2009; Shiga et al., 2011).

The mechanistic role of TGF-β signaling in the pathogenesis of CARASIL, is a debatable topic. HRTA1 is a serine protease which is strongly expressed in ECs, vessel SMCs and pericytes (De Luca et al., 2003). HtrA1 knockout mice display a significantly decreased retinal vascular density which coincides with patients presenting reduced cerebral small vessels (Zhang et al., 2012). Mechanistically, HTRA1 cleaves the pro-domain of proTGF-β1 and TGF-β receptors to antagonize TGF-β signaling (Oka et al., 2004; Shiga et al., 2011; Graham et al., 2013). Consistently, HtrA1 knockout either in vivo or in cultured cells induces the expression of TGF-β ligands and activates pSMAD2/3 signaling (Zhang et al., 2012; Klose et al., 2019). All these results indicate that abnormal activation of TGF-β signaling contributes to the pathogenesis of CARASIL. However, there are studies showing that impaired TGF-β signaling is involved in CARASIL pathogenesis (Beaufort et al., 2014; Fasano et al., 2020). Loss of HtrA1 leads to defective HTRA1-mediated LTBP-1 processing and reduced TGF-β signaling (Beaufort et al., 2014), and fibroblasts derived from *HTRA1* mutation carriers exhibit no significant change in pSMAD2/3 expression (Fasano et al., 2020). The possible reasons for the discrepancy might partially be due to different *HTRA1* mutations leading to different outcomes (Verdura et al., 2015; Lee et al., 2018). Future studies should identify the natural characteristics of CARASIL associated mutations and develop animal models that accurately mimic all pathological and molecular aspects of human CARASIL patients, which will help uncover the mechanisms of CARASIL and discover new therapeutic targets.

POTENTIAL THERAPIES TARGETING TGF-β SIGNALING

Current therapies for CCM, HHT, and CARASIL patients mainly rely on surgery or relieving complications (Tikka et al., 2014; Kritharis et al., 2018; Stapleton and Barker, 2018). Recent advances in understanding the mechanisms of dysfunctional TGF-β signaling which results in cerebrovascular diseases has provided hope to develop pharmacological and genetic therapies for these diseases.

Activated TGF-β/BMP signaling has been demonstrated to contribute to the onset and progression of CCMs in patients and mouse models (Maddaluno et al., 2013; Bravi et al., 2016; Cuttano et al., 2016). Therefore, it is expected that therapeutics targeting TGF-β/BMP signaling would be beneficial for CCMs. Indeed, TGFBR1/pSMAD inhibitors LY364947 and SB431542 as well as BMPR1 inhibitor dorsomorphin (DMH1) strikingly reduce the level of pSMAD1 and pSMAD3, prevent the expression of EndMT markers, and decrease the number and size of vascular malformation lesions in CCM1 mutant mice (Maddaluno et al., 2013). KLF4 has been shown to be a good therapeutic target for CCM. Ccm1 knockout results in MEKK3-MEK5-ERK5-MEF2 signaling dependent activation of KLF4 which promotes the expression of Bmp6. A specific MEK5 inhibitor BIX-02189 (Tatake et al., 2008) significantly decreases pERK5 and KLF4 expression, inhibits Bmp6 upregulation and EndMT in CCM1 deficient ECs (Cuttano et al., 2016), indicating that inhibitors of the MEKK3-MEK5-ERK5-MEF2 axis might be useful for suppressing BMP signaling and EndMT in the pathogenesis of CCM. There are several novel drugs targeting TGF-β signaling, developed through preclinical trials and further tested in clinical trials, including anti-ligand antisense oligonucleotides (ASOs), ligand-competitive peptides, antibodies targeting ligands, receptors or associated proteins, and inhibitors against TGF-β receptor kinases for various diseases (Akhurst and Hata, 2012; Graham et al., 2013; Kemaladewi et al., 2014; Aykul and Martinez-Hackert, 2016; Wu et al., 2017; Holmgaard et al., 2018). It is worth examining whether these candidate drugs that target TGF-β signaling could inhibit the progress of CCMs.

The majority of HHT patients have pathogenic loss of function mutations in TGF-β signaling. Although many studies have uncovered the molecular mechanisms underlying HHTs caused by dysfunctional TGF-β signaling, there is currently no efficient drug for HHT treatment. Current drug therapy regimens mainly focus on interfering with the downstream core signaling pathway such as activated VEGF and PI3K/Akt signaling (Alsina-Sanchis et al., 2018; Ola et al., 2018). Since haploinsufficiency of endoglin and ALK1 have been identified as the causes of HHT1 and HHT2, a better understanding of the regulation of their expression

levels at the transcriptional level or post-transcriptional level will help developing therapeutic strategies targeting endoglin and ALK1 expression or function. Indeed, a very recent study shows that ALK1-overexpression could normalize SMAD and NOTCH target gene expression, restore the effect of BMP9 on suppression of p-Akt, and inhibit the development of AVMs in Alk1- and Eng-inducible knockout mice, suggesting that ALK1 overexpression or activation might be a potential therapeutic strategy for HHT patients (Kim et al., 2020).

Genome editing may serve as the final solution. CRISPR-based genome editing has been demonstrated as a powerful tool for treating genetic diseases (Pickar-Oliver and Gersbach, 2019). The CRISPR-Cas9 system has been demonstrated to efficiently correct gene mutations in various mouse models of human diseases, including cataracts, muscular dystrophy and many others (Wu et al., 2013; Long et al., 2014). Recent studies show that base editing can correct mutations in human cells and in a mouse model of genetic deafness, and a newly developed template-free Cas9 editing is able to precisely correct the pathogenic mutations in human cells (Gaudelli et al., 2017; Gao et al., 2018; Shen et al., 2018). A newly developed CRISPR-CasΦ system, with a molecular weight which is only half of Cas9 or Cas12a displays expanded target recognition capabilities and is functional in human cells as well (Pausch et al., 2020), providing a new genome editing tool for treating cerebrovascular diseases. Once the causal link between mutations in TGF-β signaling and cerebrovascular diseases has been established, genome editing will likely correct these mutant genes to heal the related cerebrovascular diseases.

CONCLUSION AND PERSPECTIVES

Previous studies have demonstrated the crucial function of TGF-β signaling in cerebral vasculature development and integrity, and uncovered the causal link between the dysfunctional TGF-β signaling and the onset or progression of several cerebrovascular diseases such as CCM, HHT and CARASIL. However, the related mechanisms underlying the dysregulation of TGF-β signaling resulting in cerebrovascular diseases remains to be further elucidated. In recent years, using the rapidly developed single cell sequencing technology and advanced graphics algorithm, researchers have revealed the unappreciated heterogeneity and plasticity of human and mouse cerebral blood vessels, discovering not only new markers for different subtypes of ECs but also a new cell type adjacent to the blood vessel (Schaum et al., 2018; Vanlandewijck et al., 2018; Kalucka et al., 2020). Further investigation of the role and mechanism of TGF-β signaling in the regulation of cerebrovascular heterogeneity and plasticity will help to understand the function of TGF-β signaling in the occurrence and development of cerebrovascular diseases.

There are not many animal models that can accurately mimic the genetic and pathological characteristics of human cerebrovascular diseases. Rapid advances in genome editing technologies based on CRISPR-Cas systems provide powerful tools for generating animal models carrying genomic mutations precisely mimicking the ones in human patients (Pickar-Oliver and Gersbach, 2019). Studies using cell lineage tracing technology combined with single cell sequencing in animal models of human cerebrovascular diseases will help reveal the cellular and molecular mechanisms of cerebrovascular diseases and discover new therapeutic targets. In addition, human cortical organoids with functional cerebral vessels will provide valuable models for dissecting the roles of TGF-β signaling in the development and progression of human cerebrovascular diseases (Cakir et al., 2019).

Although recent advances have indicated that targeting TGF-β signaling will be a potential strategy for the treatment of cerebrovascular diseases, clinical transformation is still challenging. Considering the cell context-dependent pleiotropic roles of the TGF-β signaling pathway, the selectivity and dosage of targeted drugs may be crucial for the desired therapeutic effects. Previous research has identified various TGF-β inhibitory drugs involving almost every level in the TGF-β signaling cascade, some of which have been proved safe and effective for treating systemic sclerosis, cancers or idiopathic pulmonary fibrosis in clinical trials (Rice et al., 2015; Yingling et al., 2018; Joyce et al., 2019; Kelley et al., 2019; Papachristodoulou et al., 2019; Santini et al., 2019). These existing TGF-β inhibitory drugs provide potential therapeutic opportunities for treating cerebrovascular diseases with activated TGF-β signaling. For cerebrovascular diseases with loss-of-function mutations in TGF-β signaling, somatic genome editing may provide tools to correct the mutations or enhance TGF-β signaling.

Increased clinical research data shows that there is a close correlation between cerebrovascular and central nervous system diseases. Abnormal cerebrovascular structure and function are closely related to brain atrophy, dementia and various neurodegenerative disorders and cognitive impairment (Turner et al., 2016; Yang et al., 2017; Iadecola and Gottesman, 2018; Kummer et al., 2019). Dysregulated TGF-β signaling has been observed in neurodegenerative diseases accompanied by cerebrovascular abnormalities (von Bernhardi et al., 2015). Further studying the synergistic mechanisms by which TGF-β signaling maintains the homeostasis of the cerebrovascular and central nervous system might be very helpful in uncovering the direct causal link between cerebrovascular and central nervous system diseases, providing new theoretical basis and treatment strategies for joint preventing and treating cerebrovascular and central nervous system diseases.

AUTHOR CONTRIBUTIONS

YZ and XY wrote the review. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Metformin Attenuates Renal Fibrosis in a Mouse Model of Adenine-Induced Renal Injury Through Inhibiting TGF-β1 Signaling Pathways

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It is well-known that all progressive chronic kidney disease (CKD) is pathologically characterized by tubulointerstitial fibrosis process. Multiple studies have shown the critical role of inflammation and fibrosis in the development of CKD. Hence strategies that target inflammatory and fibrotic signaling pathways may provide promising opportunities to protect against renal fibrosis. Metformin has been used as the first-line glucose-lowering agent to treat patients with type 2 diabetes mellitus (T2DM) for over 50 years. Accumulating evidence suggests the potential for additional therapeutic applications of metformin, including mitigation of renal fibrosis. In this study, the anti-fibrotic effects of metformin independent of its glucose-lowering mechanism were examined in an adenine -induced mouse model of CKD. Expressions of inflammatory markers MCP-1, F4/80 and ICAM, fibrotic markers type IV collagen and fibronectin, and the cytokine TGF-β1 were increased in adenine-induced CKD when compared to control groups and significantly attenuated by metformin treatment. Moreover, treatment with metformin inhibited the phosphorylation of Smad3, ERK1/2, and P38 and was associated with activation of the AMP-activated protein kinase (AMPK) in the kidneys of adenine-treated mice. These results indicate that metformin attenuates adenine-induced renal fibrosis through inhibition of TGF-β1 signaling pathways and activation of AMPK, independent of its glucose-lowering action.

Keywords: metformin, renal fibrosis, adenine-induced renal injury, transforming growth factor β1 signaling pathways, animal model

INTRODUCTION

Chronic kidney disease (CKD) is a global public health problem. All patients with CKD gradually lose kidney function, with the rate of functional decline varying depending on the disease and patient co-morbidity. When kidney impairment becomes evident there are limited effective treatments available. Current strategies slow the progression of CKD by controlling the underlying cause, including glucose control in Type 1 or type 2 diabetes, treatment of high blood pressure,

specific therapies for glomerulonephritis, interstitial nephritis, polycystic kidney disease, relief of obstruction of the urinary tract and treatment of recurrent kidney infection, etc. When CKD progresses to end-stage kidney failure, dialysis and kidney transplantation are required which usually results in significant associated health and social needs, personal loss of independence, a decline in functional capacity and burdens on the health, and societal support systems. Despite tremendous efforts focused on finding efficient therapies that target the progression of tubulointerstitial fibrosis, few therapies are available.

Metformin is the most widely accepted first-line treatment to lower blood glucose levels in patients who have type 2 diabetes mellitus. In addition to its role in lowing blood glucose levels, recent reports suggested it has anti-oncogenic (Leone et al., 2014), cardio-protective (Xiao et al., 2010), and anti-inflammatory effects (Kita et al., 2012). Metformin can attenuate cyclosporine A-induced renal fibrosis in rats (Lin et al., 2019), modulate immune cell infiltration into the kidney during unilateral ureteral obstruction (UUO) in mice (Christensen et al., 2019), significantly reduces renal fibrosis induced by folic acid (Lee et al., 2018; Yi et al., 2018) and ameliorates diabetic nephropathy in a rat model of low-dose streptozotocin-induced diabetes (Zhang et al., 2017), which suggest an antifibrotic effect as well as superior safety and relatively low risk of side effects. However, multiple complex mechanisms as well as different amplifying risk factors are involved in the development of renal fibrosis. Metformin use is currently limited to patients with Type 2 diabetes mellitus and normal renal function or stage 1–3 CKD. Hence there is a need to more fully understand the benefits of metformin in CKD independent of diabetes mellitus and in the presence of significant renal pathology where the magnitude of benefit may be even greater. The adenine induced CKD animal model was developed by Yokozawa et al. (1982). It is well-reported that oral administration of adenine in mice causes classic morphological, biochemical and histopathological alterations in the kidneys, which mimic pathological changes of CKD in humans (Ortiz et al., 2000; Eddy et al., 2012). Previous studies have shown that adenine induces renal functional impairment, myofibroblast activation, sterile inflammatory responses and accumulation of cellular matrix proteins (including collagens and fibronectin) in the renal interstitium. Its mechanism of toxicity has been extensively evaluated with many similarities to human tubulointerstitial pathology (Kashioulis et al., 2018; Abellán et al., 2019; Gong et al., 2019; Ichida et al., 2020; Neven et al., 2020; Sieklucka et al., 2020). Hence this model has been widely used as an animal model of tubulointerstitial kidney disease (Jia et al., 2013; Mishima et al., 2015; Bobeck et al., 2017). This study was undertaken to define the renal protective role of metformin in adenine-induced renal injury. Given the robustness of the development of tubulointerstitial fibrosis, it is an ideal model to assess glucose-independent mechanisms of metformin induced renoprotection and adds to the body of knowledge regarding the benefit of metformin in CKD. Hence, in this study, the renoprotective properties of metformin were explored in a mouse model of adenine-induced renal injury.

MATERIALS AND METHODS

Animal Studies

Eight-week-old male C57BL/6 mice (Kearns Facilities, Kolling Institute), weighing ∼20–25 g, were randomly divided into four experimental groups: (1) Control group, (2) Control with Metformin, (3) Adenine, and (4) Adenine with Metformin. Mice were assigned to receive either 4 mg adenine in 200 μl water every day for 21 days, or water alone by oral gavage. Adenine was delivered to mice through oral gavage to avoid the variability of the effect of adenine due to the differential food intake amongst mice. The mice received metformin (0.4 mg/ml) in their drinking water immediately coincident with adenine treatment. The consumption of daily intake of water for each mouse was recorded. No significant differences in water intake were noted between the groups. After treatment with/without metformin for 21 days, a 24-h urine was collected before the animals were sacrificed. The Albuwell M kit and the Creatinine Companion kit (Exocell Inc., Philadelphia, PA) were used to analyze the 24-h urine albumin and creatinine (Philadelphia, PA).

This experiment was conducted according to the recommendations of the National Health and Medical Research Council of Australia and was approved by the Northern Sydney Local Health District Animal Ethics Committee (RESP/17/163).

Histology and Immunostaining

Paraffin-embedded kidney sections were used for immunohistochemistry staining. After blocking at room temperature for 10 min, the sections were incubated with the diluted primary antibodies (Dako CA) against anti-type IV collagen (1:500), anti-fibronectin (1:500), and anti-TGF-β1(1:500) at 4°C overnight. After incubation with secondary antibodies, sections were developed with DAB (Dako, CA) before being counterstained with hematoxylin. The sections were then quantified using Image J software (Huang et al., 2014). Masson's trichrome staining (American MasterTeck, Lodi, CA) was used to assess tubulointerstitial injury, which was blindly scored using Photoshop software. Interstitial fibrosis, tubular dilation, atrophy, cast formation, or inflammatory cell infiltration were considered as being indicative of interstitial fibrosis (Farris et al., 2011; Martin-Sanchez et al., 2017).

RNA Isolation and RT-PCR Analysis

Bioline RNA Mini Kit (Bioline, NSW) was used to extract total RNA from mice kidney tissues. The iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize the cDNA, which was used for quantitative real-time PCR using the SYBR green PCR master mix kit (Invitrogen, CA) with the intron-spanning primers as shown in **Table 1**. The qPCR was run on ABI-Prism-7900 Sequence Detection System (Applied Biosystems). The quantitation of the mRNAs was performed using the $2^{-\Delta\Delta Ct}$ method with β-actin as the internal control (Livak and Schmittgen, 2001).

Western Blotting Analysis

Kidney tissue lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham). After incubation with primary antibodies including type IV collagen

TABLE 1 | Nucleotide sequences of the primers used for quantitative real time PCR.

Target	Forward (5′-3′)	Reverse (5′-3′)
Type IV collagen	TTAAAGGACTCCAGGGACCAC	CCCACTGAGCCTGTCACAC
Fibronectin	CCCTATCTCTGATACCGTTGTCC	TGCCGCAACTACTGTGATTCGG
MCP-1	GCCTGCTGTTCACAGTTGC	CAGGTGAGTGGGGCGTTA
F4/80	CCTGGACGAATCCTGTGAAG	GGTGGGACCACAGAGAGTTG
ICAM1	GTGGCGGGAAAGTTCCTG	CGTCTTGCAGGTCATCTTAGGAG
TGF-β1	TCAGACATTCGGGAAGCAGT	ACGCCAGGAATTGTTGCTAT
β-actin	CAGCTGAGAGGGAAATCGTG	CGTTGCCAATAGTGATGACC

(1:5,000) (Abcam), fibronectin (1:5,000) (Abcam), α-tubulin (1:10,000) (Sigma-Aldrich), p-p38 (1:500) (Cell Signaling), p38 (1:500) (Cell Signaling), ERK1/2 (1:500) (Cell Signaling), p-ERK1/2 (1:500) (Cell Signaling), p-Smad3 (1:500) (Cell Signaling), and p-AMPK (Cell Signaling) at 4°C overnight, the membranes were incubated with HRP-conjugated secondary antibody (1:5,000) (Amersham, Little Chalfont, United Kingdom) for 1 h. The bands were visualized with ECL and analyzed quantitatively by densitometry using LAS-4000 Imaging System (FUJIFILM, Japan).

Statistical Analysis

Data were expressed as mean ± SEM and analyzed by one-way ANOVA, followed by Tukey post-test analysis for comparison among multiple groups. *P*-values of $p < 0.05$ were considered statistically significant.

RESULTS

Metformin Attenuates Adenine-Induced Renal Injury

To determine the effect of metformin on biomarkers of renal pathology, 24-h urine was collected to assess the urinary albumin and albumin to creatinine ratio (UACR). **Figure 1A** shows an increase in urinary albumin in the adenine exposed group (57.23 ± 0.54 mg/24 h) compared to the control group (33.33 ± 0.93 mg/ 24 h; $P < 0.001$), which was significantly attenuated by metformin treatment (44.43 ± 0.72 mg/24 h) (**Figure 1A**, $p < 0.001$ vs. adenine alone). Similarly, UACR was significantly increased in adenine exposed group (11.93 ± 0.40 mg/g) compared to control group (6.83 ± 0.05 mg/g) (**Figure 1B**, $p < 0.001$), which was reduced by metformin treatment (8.20 ± 0.26 mg/g) (**Figure 1B**, $p < 0.01$ vs. adenine treatment). These data indicated that metformin attenuates adenine-induced renal injury.

Metformin Reduces Extracellular Matrix Deposition and Tubulointerstitial Fibrosis in a Mouse Model of Adenine-Induced Renal Injury

To define the effect of metformin in fibrotic responses induced by adenine, extracellular matrix type IV collagen and fibronectin mRNA and protein were assessed. RT-PCR analyses showed that the mRNA expression of type IV collagen and fibronectin were significantly increased in kidneys of mice administrated adenine compared to the control group, which was attenuated by metformin treatment (**Figures 2A,B**, $p < 0.001$). Consistently, immunohistochemistry analyses and western blotting results showed a significantly increased staining of type IV collagen and fibronectin (**Figures 2E–H**, $p < 0.001$) in kidneys of mice administrated adenine compared to the control group. Metformin treatment reduced type IV collagen and fibronectin deposition in kidneys of adenine exposed mice compared with control mice (**Figures 2E–H**, $p < 0.001$). Interstitial extracellular matrix deposition was examined by Masson's trichrome staining. A significant increase in tubulointerstitial injury was observed in kidneys of mice administrated adenine compared to the control group ($p < 0.001$), which was attenuated by metformin treatment (**Figures 2C,D**, $p < 0.001$). These results indicate that metformin reduces extracellular matrix overproduction and renal fibrosis in a model of adenine-induced renal injury.

Metformin Prevents Inflammatory Responses in a Mouse Model of Adenine-Induced Renal Injury

It is well-known that chronic inflammation promotes the development of tissue fibrosis. The expression of inflammatory markers including macrophage chemotactic protein (MCP-1) and macrophage activation markers F4/80 and intercellular adhesion molecule 1 (ICAM1) was used to determine the role of metformin in the regulation of inflammation. MCP-1 is considered a critical marker of renal inflammation in models of kidney injury (Doi et al., 2006). The qRT-PCR result showed that MCP-1 level was increased by 32.12-fold in the kidneys of mice administrated with adenine compared to control (**Figure 3A**, $p < 0.001$). Metformin treatment attenuated adenine induced MCP-1 level in kidneys (**Figure 3A**, $p < 0.001$). The expression of F4/80 and ICAM1 was increased in the adenine exposed mice compared to the control mice (**Figures 3B,C**, $P < 0.001$), which was attenuated by metformin treatment ($P < 0.05$). These results suggest that metformin prevents inflammation in adenine induced kidney injury by inhibiting proinflammatory cytokine production and macrophage infiltration.

Metformin Suppresses Upregulation of TGF-β1 in a Mouse Model of Adenine-Induced Renal Injury

TGF-β1, a key profibrotic growth factor, is crucial in the development of most forms of kidney disease. Hence TGF-β1 expression was examined in the kidneys of mice exposed to adenine +/− metformin. mRNA expression of TGF-β1 was significantly upregulated in kidneys of mice exposed to adenine compared with the control group (**Figure 4A**, $p < 0.001$), which was attenuated in metformin-treated mice (**Figure 4A**, $p < 0.001$). Consistently, immunohistochemical analyses also demonstrated that TGF-β1 protein expression was significantly increased in kidneys of mice exposed to adenine compared to the control group (**Figures 4B,C**, $p < 0.001$), which was limited

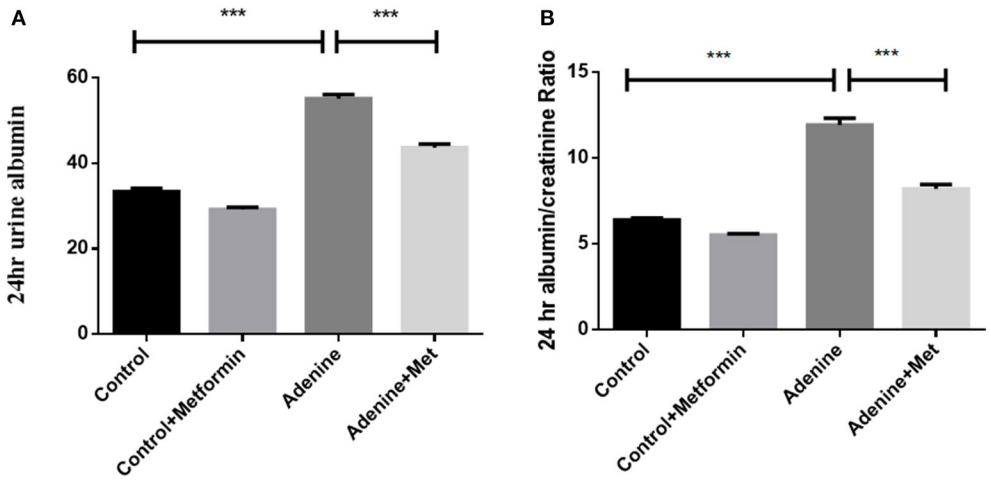

FIGURE 1 | Metformin attenuates adenine-induced renal injury. The 24-h urinary albumin excretion **(A)** and UACR **(B)** were significantly increased in adenine-induced mice compared to the control group, which were attenuated by metformin treatment. Data are expressed as mean ± SEM. $^{***}P < 0.001$, $n = 8$.

by metformin treatment (**Figures 4B,C**, $p < 0.001$). The results demonstrate that metformin inhibits overexpression of TGF-β1 mRNA and protein in the kidneys of mice with adenine induced kidney injury.

Metformin Suppresses TGF-β1 Signaling Pathways Through Inhibiting Activation of Smad3, ERK1/2 and p38 in a Mouse Model of Adenine-Induced Renal Injury

To examine if metformin inhibits TGF-β1 downstream signaling pathways in kidneys of mice with adenine induced kidney injury the phosphorylation of Smad3, ERK1/2, and p38 were examined in kidney tissues using western blot analyses. **Figure 5** showed that the phosphorylation of Smad3, ERK1/2, and p38 (**Figures 5A–C**, $p < 0.001$) were all significantly increased in kidneys of mice exposed to adenine compared to the control group, which was inhibited by metformin treatment (**Figures 5A,B**, $p < 0.001$, **Figure 5C**, $p < 0.01$). These data demonstrate that metformin confers renoprotection in the model of adenine-induced renal injury by inhibiting TGF-β1 signaling pathways.

Metformin Activated AMPK in a Mouse Model of Adenine-Induced Renal Injury

It is well-known that metformin acts through both AMPK-dependent and AMPK-independent mechanisms. To examine if the AMPK-dependent mechanisms were activated in mice with adenine induced kidney injury, the phosphorylation of AMPK was examined in kidney tissues using western blot analyses. **Figure 6** showed that although adenine did not reduce the phosphorylation of AMPK compared to control, metformin significantly increased p-AMPK compared to control and the adenine exposed groups (**Figure 6A**, $p < 0.001$). These data demonstrated that metformin may protect against renal injury through AMPK-dependent mechanisms.

DISCUSSION

This study was undertaken to determine if metformin attenuates renal injury in a non-diabetic model of tubulointerstitil renal injury and to elucidate the possible mechanisms. The present study uniquely demonstrates that metformin ameliorated extracellular matrix deposition and inflammation in an adenine-induced CKD mouse model. Furthermore, the study showed that metformin exerted its antifibrotic effect through suppression of TGF-β1 expression and downstream TGF-β1 signaling pathways.

Metformin, an adenosine monophosphate-activated protein kinase (AMPK) activator, is a commonly used drug to control blood glucose levels in patients with type 2 diabetes mellitus. Metformin has also been reported to limit liver, cardiac, lung and renal fibrosis (Xiao et al., 2010; Schuppan and Kim, 2013; Sato et al., 2016; Yi et al., 2018). Metformin's direct renoprotective role, independent of its glucose lowing effect, has been demonstrated in a high-fat diet, low-dose streptozotocin-induced rat model of diabetic kidney disease (Zhang et al., 2017). Metformin markedly attenuated characteristic renal pathological lesions and reduced glomerular basement membrane thickness, which was accompanied by decreased TGF-β1 expression (Zhang et al., 2017). However, the role of metformin on non-diabetic kidney disease models of renal injury is less well-studied and to date only in limited non-diabetic models of kidney disease. A prior study demonstrated that metformin suppressed macrophage infiltration, expression of markers of inflammation, extracellular matrix proteins, TGF-β1 expression, and interstitial fibroblast activation in obstructed kidneys which led to the conclusion that metformin prevents renal inflammation and fibrosis in mice (Cavaglieri et al., 2015). It has also been reported that metformin attenuated renal fibrosis in UUO mice due to inhibition of Ang-II-induced extracellular matrix production in renal fibroblasts through the inhibition of ERK signaling (Shen et al., 2016). A further study has demonstrated that the protective effects of metformin are mediated by AMPKα2-dependent

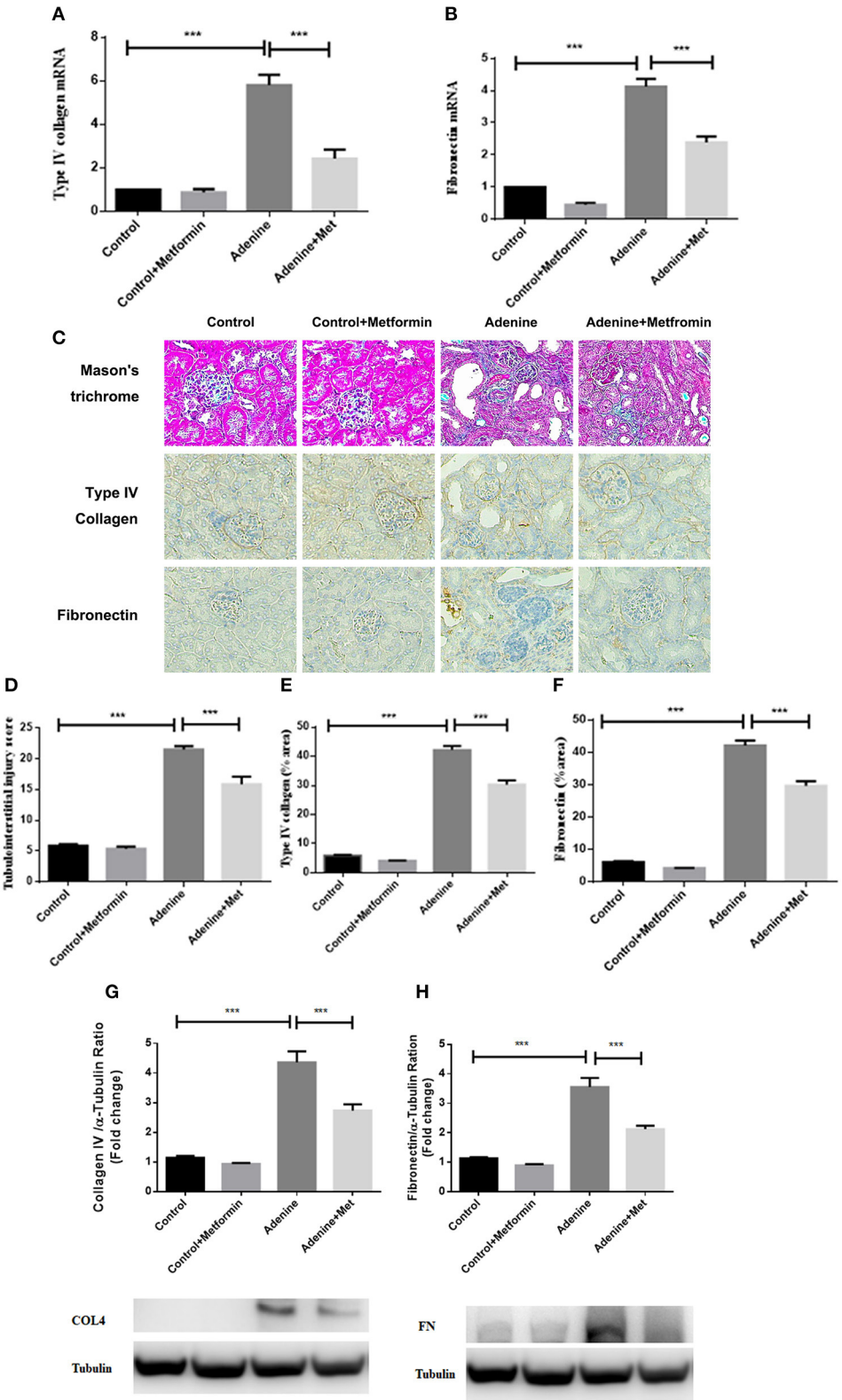

FIGURE 2 | Metformin reduces extracellular matrix deposition and tubulointerstitial fibrosis in a mouse model of adenine-induced renal injury. qRT-PCR results showed that the mRNA level of type IV collagen **(A)** and fibronectin **(B)** were significantly increased in adenine-induced mice compared to the control mice,

(Continued)

FIGURE 2 | which were reduced by metformin treatment. Masson's trichrome **(C,D)** and immunohistochemical staining **(E,F)** showed increased tubulointerstitial injury, type IV collagen, and fibronectin expression, which were reduced by metformin treatment. Western blots analysis showed that type IV collagen **(G)** and fibronectin **(H)** expression were significantly increased in adenine-induced mice compared to the control mice, which were reduced by metformin treatment. Data are expressed as mean ± SEM. $^{***}P < 0.001$, $n = 8$. Original magnification: ×200.

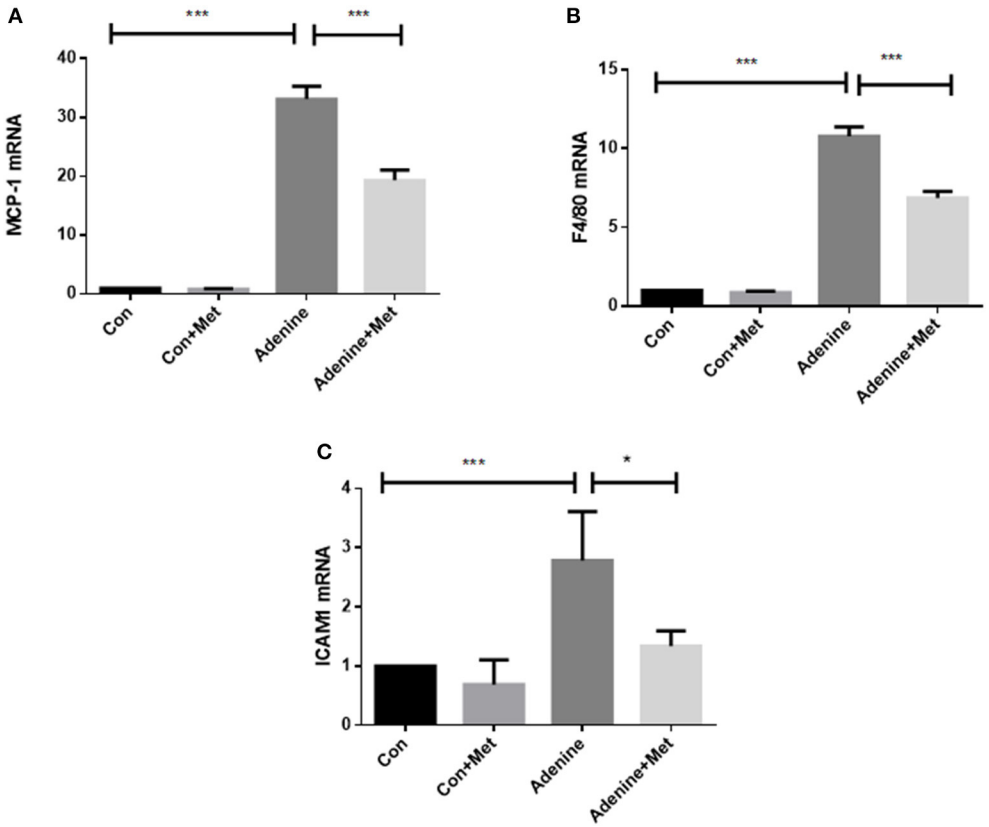

FIGURE 3 | Metformin prevents inflammatory responses in a mouse model of adenine-induced renal injury. qRT-PCR results showed that the mRNA expression of MCP-1 **(A)**, F4/80 **(B)**, and ICAM1 **(C)** were significantly increased in adenine treated mice compared to the control mice, which were reduced by metformin treatment. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$, $n = 8$.

and AMPKα2-independent targeting of TGF-β1 downstream signaling (Feng et al., 2017). Recently, metformin has been shown to modulate immune cell infiltration into the kidney in UUO mice, which limits subsequent fibrotic responses (Christensen et al., 2019). Metformin has also been shown to inhibit lipid accumulation and fibrosis in the kidneys of mice with nephropathy, and to increase fatty acid oxidation via modulation of Acetyl-CoA carboxylase by AMPK (Lee et al., 2018). Our previous study has also demonstrated that metformin treatment attenuated TGF-β1 induced inflammatory and fibrotic responses in human proximal tubular cells (HK2 cells) and folic acid-induced renal injury in C57BL mice (Guan et al., 2018; Malsin and Kamp, 2018; Wu et al., 2018; Yi et al., 2018; Yoshida et al., 2019).

Collectively, the anti-fibrotic role of metformin in diabetic kidney disease, obstructive nephropathy, and folic acid-induced nephropathy has been well-documented. Renal fibrosis is the common pathological endpoint of end-stage chronic kidney disease. The mechanisms of chronic kidney disease are complex due to different upstream causes and impacted upon by co-morbidities in an individual. The glucose independent role of metformin in the development of adenine-induced nephropathy in mice was assessed and confirmed in our study. Adenine significantly upregulated expression of type IV collagen, and fibronectin, and overall extracellular matrix in kidneys of mice with adenine-induced renal injury, which were significantly reversed by metformin treatment. These results add to the literature suggesting that metformin exerts anti-fibrotic effects in chronic kidney disease (Zhang et al., 2017; Lee et al., 2018; Yi et al., 2018; Christensen et al., 2019).

Sterile inflammation has an important role in initiating renal fibrosis (Lv et al., 2018). MCP-1 is the most studied mediator of renal inflammation (Tesch, 2008). MCP-1 promotes proliferation, infiltration, and production of more cytokines and

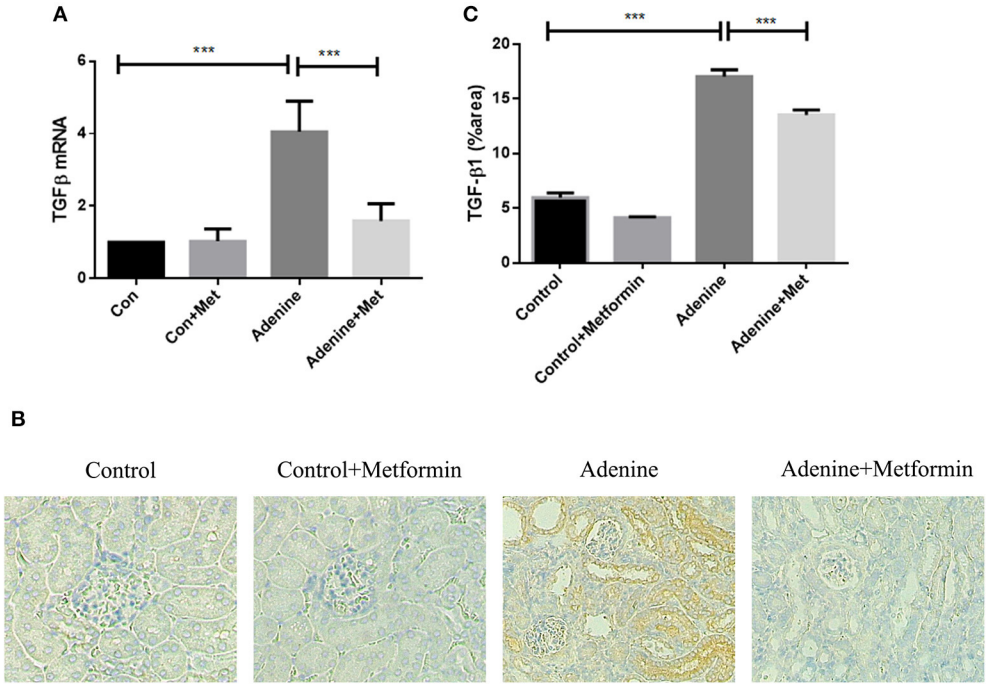

FIGURE 4 | Metformin suppresses the upregulation of TGF-β1 expression in the mouse model of adenine-induced renal injury. qRT-PCR **(A)** and immunohistochemical staining **(B,C)** results demonstrated that the expression of TGF-β1 was significantly increased in adenine-induced mice compared to the control mice, which was suppressed by metformin treatment. Data are expressed as mean ± SEM. ***$P < 0.001$, $n = 8$. Original magnification: ×200.

chemokines of inflammation cells. F4/80 is widely used as a marker of macrophage infiltration. The increased expression of F4/80 in kidneys indicates active inflammatory responses and has been well-accepted as being inherent in renal injury (Cao et al., 2015; Wang et al., 2017). Intracellular adhension molecule-1 (ICAM1), which is also known as cluster of differentiation 54 (CD54), is a protein with a signal-transducing function considered to increase proinflammatory pathways. Activation of ICAM1 recruits inflammatory immune cells such as macrophages to maintain a pro-inflammatory environment for leukocyte infiltration. Thus, ICAM1 is an important marker for macrophage infiltration in kidney tissue. In this study, metformin inhibited adenine-induced overexpression of MCP-1, F4/80, and ICAM1 in kidneys, which confirmed the anti-inflammatory role of metformin in adenine-induced renal injury.

It is well-documented that TGF-β1 signaling pathways play a central role in renal interstitial fibrosis. The upregulation of TGF-β1 signaling pathways has been considered to be fundamental to all causes of kidney disease in both experimental models and human kidney disease (Bottinger and Bitzer, 2002; Wang et al., 2005). The TGF-β1 signaling pathways include Smad and non-Smad pathways, which are involved in *many* cellular processes. Smad signaling is the major pathway of TGF-β1 signaling in renal fibrosis, and Smad3 has been shown to mediate renal fibrosis in various mouse models of chronic kidney diseases (Meng et al., 2015; Chen et al., 2018). TGF-β1 non-Smad pathways signaling molecules include the extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinase (JNK), p38 the mitogen-activated protein kinase (MAPK), the IκB kinase (IKK), phosphatidylinositol-3 kinase (PI3K) and Akt, as well as Rho family GTPases. The non-Smad signaling molecules contribute to the physiological responses as stand-alone pathways or together with Smads (Zhang, 2009, 2017). It is well-documented that in addition to regulating transcription through the phosphorylation of Smad2 and Smad3 and formation of a Smad2/3/4 complex, TGF-β1 also mediates other non-Smad signaling pathways (Meng et al., 2016). TGF-β1 activates ERK, p38 MAPK, and JNK to mediate renal fibrosis (Meng et al., 2016), which is independent of the Smad pathway. In our study, the results showed that metformin inhibited TGF- β1 expression as well as Smad and non-Smad signaling pathways as demonstrated by inhibition of phosphorylation of Smad3, ERK1/2, and p38 in kidneys of mice with adenine-induced renal injury. These data indicate that the antifibrotic effects of metformin are at least mediated through TGF-β1 and its downstream Smad and non-Smad signaling pathways.

It is well-known that AMPK is a pivotal molecule that prevents or delays the process of fibrogenesis and AMPK exerts comprehensive protective effects against fibrosis in various organs and tissues (Jiang et al., 2017). The AMPK-dependent and AMPK-independent mechanisms of metformin have been well-studied (Kalender et al., 2010; Vincent et al., 2015). The data in this study have shown that metformin inhibits the TGFb pathway

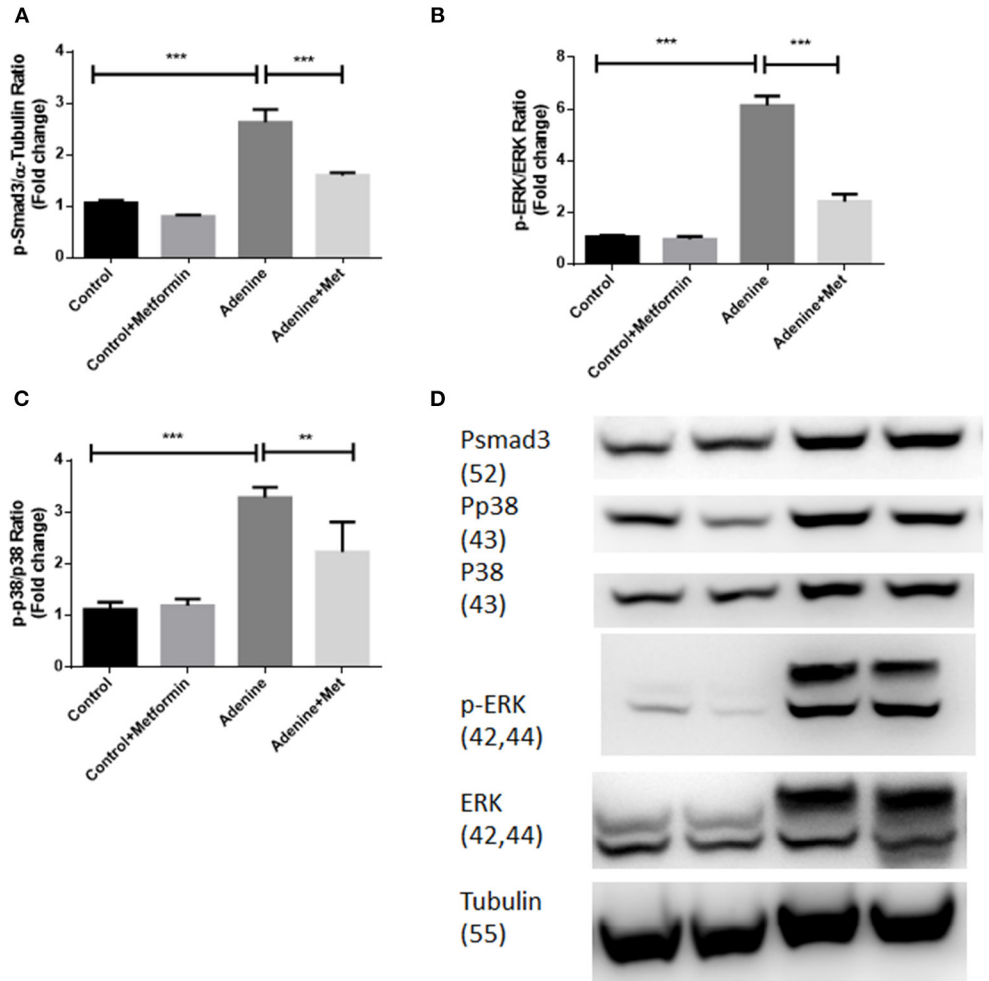

FIGURE 5 | Metformin suppresses TGF-β1 signaling pathways through inhibiting activation of Smad3, ERK1/2, and p38 in a mouse model of adenine-induced renal injury. Western blot results demonstrated that metformin suppressed adenine induced phosphorylation of p-Smad3 **(A,D)**, p-ERK1/2 **(B,D)**, and p-P38 expression **(C,D)** in adenine treated mice. Data are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 8$.

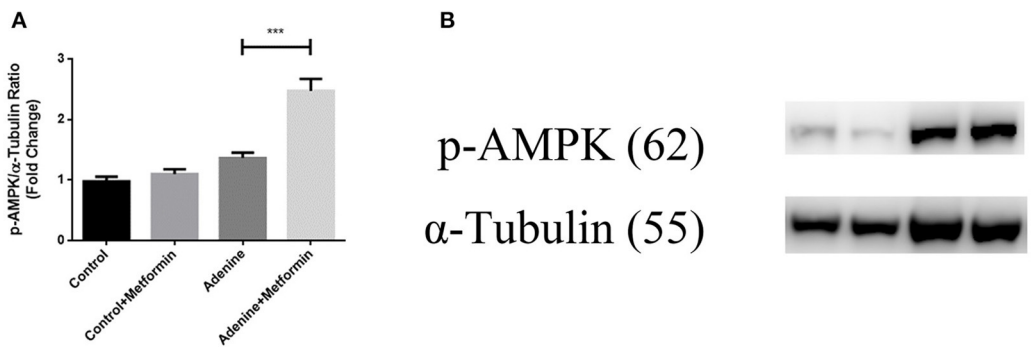

FIGURE 6 | Metformin activated AMPK in a mouse model of adenine-induced renal injury. The result of western blot results showed that metformin activated AMPK **(A,B)** in metformin-treated mice. Results are presented as mean ± SEM. $^{***}P < 0.001$, $n = 8$.

through suppressing both smad and non-smad pathway, which indicated the effects were AMPK-independent. Furthermore, a recent study has also shown that metformin could inhibit TGF-β-induced collagen production in mice by activating AMPK (Lu et al., 2015). Collectively these data are consistent with the demonstrated in this study.

In conclusion, the present study suggests that metformin can improve renal function and protect against chronic renal injury induced by adenine through inhibiting TGF-β1 signaling pathways and potentially by increasing the phosphorylation of AMPK. These results, together with other studies (Zhang et al., 2017; Lee et al., 2018; Yi et al., 2018; Christensen et al., 2019), confirm metformin exerts renoprotection independent of its glucose lowing effect in non-diabetic kidney disease. These results suggest an antifibrotic role for metformin in diverse forms of chronic kidney disease, thus warranting therapeutic evaluation in clinical settings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee Northern Sydney Local Health District.

AUTHOR CONTRIBUTIONS

HY, CP, and X-MC did conception and design of experiments. HY, CH, YS, and QC performed experiments and analyzed data. JC helped to analyses the IHC images. HY drafted the manuscript. All authors contributed to manuscript revision, read and approved the final version of manuscript.

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KCa3.1 Mediates Dysregulation of Mitochondrial Quality Control in Diabetic Kidney Disease

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Mitochondrial dysfunction is implicated in the pathogenesis of diabetic kidney disease. Mitochondrial quality control is primarily mediated by mitochondrial turnover and repair through mitochondrial fission/fusion and mitophagy. We have previously shown that blockade of the calcium-activated potassium channel KCa3.1 ameliorates diabetic renal fibrosis. However, the mechanistic link between KCa3.1 and mitochondrial quality control in diabetic kidney disease is not yet known. Transforming growth factor β1 (TGF-β1) plays a central role in diabetic kidney disease. Recent studies indicate an emerging role of TGF-β1 in the regulation of mitochondrial function. However, the molecular mechanism mediating mitochondrial quality control in response to TGF-β1 remains limited. In this study, mitochondrial function was assessed in TGF-β1-exposed renal proximal tubular epithelial cells (HK2 cells) transfected with scrambled siRNA or KCa3.1 siRNA. *In vivo*, diabetes was induced in KCa3.1+/+ and KCa3.1−/− mice by low-dose streptozotocin (STZ) injection. Mitochondrial fission/fusion-related proteins and mitophagy markers, as well as BCL2 interacting protein 3 (BNIP3) (a mitophagy regulator) were examined in HK2 cells and diabetic mice kidneys. The *in vitro* results showed that TGF-β1 significantly inhibited mitochondrial ATP production rate and increased mitochondrial ROS (mtROS) production when compared to control, which was normalized by KCa3.1 gene silencing. Increased fission and suppressed fusion were found in both TGF-β1-treated HK2 cells and diabetic mice, which were reversed by KCa3.1 deficiency. Furthermore, our results showed that mitophagy was inhibited in both *in vitro* and *in vivo* models of diabetic kidney disease. KCa3.1 deficiency restored abnormal mitophagy by inhibiting BNIP3 expression in TGF-β1-induced HK2 cells as well as in the diabetic mice. Collectively, these results indicate that KCa3.1 mediates the dysregulation of mitochondrial quality control in diabetic kidney disease.

Keywords: diabetic kidney disease, mitochondrial quality control, mitochondrial dynamics, mitophagy, transforming growth factor β1, KCa3.1

INTRODUCTION

Mitochondria are responsible for the main site of adenosine triphosphate (ATP) synthesis via oxidative phosphorylation (Alpers and Hudkins, 2011). Mitochondria have also been shown to play a crucial role in calcium signaling, reactive oxygen species (ROS) generation, apoptosis, necrosis, and innate immunity (Galluzzi et al., 2012; Suarez-Rivero et al., 2016). Mitochondrial dysfunction is characterized by a decrease in ATP production and increase in ROS generation leading to oxidative stress (Suarez-Rivero et al., 2016). Hence, maintaining optimal function of the mitochondria is important for maintaining cell survival, regulating cell death and cellular metabolic homeostasis (Sharma et al., 2003).

Mitochondrial quality control is exquisitely regulated to maintain functional mitochondria (Sharma et al., 2003). Mitochondrial quality control mechanisms are mainly regulated by mitochondrial dynamics and mitophagy (Ranjit et al., 2016). Mitochondrial dynamics include fission and fusion to repair or delete damaged components of the mitochondria. Mitochondrial fission allows for the segregation of damaged mitochondria, while mitochondrial fusion facilitates the exchanging of material between healthy mitochondria. Imbalanced mitochondrial fission and fusion are detrimental to mitochondrial function and cellular survival. Mitochondrial dynamics are regulated by several different GTPase proteins. Mitochondrial fission is regulated by dynamin-related protein 1 (Drp1) and mitochondrial fission protein 1 (Fis1). Mitochondrial fusion is mediated by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (Opa1) proteins. Mfn1 and Mfn2 are localized on the mitochondrial outer membrane (MOM) and mediate tethering of MOM of adjacent mitochondria to promote the fusion of MOM, whereas Opa1 is responsible for mitochondrial inner membrane (MIM) fusion (Anand et al., 2014). Abnormalities in these mitochondrial dynamic proteins lead to severely altered mitochondrial morphology, defective mitochondrial function, and eventually cell death (Zhan et al., 2013). Mitophagy is selective autophagy to degrade and recycle dysfunctional or damaged mitochondria. Recent studies suggest that mitochondrial priming is mediated either through the Pink1/Parkin signaling pathway or the mitophagic receptors such as BCL2 interacting protein 3 (BNIP3), BNIP3 like (BNIP3L/NIX), and FUN14 domain containing 1 (FUNDC1) (Li et al., 2018; Wang J. et al., 2020). Disruption of mitochondrial networks prevents the elimination of damaged mitochondria and exacerbates ATP deficits, which is then implicated in a variety of diseases including diabetic kidney disease (Forbes and Thorburn, 2018; Suomalainen and Battersby, 2018). Although dysfunctional mitochondria are increasingly recognized to be central to the pathogenesis of diabetic kidney disease (Saxena et al., 2019), the understanding of the mechanism of mitochondrial quality control and its regulatory signaling pathways in diabetic kidney disease remains limited.

KCa3.1 (also known as IK1, SK4, or KCNN4) belongs to the calcium-activated potassium channel (KCa) family, which is localized in the plasma membrane, nucleus, and inner mitochondrial membranes (De Marchi et al., 2009; Chachi et al., 2013). KCa3.1 channels regulate calcium entry into cells through modulating calcium-signaling processes, which is necessary for maintaining various cellular activation processes such as proliferation, migration, and cytokine production (Cruse et al., 2006; Wulff and Castle, 2010; Chen et al., 2011). Hence, KCa3.1 has been proposed as a potential therapeutic target for sickle cell anemia, autoimmunity, and atherosclerosis (Wulff et al., 2007; Chou et al., 2008; Wulff and Castle, 2010). Recently, we have demonstrated an important role of KCa3.1 in diabetic kidney disease. Our studies have demonstrated that blockade of KCa3.1 alleviated renal fibrosis and inflammation in diabetic mice through inhibition of the TGF-β1 signaling pathway and fibroblast activation (Huang et al., 2013, 2014b). Furthermore, our results showed that blockade of KCa3.1 is likely to exert its anti-fibrotic effects through the restoration of dysregulated tubular autophagy (Huang et al., 2016a). However, the mechanism by which KCa3.1 mediates mitochondrial quality control in diabetic kidney disease remains unknown.

It is well accepted that transforming growth factor β1 (TGF-β1) plays a central role in the development of diabetic kidney disease. Recent observations indicate an emerging role of TGF-β1 in the regulation of mitochondrial function (Pozdzik et al., 2016; Choi et al., 2019). However, the molecular mechanism mediating mitochondrial quality control in response to TGF-β1 remains limited. In this study, we investigated the effect of KCa3.1 silencing on mitochondrial function in TGF-β1 stimulated human renal proximal tubular cells. We also assessed the role of KCa3.1 in mitochondrial dynamics and mitophagy as well as the underlying signaling pathways in both *in vitro* and *in vivo* models. Our results demonstrated that KCa3.1 deficiency was able to reverse diabetes-induced mitochondrial dysfunction by normalizing the disrupted mitochondrial quality control, which was likely mediated through inhibition of BNIP3 expression.

MATERIALS AND METHODS

Materials

Tissue culture medium and Lipofectamine 2000 were provided from Invitrogen Life Technologies (Carlsbad, CA, United States). Anti-LC3, anti-P62, anti-Cox4, anti-Mfn2, and anti-BNIP3 antibodies were purchased from Abcam (Cambridge, MA, United States), and anti-α-tubulin antibody was from Sigma (St. Louis, MO, United States). Anti-phospho-Drp1 and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). Anti-Fis1 antibody was purchased from Proteintech (Rosemont, IL, United States), and Anti-Opa1 antibody was purchased from Novus Biologicals (Centennial, CO, United States). Alexa Fluor 488-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA, United States).

Animal Studies

Male KCa3.1+/+ mice and KCa3.1−/− mice (6–8 weeks old) weighing approximately 20–25 g were used in the study. Mice were intraperitoneally injected with either 55 mg/kg of STZ (Sigma, St. Louis, MO, United States) diluted in 0.1 M citrate buffer, pH 4.5, or citrate buffer alone as described previously

(Huang et al., 2013). Mice were weighed, and blood glucose level was determined using the Accu-chek glucometer (Roche Diagnostics). Mice with blood glucose greater than 16 mmol/l were considered to have diabetes.

This study was approved by the Animal Research Ethics Committee of Royal North Shore Hospital (1101-001A). Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia's Code for the Care and Use of Animals for Scientific Purposes.

Cell Culture and KCa3.1 Gene Silencing

Immortalized human renal proximal tubular cells (HK2 cells), obtained from ATCC (Manassas, VA, United States), were grown in keratinocyte serum-free media (Invitrogen, Carlsbad, CA, United States). All experiments were performed at passages 5–15.

HK2 cells were transfected with either KCa3.1 siRNA or scrambled control siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instruction. The transfected cells were then incubated with TGF-β1 (2 ng/ml) for 48 h. The siRNA sequence for KCa3.1 is 5′-GCACCUUUCAGACACACUU-3′ (GenePharma, Shanghai).

Mitochondrial ATP Production Rate

Mitochondrial ATP production rate was determined using the ATP bioluminescence assay kit (Roche Diagnostics, Switzerland) according to the protocol described previously (Huang et al., 2016b). ATP production was induced by incubation of the cell suspension with substrate buffer at 37°C for 10 min, which was then stopped by addition of boiling quenching buffer at 100°C for 2 min. The reaction mixture diluted 1:10 in quenching buffer was measured using an FB10 luminometer (Berthold Detection Systems, Germany) to determine the ATP level.

Mitochondrial Superoxide Quantification

Mitochondrial superoxide was detected by MitoSOX Red staining (Molecular Probes-Invitrogen) as described previously (Li et al., 2019). Briefly, the treated cells were incubated with 5 μM MitoSOX Red for 15 min at 37°C. After washing with warm buffer, the stained cells were then visualized under confocal fluorescence microscopy (Leica Microsystems, Mannheim, Germany). The results were expressed as the fluorescence intensity normalized to the control group.

Transmission Electron Microscopy

The cell samples were prepared for transmission electron microscopic analysis as previously reported (Huang et al., 2014a). Briefly, after washing with pre-warmed PBS, the cells were next fixed in 2% glutaraldehyde for 1 h. Subsequently, the fixed cells were postfixed with 1% osmium tetroxide for 1 h after briefly washing with PBS. The samples were rinsed in distilled water, stained with 1% tannic acid, dehydrated in a gradient of ethanol, and embedded in Epon. Sections of 70 nm were generated with an ultramicrotome (Ultracut 7, Leica) and post-stained with 2% aqueous uranyl acetate and Reynold's lead citrate for 10 min each. The specimens were examined under a transmission electron microscope operating at 200 kV (JEM-2100, JEOL, Japan). Mitochondrial Feret's diameter (maximum and minimum), the distance between two parallel tangential lines within the selected mitochondrion, was determined using Image J (Demeter-Haludka et al., 2018; Lomash et al., 2019).

Immunocytofluorescence Staining

To monitor mitophagy, HK2 cells were stained with 1 nM of MitoTracker Deep Red FM for 15 min at 37°C (Huang et al., 2016b). After fixation and blocking, the cells were incubated with primary antibodies against LC3 or P62 in 2% BSA in PBS for 1 h, followed with Alexa Fluor-488 conjugated secondary antibodies for 40 min. The cells were then counterstained and mounted with 4′,6-diamidino-2 phenylindole (DAPI)-mounting medium (Invitrogen). The fluorescent signals were collected and analyzed by confocal fluorescence microscopy (Leica Microsystems, Mannheim, Germany).

Mitochondrial Isolation

Mitochondria were isolated from mice renal cortex as described previously (Nguyen et al., 2015). Briefly, tissue samples were homogenized in HEPES buffer (20 mM, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). The homogenate was centrifuged at 1,500 × *g* for 5 min at 4°C. The supernatant was collected and then centrifuged at 10,000 × *g* for 15 min at 4°C to pellet the mitochondria, which were resuspended in HEPES buffer for analyses. The supernatants were collected as the cytosol fraction. The total protein concentration of the isolated mitochondrial and cytosol fraction was determined by the BCA Protein Assay Kit (Thermo Scientific).

Western Blotting

An equal amount of cell and tissue lysate samples was separated by SDS-PAGE, and then transferred to Hybond ECL nitrocellulose membrane (Amersham, United States). The membranes were blocked and then probed with primary antibodies (LC3, P62, p-Drp1, Fis1, Opa1, Mfn2, Cox4, BNIP3, and α-Tubulin) at 4°C overnight followed with HRP-conjugated secondary antibody (Amersham, United States). The membrane blots were detected and quantified using LAS-4000 Imaging System (FUJIFILM, Japan).

Statistical Analysis

The data were expressed as mean ± SEM. Statistical analysis between two groups was evaluated by two-tailed *t*-test. Comparison of the results from multiple groups was performed by one-way ANOVA, followed by Tukey post-test. A P-value < 0.05 was considered as statistically significant.

RESULTS

KCa3.1 Gene Silencing Reversed TGF-β1-Induced Mitochondrial Dysfunction in HK2 Cells

To determine the role of KCa3.1 in mitochondrial function, mitochondrial ATP production rate was first examined in

HK2 cells exposed to TGF-β1 with or without KCa3.1 siRNA. As shown in **Figure 1A**, compared to the controls, TGF-β1 significantly inhibited mitochondrial ATP production rate in HK2 cells transfected with scrambled siRNA (19.08 ± 0.82 for the control and 12.77 ± 0.56 for TGF-β1 + scrambled siRNA, $P < 0.01$, **Figure 1A**). Inhibition of KCa3.1 with KCa3.1 siRNA reversed TGF-β1-induced inhibition of ATP production rate (15.84 ± 0.29, $P < 0.01$, **Figure 1A**).

Mitochondrial ROS (mtROS) production in HK2 cells was then examined by fluorescence staining with MitoSOX Red, which is designed for highly selective detection of superoxide in mitochondria. As shown in **Figure 1B**, a low level of fluorescence was found in the control cells, indicating normal basal levels of mtROS production. Compared to the control cells, TGF-β1 induced increased mtROS production, characterized by the elevated fluorescent intensity of MitoSOX in HK2 cells. KCa3.1 gene silencing significantly reduced TGF-β1-induced mtROS generation ($P < 0.01$, **Figure 1C**). These data collectively demonstrate that TGF-β1 impaired mitochondrial function through a KCa3.1-related mechanism in HK2 cells and KCa3.1 gene silencing reversed TGF-β1-induced mitochondrial dysfunction.

KCa3.1 Gene Silencing Attenuated TGF-β1-Induced Increased Fission and Suppressed Fusion in HK2 Cells

To determine whether KCa3.1 has any effect on mitochondrial fission and fusion processes, mitochondrial fission-related protein Drp1, Fis1, and mitochondrial fusion-related protein Opa1, Mfn2 were examined in HK2 cells exposed to TGF-β1 with or without KCa3.1 gene silencing. As shown in **Figure 2A**, TGF-β1 significantly increased the level of pro-fission protein Drp1 expression compared to the control group ($P < 0.01$). This increase was attenuated by KCa3.1 gene silencing ($P < 0.05$, **Figure 2A**). In response to TGF-β1, the levels of profusion protein Opa1 expression in HK2 cells were significantly decreased compared to the control group ($P < 0.05$, **Figure 2C**), which was attenuated by KCa3.1 gene silencing ($P < 0.05$, **Figure 2C**). Interestingly, the expression of Fis1 and Mfn2 was not obviously altered by TGF-β1 stimulation (**Figures 2B,D**).

KCa3.1 Gene Silencing Reversed TGF-β1-Induced Inhibition of Mitophagy

The mitochondrial shape is maintained through the processes of mitochondrial fission and fusion (Zhang et al., 2019). To investigate the role of KCa3.1 in mitochondrial morphology, we employed transmission electron microscopy to assess the fine structure of mitochondria at high resolution. The control group cells exhibited healthy, normal appearing mitochondria with well-developed cristae (**Figure 3A**). In contrast, an abundance of mitochondria with severely disrupted cristae was found in HK2 cells exposed to TGF-β1, which was attenuated by KCa3.1 gene silencing. As shown in **Figures 3B,C**, compared to the control group, exposure to TGF-β1 resulted in a significant reduction in maximum and minimum Feret's diameter of the mitochondria, indicating that the mitochondria became smaller following the TGF-β1 insult. These alterations were significantly recovered by KCa3.1 gene silencing.

Mitochondrial autophagy was further studied by co-localization of autophagy markers LC3 and P62 with MitoTracker Deep Red stained mitochondria. As shown in **Figures 3D,E**, the intensity of LC3 that colocalized with MitoTracker Deep Red stained mitochondria was significantly increased in HK2 cells exposed to TGF-β1 when compared to the control ($P < 0.01$, **Figure 3E**), which was significantly attenuated by KCa3.1 gene silencing ($P < 0.05$, **Figures 3D,E**). Similarly, exposure of cells to KCa3.1 siRNA significantly suppressed TGF-β1-induced increased intensity of P62 colocalized with the mitochondria ($P < 0.05$, **Figures 3F,G**). These data indicate that KCa3.1 gene silencing reversed TGF-β1-induced inhibition of mitophagy in HK2 cells.

KCa3.1 Deficiency Attenuated Diabetes-Induced Increased Fission and Suppressed Fusion in Diabetic Mice

To further confirm the effect of KCa3.1 on diabetes-related mitochondrial dynamics, mitochondrial fission- and fusion-related proteins were assessed in mice kidneys. As shown in **Figure 4**, diabetes significantly increased the level of pro-fission protein Drp1 and suppressed the level of pro-fusion protein Opa1 in diabetic KCa3.1 wild-type mice (K+/+ DM) compared to non-diabetic control mice (K+/+ control) ($P < 0.05$, **Figures 4A,C**). However, the changes were attenuated in diabetic KCa3.1 deficient mice (K−/− DM) ($P < 0.05$, **Figures 4A,C**). Conversely, the levels of Fis1 and Mfn2 were not notably changed in mice kidneys (**Figures 4B,D**). The findings suggested that KCa3.1 regulates diabetes-induced imbalance in mitochondrial dynamics by enhancing fission and reducing fusion.

KCa3.1 Deficiency Attenuated Diabetes-Induced Inhibition of Mitophagy in Diabetic Mice

To determine whether KCa3.1 deficiency attenuates diabetic renal fibrosis via regulating mitophagy, the autophagy markers LC3 and P62 were assessed in mitochondria from diabetic kidney tissues using western blot analysis. As shown in **Figure 5A**, increased expression of LC3 in mitochondria was observed in diabetic KCa3.1 wild-type mice (K+/+ DM) when compared to the non-diabetic controls (K+/+ control) ($P < 0.05$). KCa3.1 deficiency significantly attenuated diabetes-induced upregulation of LC3 expression in mitochondria from diabetic KCa3.1 deficient mice (K−/− DM) ($P < 0.05$, **Figure 5A**). In line with the LC3 findings, western blot analysis results showed that P62 expression in mitochondria was significantly increased in diabetic kidneys as compared to the non-diabetic controls ($P < 0.05$, **Figure 5B**), which was inhibited in diabetic KCa3.1 deficient mice ($P < 0.05$, **Figure 5B**). Collectively, these results indicate

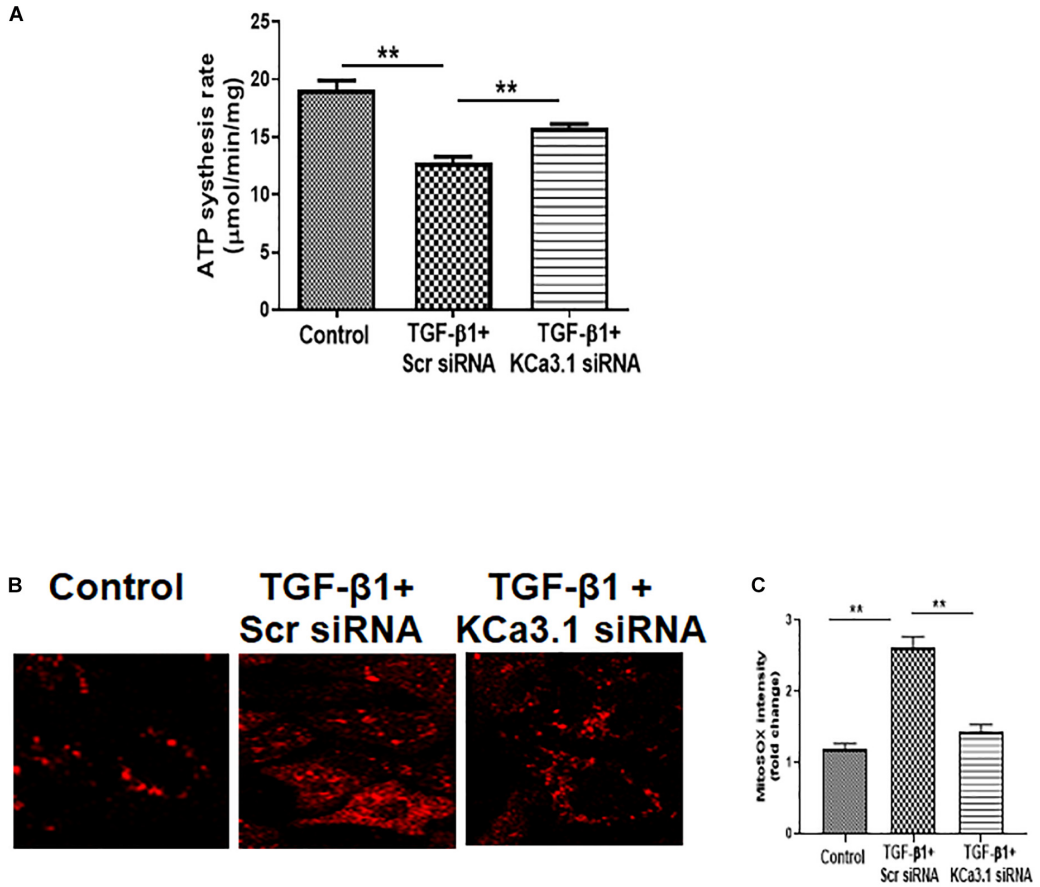

FIGURE 1 | KCa3.1 gene silencing reversed TGF-β1-induced mitochondrial dysfunction in HK2 cells. **(A)** Mitochondrial ATP production rate was assessed to detect mitochondrial function. KCa3.1 silencing significantly increased TGF-β1-induced inhibition of ATP production rate. **(B)** Mitochondrial reactive oxygen species (mtROS) production was assessed by MitoSOX Red staining. KCa3.1 silencing significantly reduced TGF-β1-induced mtROS overproduction. **(C)** Quantification of MitoSOX Red fluorescence intensity normalized to the control group in HK2 cells. Results are presented as mean ± SEM. $^{**}P < 0.01$. $N = 3$. Original magnification: ×600.

that KCa3.1 deficiency attenuates diabetes-induced inhibition of mitophagy in diabetic mice.

KCa3.1 Deficiency Suppressed Diabetes-Induced Upregulation of BNIP3 Expression in HK2 Cells and Diabetic Mice

To investigate the mechanism whereby KCa3.1 regulates mitophagy, BNIP3, a regulator of mitophagy, was examined in HK2 cells exposed to TGF-β1 as well as diabetic mice kidneys. As shown in **Figure 6A**, the expression of BNIP3 was significantly increased by TGF-β1 in HK2 cells ($P < 0.05$, **Figure 6A**), which was attenuated by KCa3.1 gene silencing ($P < 0.01$, **Figure 6A**). Similarly, the western blot analysis confirmed a marked induction of BNIP3 in diabetic KCa3.1 wild-type mice (K+/+ DM) when compared to non-diabetic control mice (K+/+ control) ($P < 0.05$, **Figure 6B**). KCa3.1 deficiency significantly attenuated diabetes-induced upregulation of BNIP3 expression in diabetic KCa3.1−/− mice (K−/− DM) ($P < 0.05$, **Figure 6B**). Together, these results suggest that KCa3.1-mediated dysregulation of mitophagy is associated with upregulation of BNIP3 expression.

DISCUSSION

This study was undertaken to define the role of KCa3.1 in regulating mitochondrial quality control in diabetic kidney disease as depicted in **Figure 7**. The study demonstrated that TGF-β1 resulted in mitochondrial dysfunction and subsequent mtROS overproduction as well as inhibition of mitophagy, which leads to the disruption of the mitochondrial quality control, eventually causing tubular cell injury. KCa3.1 deficiency restored abnormal mitochondrial dysfunction and mitochondrial quality control by improving BNIP3-mediated mitophagy in TGF-β1-induced renal proximal tubular cells as well as in STZ-induced diabetic mice.

Transforming growth factor β1, the most abundant isoform of TGF-β family members, can be secreted by all types of renal cells and infiltrating inflammatory cells. It is well established that TGF-β1 acts as a pivotal mediator in diabetic kidney

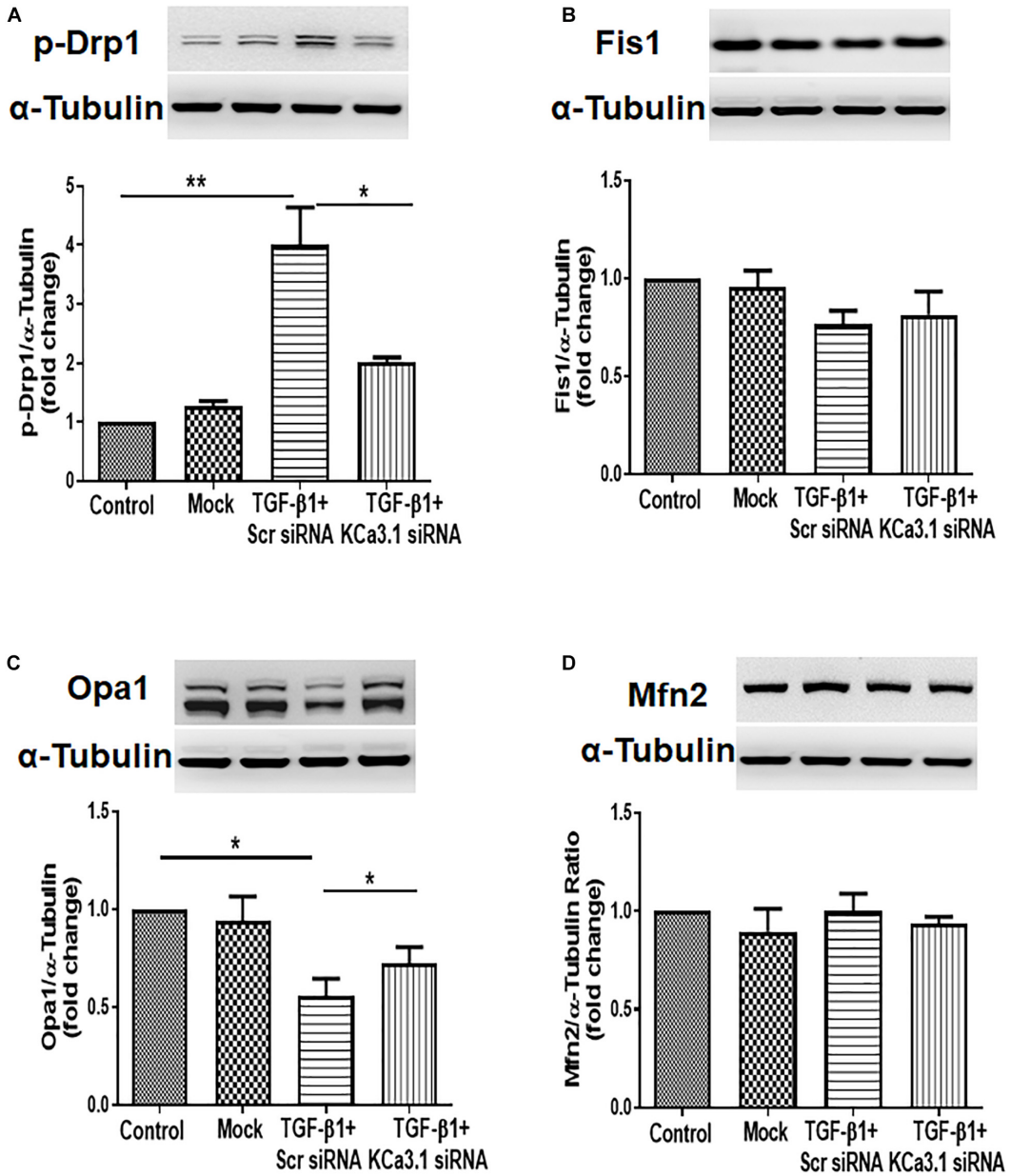

FIGURE 2 | KCa3.1 gene silencing attenuated TGF-β1-induced increased fission and suppressed fusion in HK2 cells. Mitochondrial pro-fission proteins (Drp1 and Fis1) and pro-fusion mediators (Opa1 and Mfn2) were examined by western blotting. Western blot analyses revealed an increased expression of Drp1 **(A)** and a reduced expression of Opa1 **(C)** in TGF-β1-induced HK2 cells, which were reversed by KCa3.1 gene silencing. There were no changes in the expression of Fis1 **(B)** and Mfn2 under TGF-β1 stimulation **(D)**. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $N = 3$.

disease given its involvement in renal fibrosis, inflammation, cell growth, apoptosis, and differentiation (Bottinger, 2007; Hills and Squires, 2011; Lan, 2012; Meng et al., 2013). A growing body of evidence indicates that mitochondrial dysfunction may be important in the development and progression of diabetic kidney disease (Forbes and Thorburn, 2018; Saxena et al., 2019). Recent studies have revealed a link between TGF-β1 and mitochondrial dysfunction. *In vitro* studies demonstrated that TGF-β1-induced mitochondria dysfunction has been found in various types of cells including lung epithelial cells (Patel et al., 2015), alveolar macrophages (Grunwell et al., 2018), and subepithelial fibroblasts (Sun et al., 2019) as well as renal cells (Yu et al., 2016; Wang Y. et al., 2020). Yu et al. (2016) reported that a TGF-β1-induced fibrotic phenotype was associated with significant mitochondrial dysfunction in mouse renal tubular cells, which was markedly improved by MnTBAP (a cell-permeable mimic of superoxide dismutase) treatment. Recently, mitochondrial dysfunction was found in rat kidney fibroblast cells under TGF-β1 challenge

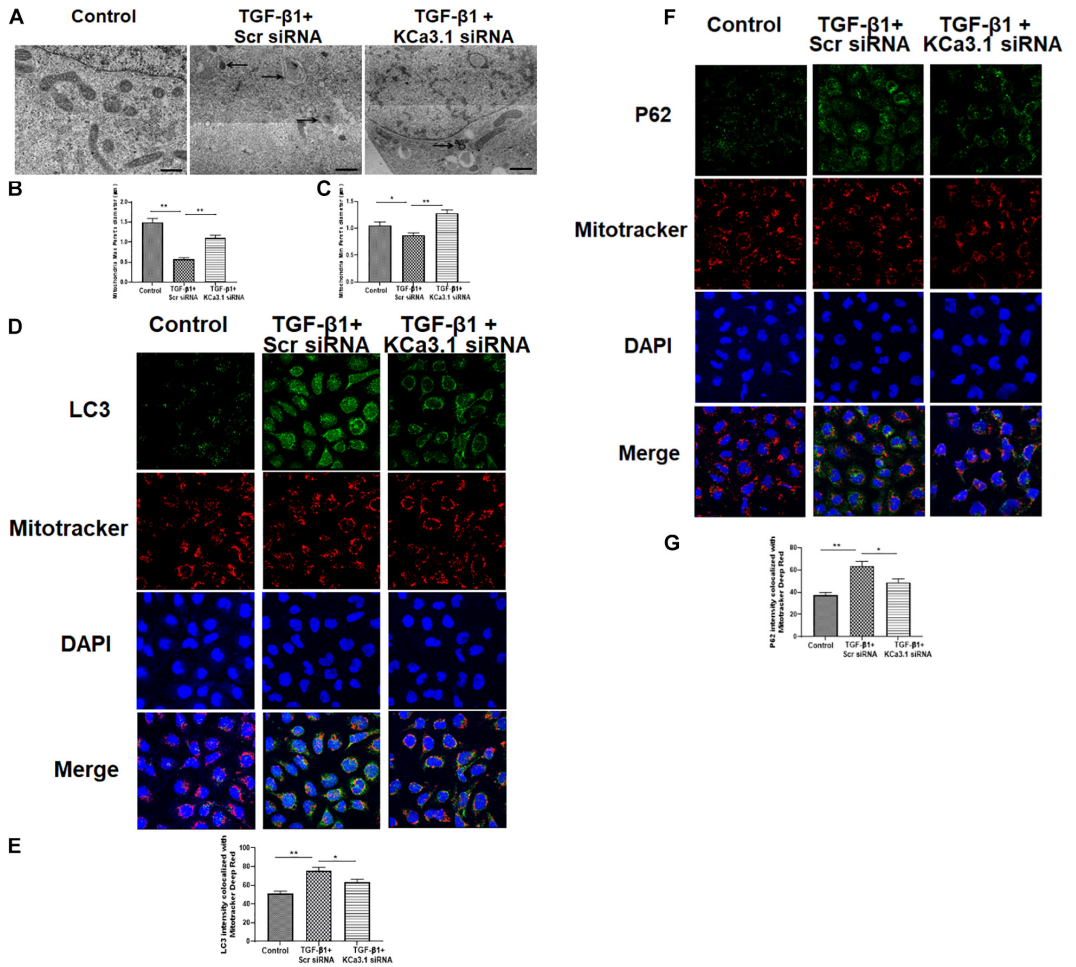

FIGURE 3 | KCa3.1 gene silencing reversed TGF-β1-induced inhibition of mitophagy in HK2 cells. **(A)** Representative electron micrographs of mitochondrial morphology from HK2 cells. The arrow displays abnormalities in mitochondrial morphology indicative of mitophagy in the groups exposed to TGF-β1. Quantification of maximum **(B)** and minimum **(C)** Feret's diameter in HK2 cells. Scale bars, 200 nm. Confocal microscopy of MitoTracker Red-labeled mitochondria and LC3 staining **(D)** and P62 **(F)**. Quantification of fluorescence intensity of LC3 colocalized with mitochondria **(E)** and P62 colocalized with mitochondria **(G)** in HK2 cells. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $N = 3$. Original magnification: ×600.

together with fibroblast activation (Wang Y. et al., 2020). Similarly, *in vivo* studies have also demonstrated that increasing TGF-β1 activity is associated with mitochondrial dysfunction and increasing mtROS synthesis in various diseases including diabetic kidney disease (Lee et al., 2017). In line with previous studies, the interaction between TGF-β1 signaling and mitochondria has been demonstrated in the current study. Our previous study showed that the anti-fibrotic effect of KCa3.1 inhibition was likely mediated by antagonizing TGF-β1 signaling through suppression of TGF-β1 and TGF-β receptor II expression and the downstream Smad2/3 pathway in diabetic kidney disease (Huang et al., 2013). Our current results show that TGF-β1 induces mitochondrial dysfunction, as indicated by suppressed ATP production and increased mtROS production in renal proximal tubular cells (**Figure 1**). Furthermore, our results demonstrate that TGF-β1 exposure leads to altered mitochondrial morphology and increased accumulation of LC3 and P62 colocalized with mitochondria by immunofluorescence staining in renal proximal tubular cells, suggesting that TGF-β1 impaired mitochondrial function and mitophagy flux in renal tubular cells (**Figure 3**). Taken together, our previous and current studies demonstrate that activation of the TGF-β1 signaling pathway (Huang et al., 2013) and mitochondrial dysfunction are both recovered by KCa3.1 deficiency, indicating that improving mitochondrial function may be a key mechanism by which inhibition of KCa3.1 protects the kidney from diabetes-induced fibrosis.

Mitochondria are dynamic organelles that are constantly undergoing fission and fusion to repair damaged components of the mitochondria and maintain the homeostasis of cells. During mitochondrial fission, Drp1 is recruited from the cytosol onto the MOMs to interact with various receptors, such as Fis1, mitochondrial fission factor (MFF), and mitochondrial dynamic proteins of 49 and 51 kDa (MiD49 and MiD51). Opa1, a dynamin protein, is involved in mitochondrial fusion,

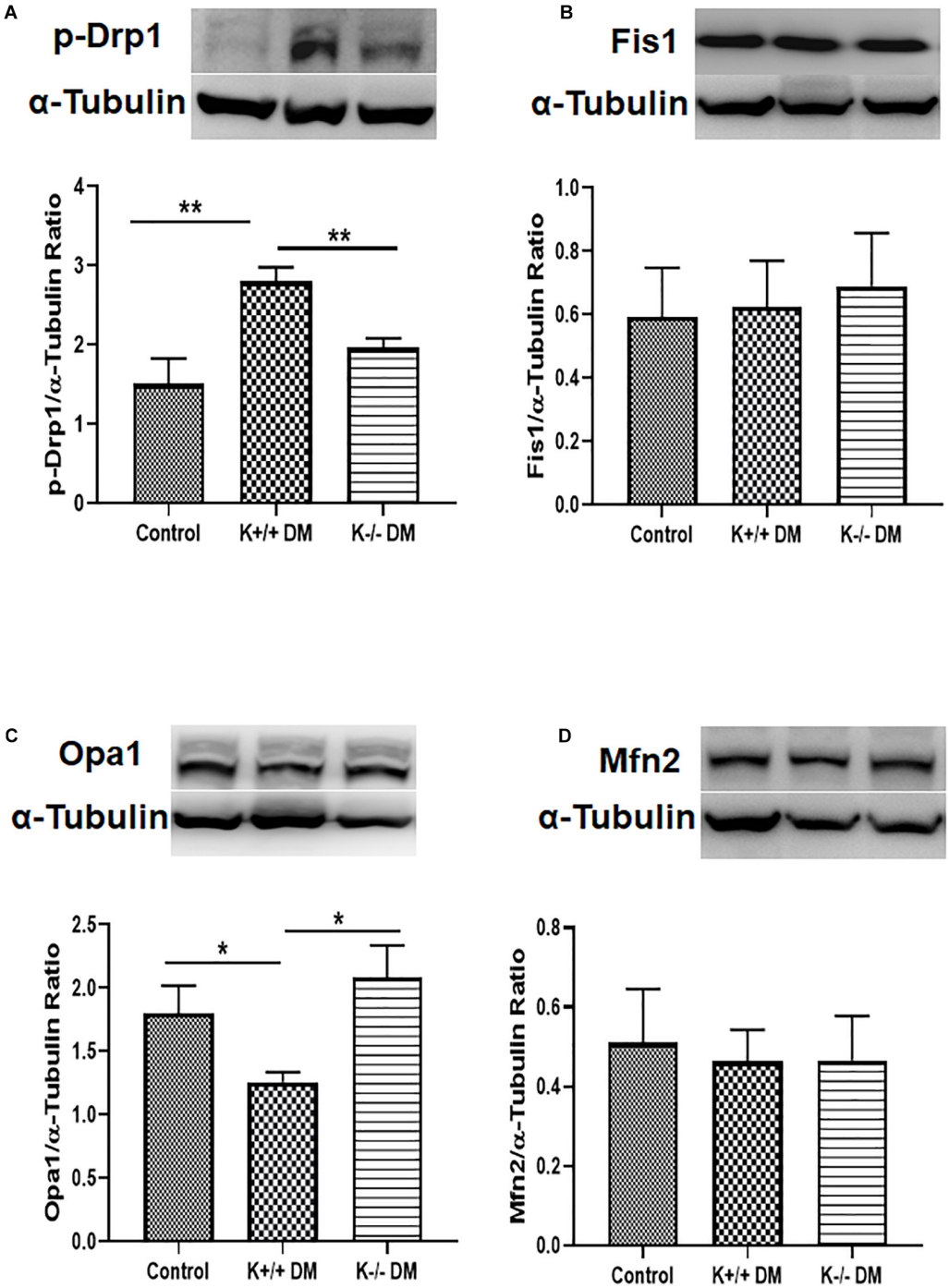

FIGURE 4 | KCa3.1 deficiency attenuated diabetes-induced increased fission and suppressed fusion in diabetic mice. Mitochondrial pro-fission proteins (Drp1 and Fis1) and pro-fusion mediators (Opa1 and Mfn2) were examined by western blotting in kidney tissues. Western blot analysis revealed an increased expression of Drp1 **(A)** and a reduced expression of Opa1 **(C)** in diabetic KCa3.1+/+ mice, which were reversed in KCa3.1 deficient mice (K−/− DM). The levels of Fis1 **(B)** and Mfn2 **(D)** were not notably changed in mice kidneys. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $N = 5$.

cristae structure maintenance, and apoptosis (Del Dotto et al., 2018). Opa1 has eight alternatively spliced isoforms, which can be further processed by proteases yeast mitochondrial escape 1 like 1 ATPase and metalloendopeptidase OMA1 to convert the long Opa1 (L-Opa1) into a cleaved short Opa1 (S-Opa1) form (Anand et al., 2014). L-Opa1 is competent for mitochondrial fusion, while the function of S-Opa1 is still not clear. However, both forms are essential for the

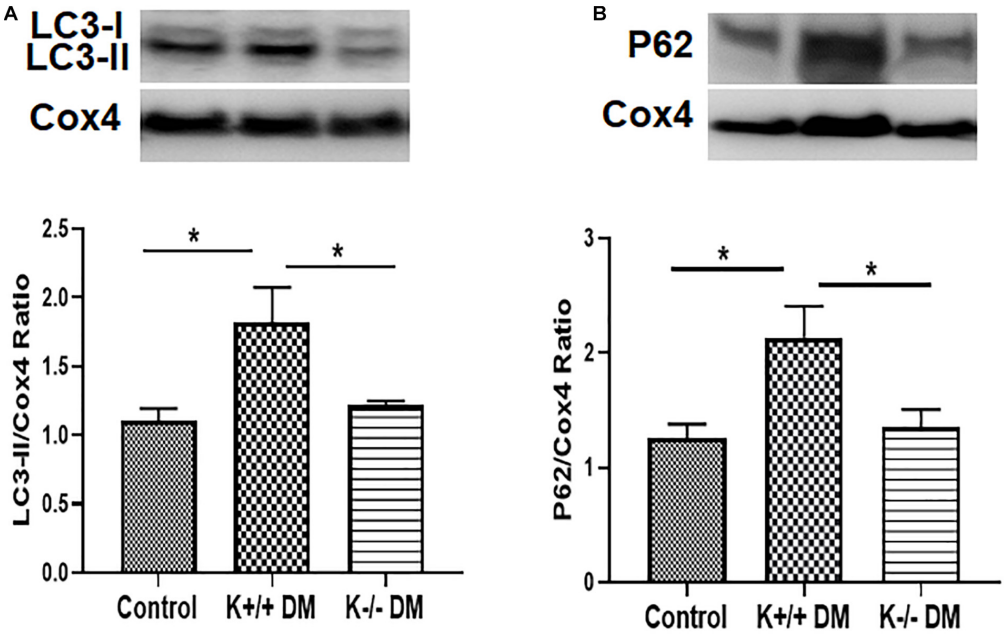

FIGURE 5 | KCa3.1 deficiency attenuated diabetes-induced inhibition of mitophagy in diabetic mice. The autophagy markers LC3 and P62 were assessed in mitochondria from diabetic kidney tissues using western blot analysis. Western blot analyses revealed an increased expression of LC3 **(A)** and P62 **(B)** in diabetic KCa3.1+/+ mice, which were significantly attenuated in KCa3.1 deficient mice (K−/− DM). Results are presented as mean + SEM. $^{*}P < 0.05$, $N = 5$.

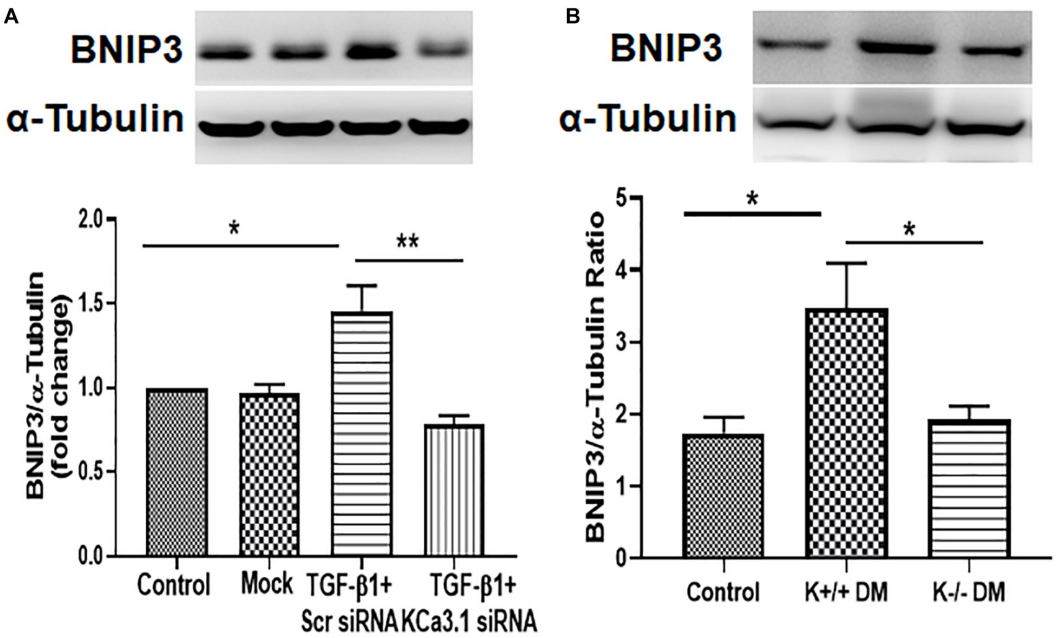

FIGURE 6 | KCa3.1 deficiency suppressed diabetes-induced upregulation of BNIP3 expression in HK2 cells and diabetic mice. **(A)** Western blot analysis showed that KCa3.1 silencing inhibited TGF-β1-induced BNIP3 expression in HK2 cells. $N = 3$. **(B)** Western blot analysis revealed an increased expression of BNIP3 in diabetic KCa3.1+/+ mice, which were significantly attenuated in KCa3.1 deficiency mice (K−/− DM). Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $N = 5$.

function of Opa1 on mitochondrial dynamics and architecture (Del Dotto et al., 2018). The imbalance in mitochondrial fission and fusion largely contributes to tissue pathology in a variety of metabolic conditions, including kidney diseases (Sun et al., 2017; Cassina et al., 2020; Wang Y. et al., 2020; Zhang et al., 2020). Sun et al. (2017) showed expression of

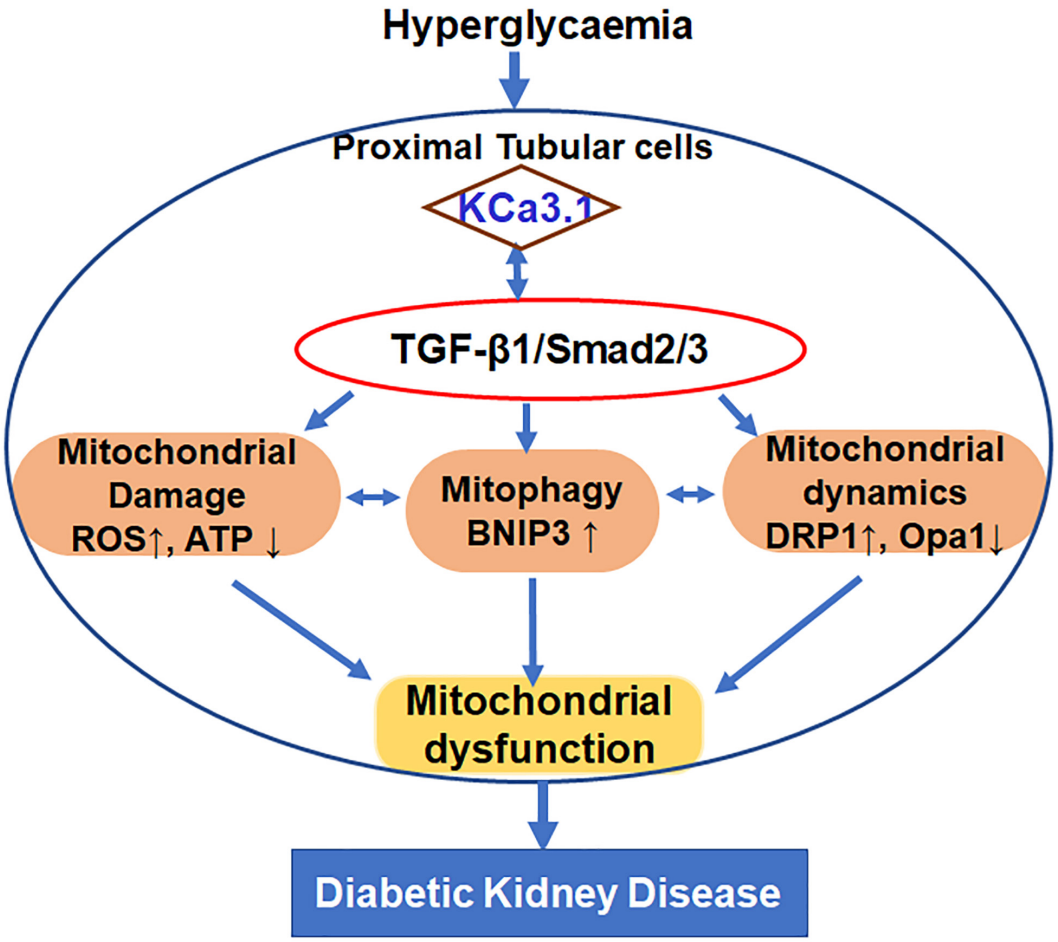

FIGURE 7 | Schematic diagram depicting the conceivable cellular events by which KCa3.1 mediates dysregulation of mitochondrial quality control in diabetic kidney disease.

the mitochondrial pro-fission protein DRP1 is increased and the mitochondrial pro-fusion protein Opa1 declined in 5/6 nephrectomized (Nx) rats and TGF-β1-exposed HK2 cells. In addition, increased expression of Drp1 and downregulation of Opa1 expression has been found in other animal models of kidney diseases including autosomal dominant polycystic kidney disease, obstructive nephropathy, and STZ-induced diabetic kidney disease (Cassina et al., 2020; Wang Y. et al., 2020; Zhang et al., 2020). Restoration of the imbalanced expression of all these mitochondrial dynamics-associated proteins has been proven to exert renoprotective effects. Consistently, in the present study, excessive mitochondrial fission and decreased fusion have also been demonstrated in TGF-β1-exposed HK2 cells and in kidneys of diabetic mice, as evidenced by upregulation of Drp1 and downregulation of Opa1 (**Figures 2**, **4**). As expected, the long form of Opa1 was downregulated, suggesting decreased fusion. However, the conversion of the long form to the short form of Opa1 was not observed in our study, as indicated by a reduction in the short form of Opa1. The other protease systems such as the ubiquitin proteasome pathway may also be involved in the model of diabetic kidney disease, which deserves further investigation. In our study, KCa3.1 deficiency normalized the expression of mitochondrial dynamic proteins to mitigate the altered mitochondrial dynamics, suggesting that the anti-fibrotic effects of KCa3.1 inhibition may be partly attributed to the modulation of mitochondrial dynamics.

Mitophagy is a form of selective autophagy, which eliminates damaged or defective mitochondria. Recently, mitophagy has emerged as a cytoprotective mechanism to maintain mitochondrial homeostasis and cell survival under conditions of stress. Defective mitophagy has been reported in various kidney diseases including cisplatin-induced acute kidney injury (Zhao et al., 2017), ischemia–reperfusion-induced acute kidney injury (Ishihara et al., 2013), and diabetic kidney disease (Li et al., 2017; Xiao et al., 2017). Consistently, we found that mitophagy was markedly decreased in both *in vitro* and *in vivo* studies (**Figures 3**, **5**), which was accompanied by mitochondrial dysfunction. BNIP3, a member of the Bcl2 family, has been identified as a key receptor for mitophagy via interaction with LC3 (Hanna et al., 2012). BNIP3 resides primarily on the mitochondria and is a critical regulator of

mitochondrial function and cell apoptosis (Gao et al., 2020). Specifically, increasing BNIP3 expression leads to loss of mitochondrial membrane potential and the opening of the mitochondrial permeability transformation pore, which results in mitochondrial dysfunction and cell death (Kubli et al., 2007). BNIP3 has been shown to be involved in many diseases such as hepatic, cardiovascular diseases, and cancer (Kanzawa et al., 2005; Dhingra et al., 2017; Gong et al., 2018). In kidneys, Ishihara et al. (2013) observed the induction of BNIP3 together with increased apoptosis and defective autophagy/mitophagy under hypoxic conditions in renal tubular cells and in ischemia–reperfusion injury in rats. Tang et al. (2019) further demonstrated an important role of BNIP3-mediated mitophagy in mitochondrial quality control, tubular cell survival, and renal function during ischemia–reperfusion injury. Recently, Liu et al. (2019) reported that Stanniocalcin-1 ameliorates oxidative stress and cell apoptosis in the kidneys of the db/db mice and high glucose-treated mouse proximal tubular cells by inhibiting BNIP3 expression, which is mediated by activating the AMPK/Sirt3 pathway. In our study, the increased BNIP3 expression was found to be related to dysfunctional mitochondria and abnormal mitochondrial dynamics in TGF-β1-exposed HK2 cells and STZ induced type 1 diabetic mice (**Figure 6**). KCa3.1 deficiency restored mitochondrial quality surveillance by inhibiting BNIP3 expression, indicating a potential relationship between KCa3.1 and BNIP3. It is important to point out that other mitophagy-related pathways such as PINK1/Parkin and FUNDC1-dependent mitophagy have also been reported in diabetic kidney disease (Xiao et al., 2017; Liu et al., 2020; Wei et al., 2020), which were not examined in this study. Hence, further study is warranted to better understand the role and the interaction between different mitophagy-related pathways in diabetic kidney disease.

CONCLUSION

These studies in both *in vitro* and *in vivo* models demonstrate that KCa3.1 mediates dysregulation of mitochondrial function, mitochondrial dynamics, and mitophagy in diabetic kidney disease. Functional KCa3.1 has been shown to be expressed in the inner mitochondrial membrane in addition to the plasma membrane (Leanza et al., 2014; Kovalenko et al., 2016). Although the exact regulatory mechanism of KCa3.1 is not fully understood, it is likely that KCa3.1 regulates mitochondrial quality control through the modulation of membrane potential, cell volume, or calcium influx, which are crucial for mitochondrial function, mitochondrial dynamics, and mitophagy (Mohr et al., 2019; Romero-Garcia and Prado-Garcia, 2019). The findings from the current study not only further confirm the role of mitochondrial dysfunction in diabetic kidney disease, but also offer the potential of targeting KCa3.1 to normalize mitochondrial quality control in the treatment of diabetic kidney disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the corresponding author, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research Ethics Committee of Royal North Shore Hospital.

AUTHOR CONTRIBUTIONS

CH, CP, and X-MC conceptualized and designed the experiments. CH, HY, YS, QC, YS, DC, and FB performed the experiments and analyzed the data. CH drafted the manuscript. All authors contributed to manuscript revision and read and approved the final version of the manuscript.

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REVIEW
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The Genomic Response to TGF-β1 Dictates Failed Repair and Progression of Fibrotic Disease in the Obstructed Kidney

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Tubulointerstitial fibrosis is a common and diagnostic hallmark of a spectrum of chronic renal disorders. While the etiology varies as to the causative nature of the underlying pathology, persistent TGF-β1 signaling drives the relentless progression of renal fibrotic disease. TGF-β1 orchestrates the multifaceted program of kidney fibrogenesis involving proximal tubular dysfunction, failed epithelial recovery or re-differentiation, capillary collapse and subsequent interstitial fibrosis eventually leading to chronic and ultimately end-stage disease. An increasing complement of non-canonical elements function as co-factors in TGF-β1 signaling. p53 is a particularly prominent transcriptional co-regulator of several TGF-β1 fibrotic-response genes by complexing with TGF-β1 receptor-activated SMADs. This cooperative p53/TGF-β1 genomic cluster includes genes involved in cellular proliferative control, survival, apoptosis, senescence, and ECM remodeling. While the molecular basis for this co-dependency remains to be determined, a subset of TGF-β1-regulated genes possess both p53- and SMAD-binding motifs. Increases in p53 expression and phosphorylation, moreover, are evident in various forms of renal injury as well as kidney allograft rejection. Targeted reduction of p53 levels by pharmacologic and genetic approaches attenuates expression of the involved genes and mitigates the fibrotic response confirming a key role for p53 in renal disorders. This review focuses on mechanisms underlying TGF-β1-induced renal fibrosis largely in the context of ureteral obstruction, which mimics the pathophysiology of pediatric unilateral ureteropelvic junction obstruction, and the role of p53 as a transcriptional regulator within the TGF-β1 repertoire of fibrosis-promoting genes.

Keywords: fibrosis, PAI-1, transcription, TGF-β, p53

THE CLINICAL REALITIES OF CHRONIC RENAL DISEASE

Acute kidney injury (AKI) and chronic kidney disease (CKD) comprise a rapidly growing medical and economic burden within the US as well as globally. Renal tubular epithelial trauma and subsequent cell death correlates with patient morbidity and mortality and, when severe or episodic, often progresses to CKD and eventual end-stage renal disease (ESRD) (Bonventre and Yang, 2011; Kaissling et al., 2013; Ferenbach and Bonventre, 2015; Kumar, 2018; Liu et al., 2018). Epidemiologic data suggest that CKD may be the most under-recognized public health issue impacting 1 in 7 (35 million) adults in the US with 90% of affected individuals unaware of their underlying condition (Tuot et al., 2011; Centers for Disease Control and Prevention, 2016[1]; US Renal Data System, 2018). The Global Burden of Disease Study[2] (Bowe et al., 2018; O'Brien, 2019) estimated that over the period from 2002 to 2016, deaths due to CKD rose 58%. Moreover, disability adjusted life years lost to CKD climbed 41% while years living with disability and years of life lost to CKD increased by 48 and 56%, respectively. Diabetes and hypertension are the primary and secondary drivers, respectively, of CDK and ESRD (NIDDK Health Information Website)[3]; other prominent contributors include sepsis, ischemia/reperfusion injury, obstructive nephropathy, metabolic disorders, and dietary exposure to nephrotoxins (Uchino et al., 2005; Bagshaw et al., 2008; Emlet et al., 2015; **Figure 1**). Medicare costs for patients with all stages of CKD approximated $114 billion in 2016 alone ($35 billion for ESRD and $79 billion for the treatment of individuals with CKD without end-stage organ failure). Race, age and economic disparities are prevalent in the renal disease patient population (Luyckx et al., 2018) and the overall incidence as well as expenditures continue to rise with limited effective therapies on the horizon (Ruiz-Ortega et al., 2020).

Regardless of etiology, progressive tubulointerstitial fibrosis is the final common pathway to CKD and a hallmark of ESRD (Eddy, 2005, 2014; Bonventre, 2010; Zeisberg and Neilson, 2010). Indeed, the extent of tubulointerstitial pathology (i.e., degree of inflammation, tubular dysmorphism and atrophy, progressive fibrosis) has important functional and prognostic implications (Grande et al., 2010; Truong et al., 2011; Eddy, 2014). While older individuals constitute the majority of the at-risk cohort (60% of the population >80 years have CKD), with collateral age-dependent increases in cardiovascular complications (Ruiz-Ortega et al., 2020), children are also susceptible. In 2016, approximately 5,700 pediatric patients developed ESRD due to several causative factors with a mortality incidence 30-times that of their healthy counterparts (McDonald et al., 2004; Kramer et al., 2009; Centers for Disease Control and Prevention, 2016; US Renal Data System, 2018). Indeed, the primary causes of pediatric CKD and ESRD are congenital anomalies of the kidney and urinary tract (Ingraham and McHugh, 2011; Chevalier, 2016). Unilateral ureteropelvic junction (UPJ) obstruction, with an incidence of 1:500–1,500 live births, is the most common form of obstructive uropathy associated with end-stage disease although other contributors include ureterovesical junction blockage, posterior urethral valve disease, urethral atresia or stricture and neuropathic bladder (Ucero et al., 2010; Weitz et al., 2017).

TUBULOINTERSTITIAL INJURY: THE BASICS

Extensive or recurring sublethal epithelial trauma, usually in the context of persistent transforming growth factor-β1 (TGF-β1) pathway activation, initiates and sustains a program of maladaptive repair that facilitates the progression of AKI to CKD (Friedman et al., 2013; Emlet et al., 2015; Ferenbach and Bonventre, 2015; Venkatachalam et al., 2015; Basile et al., 2016; Takaori et al., 2016; Chang-Panesso and Humphreys, 2017; Schnaper, 2017; Chung et al., 2018; Qi and Yang, 2018; Gewin, 2019; Tang et al., 2020; **Figure 2**). The major source of TGF-β1, as well as other proinflammatory cytokines, in the kidney is the injured epithelium although both resident and infiltrative macrophages are also major contributors (Bonventre and Yang, 2011; Liu et al., 2018; Black et al., 2019; Zhang et al., 2020). Repetitive tubular damage triggers renal inflammation, pericyte loss, subsequent capillary rarefaction and tissue hypoxia, epithelial dedifferentiation, G_2/M growth arrest, tubule dysfunction and nephron dropout (Basile, 2004; Fine and Norman, 2008; Yang et al., 2010; Moonen et al., 2018; Kumar, 2018; Zhang D. et al., 2018; Zhang S. et al., 2018; Liu et al., 2019). Necrotic or apoptotic renal epithelial cells also release various damage-associated molecular pattern (DAMP) factors that activate toll-like receptors and stimulate the innate immune system prolonging the inflammatory response (Liu et al., 2018).

Non-resolving inflammation precedes, and likely promotes, renal interstitial fibrosis (Bascands and Schanstra, 2005; Chevalier et al., 2010; Meng et al., 2015, 2016; Li et al., 2017). The extent of tubulointerstitial pathology (i.e., degree of inflammation, tubular dysmorphism and atrophy, progressive fibrosis) has critical functional and prognostic implications (Grande et al., 2010; Truong et al., 2011; Eddy, 2014). Increased angiotensin II and TGF-β1 levels in the injured kidney stimulates

Abbreviations: AQP2, aquaporin 2; ALK, activin-like kinase; AKI, acute kidney injury; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related serine/threonine-protein kinase; CASP, chronic kidney disease-associated secretory phenotype; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; CKD, chronic kidney disease; CTGF, connective tissue growth factor; DEG, differentially expressed genes; Dot1l, disruptor of telomeric silencing-1-like; ECM, extracellular matrix; EDA, extra domain A; ESRD, end stage renal disease; FnEDA, extra domain A splice variant of fibronectin; GARP, glycoprotein A repitious predominant; KIM-1, kidney injury molecule-1; LAP, latency-associated peptide; LTBP, latent transforming growth factor-β1 binding protein; MRTF, myocardin-related transcription factors; PAI-1, plasminogen activator inhibitor-1; PIF-alpha, pifithrin-α; RGD, arginine-glycine-aspartic acid; SASP, senescence-associated secretory phenotype; SERPIN, serine protease inhibitor; SERPINE1, serine protease inhibitor, clade E, member 1; siRNA, small interfering RNA; SRF, serum response factor; TASCC, target or rapamycin-autophagy spatial coupling components; TCF, ternary complex factors; TGF-α1, transforming growth factor-β1; TGF-βR, transforming growth factor-β receptor; TAZ, transcriptional coactivator with PDZ-binding motif; UPJ, ureteropelvic junction; UUO, unilateral ureteral obstruction; YAP, yes-associated protein.

[1] nccd.cdc.gov/ckd

[2] www.healthdata.org/gbd

[3] www.nikkd.nih.gov/health-information/kidneydisease

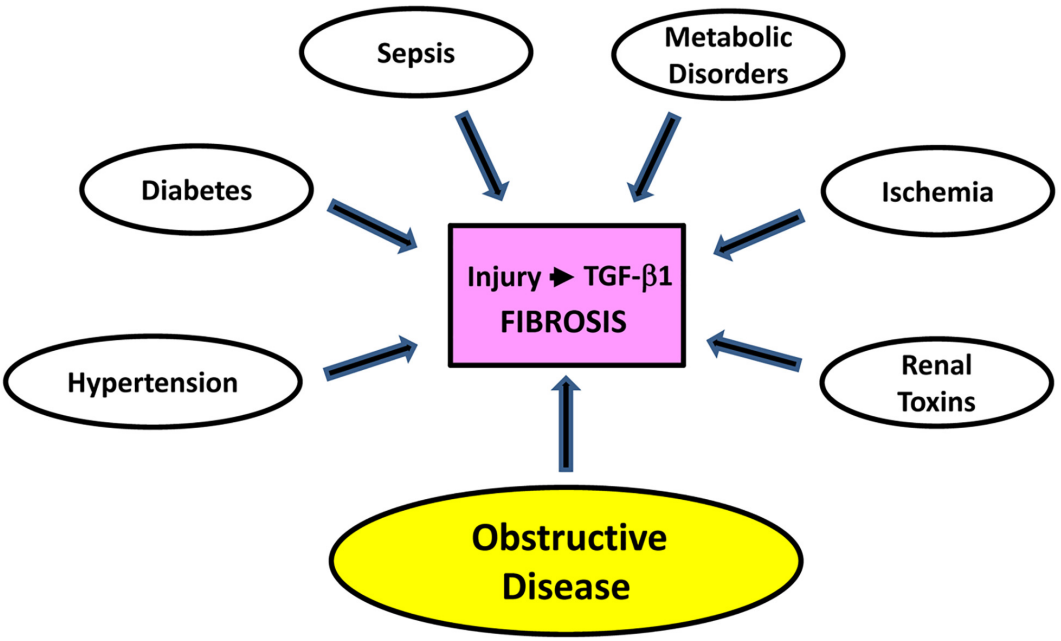

FIGURE 1 | Contributors to renal damage. Extensive trauma or episodic epithelial injury, regardless of etiology and usually in the context of persistent transforming growth factor-β1 (TGF-β1) pathway activation, initiates and sustains a program of maladaptive repair that facilitates the progression of AKI to CKD. While diabetes and hypertension are preeminent initiators of CKD, sepsis, metabolic disorders, ischemia/reperfusion injury, exposure to nephrotoxins, and obstructive nephropathy are other significant causative factors. Several animal models of renal injury lend themselves to the discovery of genes and pathways that contribute to the onset and progression of kidney fibrosis. Unilateral ureteral obstruction (UUO) in rodents (either complete or partial/reversible), for example, is one of the most widely used as it approximates the pathophysiology of human obstructive nephropathy in children and adults. Ureteral ligation is a relatively simple procedure and produces a highly reproducible pathological response over a short time course with minimal inter-animal variability. UUO provides a translationally-relevant *in vivo* platform to probe the genomic complexity of kidney injury, mechanisms underlying maladaptive repair and the efficacy of new therapeutic approaches to the management of fibrotic disease (Martínez-Klimova et al., 2019).

the conversion of activated $Gli1^+$/$FOXD1^+$ vascular pericytes and interstitial fibroblasts to matrix-producing myofibroblasts driving the pathophysiology of tissue fibrosis (Qi et al., 2006; Picard et al., 2008; Ricardo et al., 2008; Grande and Lopez-Novoa, 2009; Cook, 2010; Humphreys et al., 2010; LeBleu et al., 2013; Duffield, 2014; Gomez and Duffield, 2014; Kramann and Humphreys, 2014; Richter et al., 2015; Kramann et al., 2015; Mack and Yanagita, 2015). Pericyte mobilization in response to injury, moreover, results in their interstitial translocation, effectively promoting peritubular capillary collapse and creation of a hypoxic environment (Kramann et al., 2013; Kramann and Humphreys, 2014).

EXPERIMENTAL OBSTRUCTIVE NEPHROPATHY: A TOOL TO PROBE MECHANISMS AND PATHWAYS

Several animal models of acute and chronic renal disease are amenable to the discovery of causative factors underlying the onset and progression of kidney fibrosis while affording a platform to assess the efficacy of therapeutic interventions (Ortiz et al., 2015; Nogueira et al., 2017; Bao et al., 2018). Unilateral ureteral obstruction (UUO) in rodents (e.g., Chevalier, 2015; Martínez-Klimova et al., 2019), for example, closely mirrors (in an accelerated context) human obstructive nephropathy while bridging the pathologic features of AKI and CKD (Moller et al., 1984; Hruska, 2002; Ucero et al., 2014). Ureteral ligation provides an accessible, translationally-relevant, *in vivo* opportunity to clarify the genomic complexity of renal fibrotic disease, dissect critical pathophysiologic events underlying the kidney response to injury and identify mechanisms involved in maladaptive repair (Klahr and Morrissey, 2002; Truong et al., 2011; Eddy et al., 2012; Samarakoon et al., 2012; Arvaniti et al., 2016; Sun et al., 2016; Jackson L. et al., 2018; Jackson A. R. et al., 2018; Martínez-Klimova et al., 2019; Pavkovic et al., 2019).

Surgical interference with the flow of urine increases hydrostatic pressure initially in the collecting ducts expanding rapidly to the distal and proximal tubules (Martínez-Klimova et al., 2019). Long-term obstruction results in outer medullar ablation and tubular atrophy; a 65% decrease in proximal tubule mass becomes evident within 14 days of ureteral ligation. Tubule dilation, epithelial necrosis/apoptosis, basement membrane denudation, rapid influx of inflammatory cells, interstitial expansion with increased cellular proliferation and eventual fibrosis are prominent in the cortex of the ligated kidney (Cochrane et al., 2005; Manucha, 2007; Forbes et al., 2011, 2012; Ucero et al., 2014). The proximal tubule appears to be the predominant sensor and immediate effector of renal damage and may well orchestrate disease progression via injury-associated

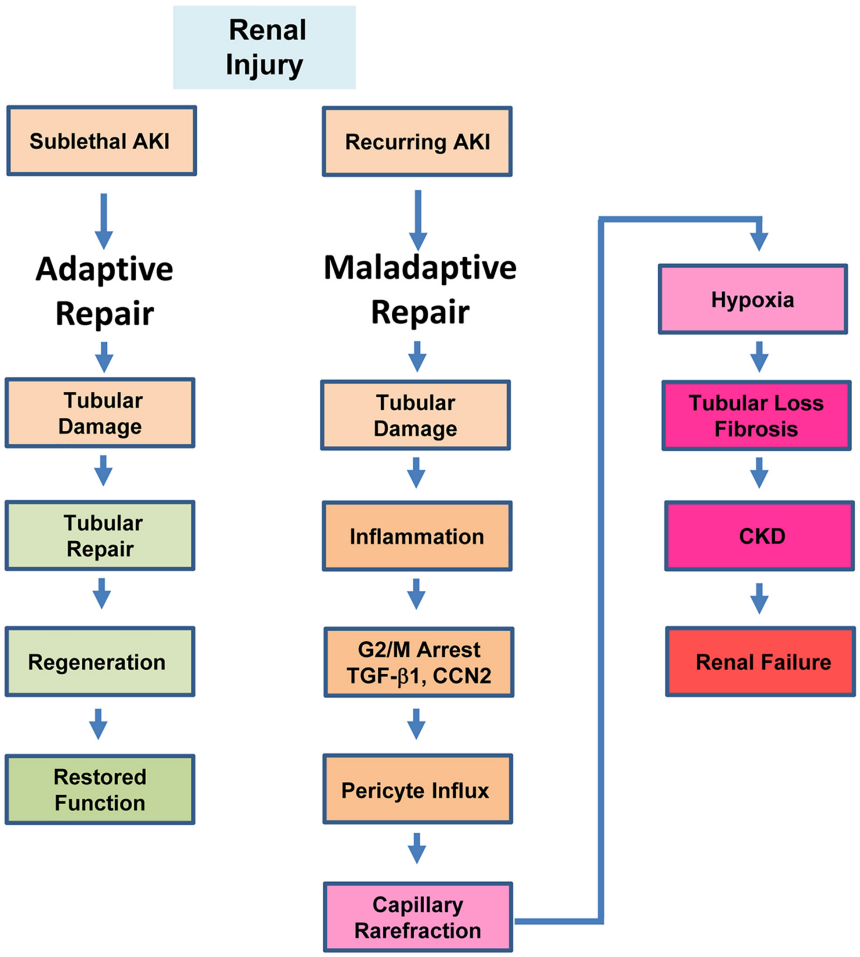

FIGURE 2 | Repair outcomes in the injured kidney. Mild or sublethal AKI initiates a process of adaptive repair that involves resolution of inflammation and restoration of tubular architecture with a regain of renal function. Recurring AKI or severe tubular trauma, in contrast, results in maladaptive repair which is characterized by a sustained inflammatory response, stalling of injured proximal tubular epithelial cells in the G_2/M stage of the cell cycle, interstitial translocation of vascular pericytes and their differentiation into ECM-producing myofibroblasts, tubular atrophy, capillary loss and failure to regenerate a functional epithelium. G_2/M-stalled proximal tubular cells express significant levels of the potent pro-fibrotic effectors TGF-β1 and CCN2 which contribute to the initiation and progression of renal fibrosis.

tubular shortening and/or paracrine mechanisms that impact several resident renal cell types (Endo et al., 2015; Tan et al., 2016; Gewin et al., 2017). A significant fraction (46%) of glomeruli, moreover, exhibit atrophic proximal tubules and 39% eventually become atubular indicating that the glomerulotubular junction tubular epithelium is particularly sensitive to UUO-induced necrosis and/or apoptosis (Chevalier et al., 2011). One suggestion is that glomerulotubular junction cell death may be a key driver of nephron loss and that the subsequent fibrotic response reflects an attempt at self-limiting tissue repair (Chevalier, 2016). Such congenital reduction in nephron density impairs recovery from obstructive injury and exacerbates the fibrotic process (Sergio et al., 2015).

Partial and complete UUO in neonatal rodents are similar except for a temporal offset in acquisition of pathologic features (Jackson L. et al., 2018; Jackson A. R. et al., 2018). UUO modeling largely focuses on the proximal tubular compartment due to its high mitochondrial load, dependency on oxidative phosphorylation, susceptibility to ischemic injury and relative deficiency of anti-oxidant/anti-apoptotic factors (Chevalier, 2016). The distal nephron including the collecting duct, however, also contributes significantly to the overall response of the kidney to ureteral ligation-induced injury (Hiatt et al., 2013). Tubular dilation and myofibroblast accumulation in the distal nephron increases by 2—3- and 6-fold, respectively, in the obstructed kidney and coupled to a change in cellular composition of the collecting duct. Aquaporin 2 (Aqp2)-expressing principal cells decline by 65% and intercalated cell abundance decreases by 75%. E-cadherin- and β-catenin-mediated collecting duct epithelial adhesion is also disrupted. Notably, these features are replicated in the distal and connecting tubules (Hiatt et al., 2013) confirming that the distal nephron is a major target of UUO-initiated renal disease, highlighting the utility of UUO as a model to dissect the involvement of collecting duct and distal tubule injury to kidney repair and fibrosis. Principal cells in the collecting duct are fundamental to the development of

tubulointerstitial fibrosis (Butt et al., 2007; Ivanova et al., 2008; Fujiu et al., 2011), at least in part through Notch signaling, and are subject to epigenetic regulation (Zhang et al., 2020). Mib1, an E3 ligase produced by ligand-expressing cells, is required for efficient Notch mobilization while inactivation of Mib1 in the collecting duct results in increased tubulointerstitial fibrosis and apoptosis of principal cells in response to UUO. Furthermore, CKD can be induced by connecting tubule/collecting duct-specific disruption of the β1 integrin (Mamuya et al., 2017), integrin-linked kinase (Huang et al., 2019), and histone H_3 K79 methyltransferase Dot1l (Zhang et al., 2020) or ameliorated by collecting duct-specific ablation of Krüppel-like factor 5 (Fujiu et al., 2011).

Recent genetic studies, moreover, implicate connecting tubule/connecting duct endothelin-1, a potent vasoconstrictor with proinflammatory and profibrotic properties, in not only UUO-mediated injury but also in streptozotocin-induced as well as age-related kidney disease (Zhang et al., 2020). Four groups of engineered mice including (1) those with floxed alleles of histone H_3 lysine79 (H_3K79) methyltransferase disruptor of telomeric silencing-1 (*Dot1l*$^{f/f}$) and endothelin-1 (*Edn1*$^{f/f}$); (2) *Dot1l*$^{f/f}$ *Aqp2Cre* (*Dot1l*^{AC}); (3) *Dot1l*$^{f/f}$ *Edn1*$^{f/f}$ *Aqp2Cre* (*DE*^{AC}); and (4) *Edn1*$^{f/f}$ *Aqp2Cre* (*Edn1*^{AC}) were subjected to UUO. An Aqp2 promoter-driven Cre construct provided for Cre expression specifically in the epithelial cells of the collecting duct. *Dot1l*^{AC} vs. WT or *Edn1*^{AC} mice developed severe fibrosis and renal dysfunction. *Dot1l*^{AC} phenotypes were mitigated in the double-knockout *DE*^{AC} mice with similar results evident in streptozotocin-induced diabetes and normal aging (Zhang et al., 2020). This is the first demonstration that loss of histone H_3 K79 methyltransferase Dot1l promotes renal fibrosis due, in large measure, to endothelin-1 up-regulation in the collecting duct epithelium consistent with the implication that Dot1l exerts an antifibrotic function by repressing endothelin-1 transcription. Kidney fibrosis in response to UUO, moreover, is epigenetically regulated through Dot1l action in the connecting tubule and collecting duct. It appears, therefore, that the pathophysiology of obstructive uropathy is both complex and likely involves the entire nephron. The growing appreciation for the extensive cross-talk and mutual inducibility between the TGF-β1 and endothelin-1 signaling systems in the kidney, their shared potent fibrogenic activities and ability to impact virtually all renal cell types (e.g., Eddy, 2000; Castañares et al., 2007; Dhaun et al., 2012; Wermuth et al., 2016) suggests that nephron segment-specific fibrotic factors may need to be considered in the formulation of targeted therapies.

TUBULAR REPAIR AND CELL CYCLE ARREST IN THE INJURED KIDNEY

Depending on the severity and duration of injury to the proximal tubular epithelium (a critical initiator of the tubulointerstitial fibrotic process), the response of the kidney can be adaptive (i.e., regenerative; restoration of function) or maladaptive (i.e., fibrotic; compromised function) (Grgic et al., 2012; Lee et al., 2012; Kumar et al., 2014; Ferenbach and Bonventre, 2015; Kumar, 2018; Liu et al., 2018; Qi and Yang, 2018; **Figure 2**). Following tubular cell necrosis or apoptosis, the remaining viable epithelium undergoes morphologic dedifferentiation (i.e., loss of polarity with cell spreading and migration to cover the exposed areas of the basement membrane) and subsequent proliferation as an attempt to restore the functional integrity of the nephron (Bonventre, 2003). Fate mapping studies indicate, moreover, that tubular regeneration is orchestrated by surviving epithelial cells (Humphreys et al., 2008; Berger et al., 2014; Lombardi et al., 2016). Although it is apparent that upon injury a subpopulation of renal cells exhibits significant regenerative potential, these are not likely a fixed pre-existing progenitor population but rather derive from viable dedifferentiated proximal tubular cells that acquire a specific phenotype in response to injury (Kusaba and Humphreys, 2014; Kusaba et al., 2014; Humphreys et al., 2016; Andrianova et al., 2019). Early successful repair, nevertheless, involves activation of a Sox^+/$KIM1^+$ cohort which regresses after regeneration of a functional epithelium (Kumar, 2018). Retention of the Sox^+/$KIM1^+$ phenotype, however, signals tubules with unresolved injury while *Snai1* and *Twist1* induction predispose to a more plastic phenotype, failed differentiation and accumulation of cells in G_2/M with engagement of a proinflammatory/profibrotic genomic program (Kumar, 2018).

G_1 phase arrest in the injured kidney allows for repair of DNA damage prior to replication in S phase. G_2/M-stalling provides an additional opportunity to assess DNA integrity but also mobilizes the c-JUN N-terminal kinase stress pathway resulting in the transcription of several major pro-fibrotic senescence-associated secretory phenotype (SASP)-type effectors. These include connective tissue growth factor (CTGF, CCN2), TGF-β1 and the clade E member 1 serine protease inhibitor SERPINE1, also known as plasminogen activator inhibitor-1 (PAI-1), a potent negative regulator of the pericellular proteolytic cascade (Yang et al., 2010; Sturmlechner et al., 2017; Liu et al., 2019; **Figure 3**). Cytoscape profiling, moreover, implicates SERPINE1 as a major hub gene in the genomic program of tissue fibrosis where it functions as a key interacting modulator of focalized uPA/uPAR-dependent pericellular proteolysis as well as a binding partner and activator of the signaling competent low-density lipoprotein receptor-related protein-1 (LRP1) (**Figure 4**). String Protein-Protein Interaction Network and Gene Ontology analyses confirmed the cooperative role of SERPINE1, TGF-β1 and the extracellular matrix (ECM) protein fibronectin in the more global process of normal and maladaptive wound repair (**Figure 5**).

Events underlying the coupling of G_2/M and expression of a fibrotic program, however, are complex. TGF-β1-induced G_2 phase prolongation in proximal tubular cells appears mediated, at least in part, by *Twist1* and *Snai1* since overexpression of either is sufficient for induction of the p53 target gene p21 and protracted residence in G_2 (Lovisa et al., 2015; Qi and Yang, 2018). p21, moreover, is likely involved in the increase in G_2 cells in the very initial stages of renal injury (Koyano et al., 2019). While the p53→p21 pathway contributes to G_2/M arrest and acquisition of a fibrotic program, an additional highly up-regulated p53-dependent gene (at least in aristolochic acid [AA]-induced kidney injury) is cyclin G_1 which promotes the extended duration of G_2/M and also increases

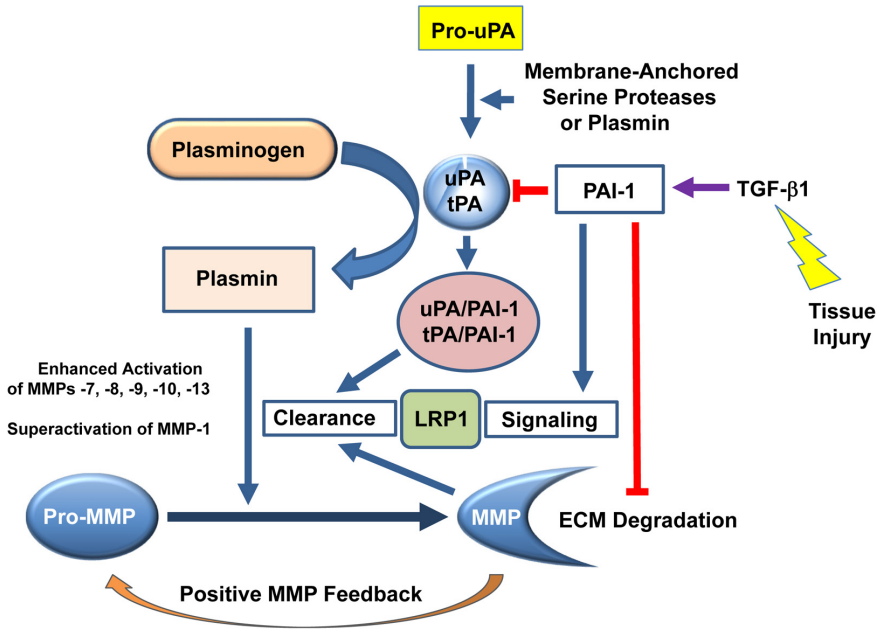

FIGURE 3 | PAI-1 (SERPINE1) is a critical factor in the regulation of the pericellular proteolytic microenvironment and fibrotic response to tissue injury. Plasminogen activators (urokinase, uPA; tissue-type, tPA) are the physiologically and pathophysiologically-relevant plasmin-generating proteinases that impact extracellular matrix (ECM) accumulation/degradation through a complex and highly interdependent proteolytic cascade. Pro-uPA is cleaved to the active enzyme uPA by membrane-anchored serine proteases (e.g., Matriptase, Hepsin, Serase-1B) or catalytically-active levels of plasmin. uPA-induced conversion of plasminogen to plasmin results in the significant downstream mobilization of several matrix metalloproteinases (MMPs). Collectively, both the plasmin-dependent and MMP proteolytic systems dictate the extent and locale of ECM remodeling. Elevated expression or bioactivity of PAI-1, generally in response to tissue injury-induced TGF-β1, facilitates ECM accumulation and inhibits ECM degradation which, if prolonged or chronic, leads to the initiation and progression of fibrotic disease.

formation of target of rapamycin (TOR)-autophagy spatial coupling components (TASCCs) stimulating, thereby, expression of SASP genes (Canaud et al., 2019). The p53 inhibitor pifithrin-α (PIF) attenuates the fraction of G_2/M-arrested epithelial cells while deletion of cyclin G_1, mTOR, LC3, or lysosomal associated membrane protein 2 (LAMP2) reduces the onset and progression of renal disease (Canaud et al., 2019).

INJURY-ASSOCIATED ACQUISITION OF A SENESCENCE-LIKE PHENOTYPE

Multiple sublethal injuries to the kidney leads to the emergence of a senescence-like state in some surviving tubular cells resulting in a failure to respond with adaptive proliferation (Ferenbach and Bonventre, 2015). Senescent epithelial cells are evident in the kidney in the pathologic context of hypertension, diabetes, IgA nephropathy and ischemia/reperfusion injury particularly in aged mice, where progressive immune system dysfunction may drive the development of CKD (Verzola et al., 2008; Satriano et al., 2010; Qi and Yang, 2018; Xiong and Zhou, 2019; Schroth et al., 2020). Indeed, aging in rodents is associated with enhanced tubular cell senescence, elevated TGF-β1, p16, and p21 expression and increasing tubulointerstitial fibrosis (Ding et al., 2001; Knoppert et al., 2019). While reparative $CD24^+/CD133^+$ epithelial cells contribute to healing and functional recovery, exogenous delivery of even a small number of senescent cells induces inflammation and fibrosis (Kim et al., 2020).

The maladaptive tubular repair and the cellular senescence programs (e.g., G_2/M stalling, expression of proinflammatory/profibrotic factors) both involve p53 and transcription of the p53 target genes p21 and PAI-1. There is, in fact, considerable overlap among the SASP, the chronic kidney disease-associated secretory phenotype (CASP) and the SASP aging and disease biomarker gene sets that includes increases in the scar-promoting proteins TGF-β1, PAI-1 (SERPINE1) and CNN2 (Wang et al., 2017; Basisty et al., 2020). A percentage of tubular epithelial cells gradually acquire a senescence-like phenotype with advancing age and express elevated levels of TGF-β1, p16, and p21 (Ding et al., 2001; Braun et al., 2012; Ferenbach and Bonventre, 2015). Indeed, senescence promotes interstitial fibrosis, tubular atrophy and renal graft deterioration limiting tubular regeneration and transplant survival (Braun et al., 2012). The elevated levels of reactive oxygen species (ROS) that accompany the DNA damage response, moreover, are likely major contributors to the initiation of the senescent phenotype (Moonen et al., 2018; Beck et al., 2020a,b). Indeed, in some cell types, TGF-β1 functions as a senescence driver via ROS-stimulated NF-κB signaling and induction of SASP factors, including PAI-1 (Kwon et al., 2017; You et al., 2019; **Figure 6**). This appears critically important in the establishment of the growth arrest state as PAI-1 is not merely a biomarker of the senescent phenotype but is necessary and sufficient for

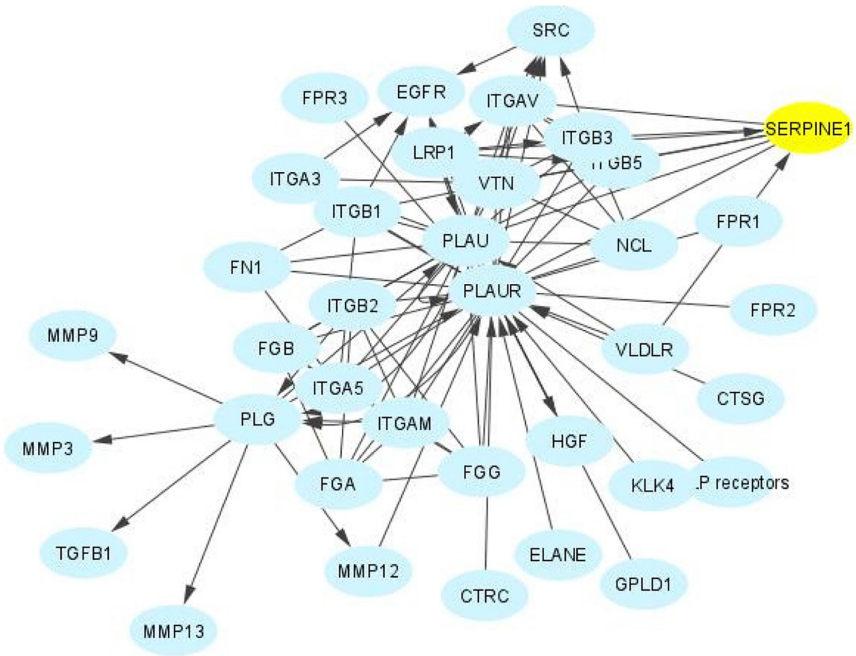

FIGURE 4 | The SERPINE1 interactome. SERPINE1 (PAI-1) is a major hub factor in the regulation of the immediate pericellular proteolytic cascade. PAI-1 titrates the conversion of plasminogen to plasmin by binding to and inhibiting the catalytic activity of urokinase plasminogen activator (PLAU), effectively attenuating stromal proteolysis while promoting matrix accumulation and the onset and progression of fibrotic disease regardless of etiology. PAI-1 also regulates cellular attachment and migration, key aspects of the injury repair program, largely by altering interaction of the PLAU-PLAU receptor (PLAUR) complex with its associated integrins and by functioning as a ligand for LRP1 to initiate post-receptor downstream signaling.

the induction of replicative senescence downstream of p53 (Kortlever et al., 2006; Hiebert et al., 2018).

Once renal repair becomes dysfunctional (i.e., elevated expression of the cell cycle arrest protein p21, down-regulation of the anti-aging factor Klotho, telomere shortening, increased oxidative stress), continued activation of the SASP and CASP programs accelerate cellular aging leading to the development of age-related pathologies (Wang et al., 2017; Andrade et al., 2018; Dai et al., 2019). Cellular senescence is evident in many forms of kidney injury (Li and Lerman, 2020) and older mice have increased senescence-associated β-galactosidase, p53, and p21 expression in response to ischemia/reperfusion injury compared to young mice (Clements et al., 2013; Valentijn et al., 2018). This is relevant to the human condition as age-associated renal scarring, and decline in kidney function, varies among ethnic groups and expression of β-galactosidase and p16 is evident even in the absence of morphologic changes (Yang and Fogo, 2010).

Although the mechanism underlying cell cycle phase-specific arrest or at least residence prolongation is unclear, activation of the p53→p21 axis, particularly in the early stages of kidney disease, likely drives renal cell stalling in both G_1 and G_2/M phases (Yang et al., 2010; Overstreet et al., 2014; Moonen et al., 2018; Wu and Prives, 2018; Liu et al., 2019). In this regard, fibrosis in response to chemotherapeutic agents, nephrotoxins, ischemia/reperfusion injury or UUO is associated with DNA damage and normal aging sensitizes tubular epithelial cells to DNA damage-induced G_2/M arrest (Yang and Fogo, 2010; Liu et al., 2018). Ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR), which function as sensors of DNA damage in the maintenance of genomic stability, are involved and alterations in their expression has consequences. ATM and ATR have several DNA repair targets in common including p53 and the cell cycle checkpoint kinases CHK1 (ATR) and CHK2 (ATM) (Bradbury and Jackson, 2003; Awasthi et al., 2015). ATR deletion in renal proximal tubular epithelial cells exacerbates maladaptive repair, increases the number of senescent cells and promotes expression of a profibrotic secretory phenotype (Kishi et al., 2019). These findings suggest that ATR provides a protective role in the injured proximal tubular epithelium to restrict or attenuate exuberant (i.e., fibrotic) repair while highlighting the role of p53 in renal disease since treatment with the p53 inhibitor PIF-α significantly reduces the fraction of G_2/M cells and mitigates the fibrotic response (Yang et al., 2010; Overstreet et al., 2014; Liu et al., 2019).

Expression of a subset of TGF-β1 target genes that contribute to growth arrest, and G_2/M stalling as well, appears to require both canonical and non-canonical signaling. To this point, TGF-β1 also upregulates the Hippo pathway effectors YAP (*yes*-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) in proximal tubular epithelial cells both *in vivo* and *in vitro*. Indeed, doxycycline-induced tubular-specific TGF-β1 expression in double-transgenic Pax8-rtTA-tet-*o*-TGF-β1 mice enhances renal TAZ levels while TGF-β1 increases TAZ levels in human proximal tubular epithelial cells; *in vitro*

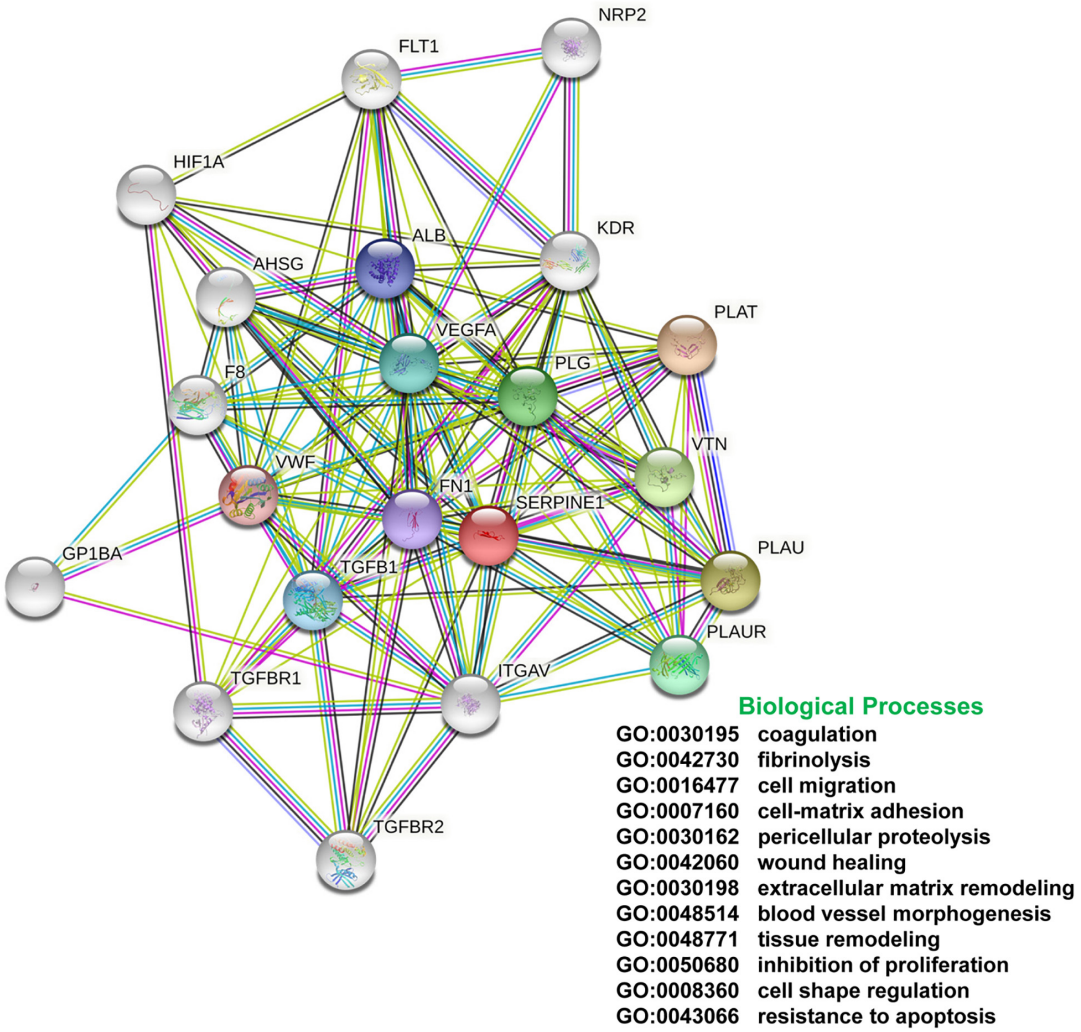

FIGURE 5 | String Network and Gene Ontology. Protein-protein interaction and GO analyses of the SERPINE1/TGF-β1/fibronectin axis indicates that SERPINE1 is a significant nodal contributor to various biological processes that impact the global program of normal and maladaptive tissue repair. These data underscore the potential clinical utility of SERPINE1 targeting in the therapy of fibrotic disease.

modeling confirmed that TAZ is necessary for TGF-β1-mediated fibrogenesis (Anorga et al., 2018). Vector-driven TAZ synthesis in human proximal tubular (HK-2) cells, or addition of conditioned medium from TAZ overproducers to control vector-transduced HK-2 cells, mimics certain aspects of the TGF-β1-induced phenotype including G_2/M arrest and acquisition of a profibrotic program (Anorga et al., 2018). Exposure of HK2 cells to hypoxic stress similarly promotes G_2/M stalling and PAI-1 induction while TAZ overexpression leads to the accumulation of HK-2 cells in G_2/M phase. TAZ is, in fact, required for maximal TGF-β1-mediated PAI-1 synthesis in proximal tubular cells (Liu et al., 2015; Samarakoon et al., 2015; Anorga et al., 2018; Bessho et al., 2019) and a similar involvement of YAP in TGF-β1-induced PAI-1 expression is evident in lung tumor cells (Kong et al., 2021). KEGG analysis confirmed that convergence of the TGF-β and Hippo signaling pathways regulates transcription of the profibrotic CCN2 and SERPINE1 genes (**Figure 7**). YAP knockdown, moreover, reduces levels of both CTGF (CCN2) and PAI-1 (SERPINE1) while introduction of the constitutively-active YAP^{S127A} construct increased PAI-1 expression (Marquard et al., 2020). Although the underlying mechanisms remain to be determined, YAP/TAZ apparently do not alter the rate of SMAD nuclear import or exit nor impact SMAD phosphorylation but may regulate SMAD nuclear levels by functioning, directly or indirectly, as retention factors and/or by changing TGF-βR activity (Labibi et al., 2020).

TGF-β/SMAD SIGNALING DRIVES FIBROSIS IN OBSTRUCTIVE NEPHROPATHY

Increased expression of the potent profibrotic cytokine TGF-β1 and the type I/II TGF-β1 receptors is a hallmark feature of

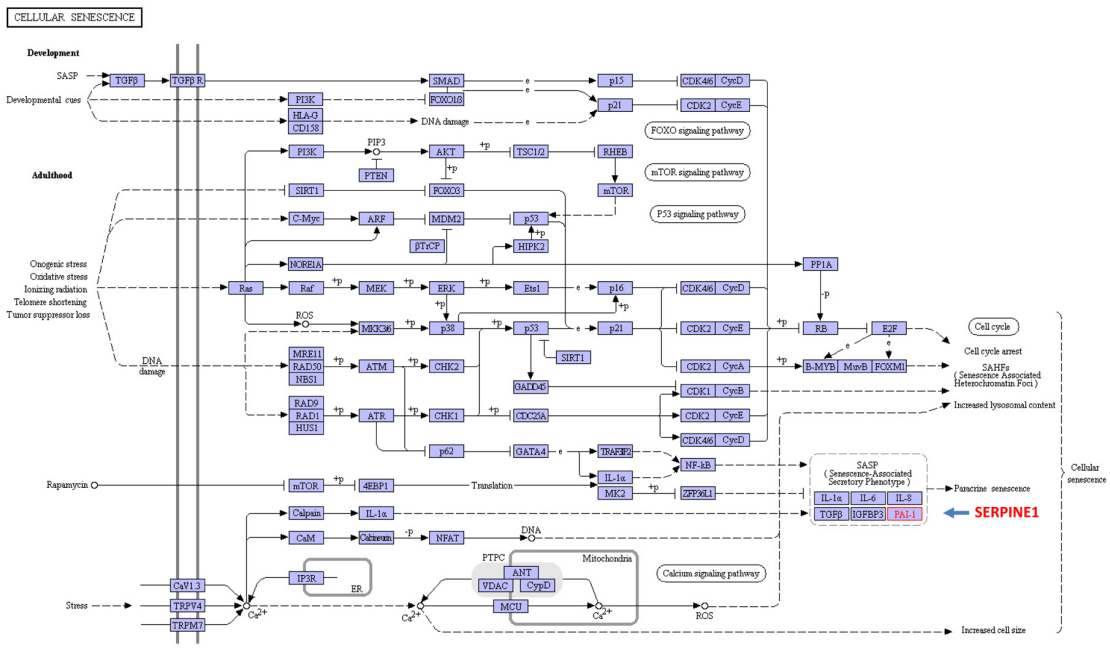

FIGURE 6 | KEGG analysis of the highly interactive program of cellular senescence. SERPINE1 and TGF-β1, two prominent activators and members of the stress-activated SASP, are key factors in the global and renal programs of proliferative arrest. Several of the involved networks include the p53, *ras* and TGF-β signaling pathways. Collectively, these regulate the expression of a spectrum of cell cycle and growth control elements (e.g., p16, p21, TGF-β1, PAI-1) (Kortlever et al., 2006, 2008).

virtually all forms of CKD (Böttinger, 2007). Tubulointerstitial pathology following experimental UUO appears largely due to elevated levels of TGF-β1, SERPINE1 and CCN2 in the injured kidney (**Figure 8**) mimicking the increased TGF-β1 expression in children with UPJ obstruction (Miyajima et al., 2000; Inazaki et al., 2004; Ucero et al., 2010). Within hours, the occluded kidney exhibits changes in hydrostatic forces and increased oxidative stress (Schreiner et al., 1988; Klahr and Morrissey, 2002; Dendooven et al., 2011). Tubular stretch further stimulates TGF-β1 expression (>20-fold), increases the epithelial apoptotic index, and leads to the development of an interstitial inflammatory infiltrate (Miyajima et al., 2000; Rohatgi and Flores, 2010). Persistently elevated renal TGF-β1 expression, even after relief of UUO (depending on the duration of obstruction and extent of pathology) frequently leads to progressive tissue injury, impaired regenerative growth, and eventual loss of organ function (Chevalier, 1999; Chevalier et al., 2009, 2010).

TGF-β1 mRNA levels steadily increase in several nephron segments as early as day-1 post-UUO followed by TGF-β1 protein upregulation (Isaka et al., 2000; Miyajima et al., 2000; Klahr and Morrissey, 2002; Yang et al., 2010; Makitani et al., 2020). TGF-β1 transcripts are most prominent in the tubular epithelia and, to a lesser extent, in a fraction of infiltrating macrophages (Kaneto et al., 1993; Fukuda et al., 2001). Attenuation of UUO-induced fibrosis upon administration of the anti-TGF-β antibody 1D11 or the TGF-β activin-like kinase 5 (ALK5) receptor signaling inhibitor SB-525334 further highlight involvement of the TGF-β pathway in ureteral obstruction-initiated renal scarring (Richards et al., 2018). The contribution of TGF-β1 to the fibrotic response, importantly, was confirmed using genetic approaches. Conditional overexpression of TGF-β1 in the tubular epithelium of Pax8-rtTA-tet-o-TGF-β1 double transgenic mice induces extensive peritubular fibrosis, focal nephron degeneration (Traykova-Brauch et al., 2008; Koesters et al., 2010) and TGF-β1-dependent loss of the SMAD phosphatase PPM1A (Tang et al., 2020). Similarly, Pax8 promoter-driven expression of a ligand-independent constitutively-active TGF-β type I receptor results in the acquisition of features typical of AKI (e.g., epithelial apoptosis, necrosis and dedifferentiation; renal inflammation) (Gentle et al., 2013). The albumin/TGF-β1 transgenic mouse (Kopp et al., 1996), moreover, recapitulates the pathophysiologic heterogeneity of CKD progression highlighting their utility in the discovery of disease progression signatures (Ju et al., 2009).

Elevated levels of TGF-β1 in the injured kidney direct the myofibroblastic differentiation of recruited vascular pericytes and resident fibroblasts while driving a program of pathologic ECM synthesis and advancing fibrosis (Bonventre, 2010; Meng et al., 2015, 2016; Sun et al., 2016; Chen et al., 2018; Feng et al., 2018; Higgins et al., 2018). Genetic deficiency of SMAD3, a major profibrotic effector of TGF-β1 signaling, or administration of the SMAD3 inhibitor SIS3 immediately after ureteral ligation, attenuates myofibroblast accumulation while suppressing deposition of collagen I and fibronectin (Sato et al., 2003; Inazaki et al., 2004; Zhang D. et al., 2018; Zhang S. et al., 2018). One mechanism may involve the SMAD3-dependent autoinduction of TGF-β1 by UUO-stimulated TGF-β1 expression (Sato et al., 2003). This has potential clinical ramifications since post-injury treatment with SIS3 also blunted

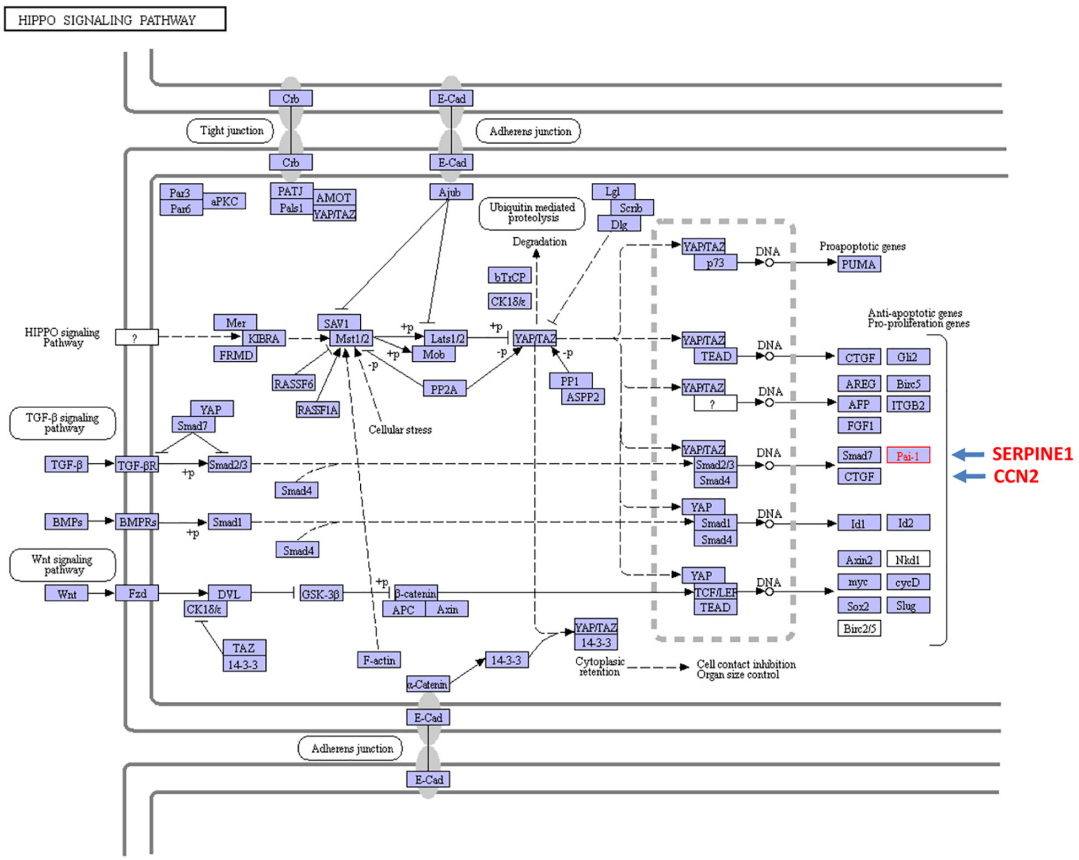

FIGURE 7 | Cross-talk between the TGF-β and the YAP/TAZ pathways impact expression of the profibrotic SERPINE1 and CCN2 genes. TGF-β activates a canonical signaling network that involves the SMAD2/3-dependent transcription of SERPINE1 and CCN2. It is also evident that non-canonical pathway engagement (e.g., Hippo) contributes to maximal TGF-β1 SERPINE1 (PAI-1) and CCN2 (CTGF) expression by stimulating YAP/TAZ nuclear translocation and interaction with the TGF-βR-phosphorylated SMAD2/3 transcriptional effectors and the shuttle SMAD4.

the subsequent fibrotic response (Zhang D. et al., 2018; Zhang S. et al., 2018) suggesting that blockade of TGF-β1→ALK5 signaling to SMAD3 has therapeutic implications. Several pre-clinical studies, in fact, targeted SMAD3 as one modality for the treatment of UUO-induced renal disease (e.g., Li et al., 2010; Ji et al., 2018; Wang et al., 2018).

Initial observations did, in fact, support the premise that interstitial fibrosis and disease progression in the obstructed kidney can be mitigated by blockade of TGF-β1 expression or function via antisense phosphorothioate oligodeoxynucleotides, small interfering RNA (siRNA) or neutralizing antibodies (Isaka et al., 2000; Miyajima et al., 2000; Gagliardini and Benigni, 2006; Hwang et al., 2006). Overexpression of the latent form of TGF-β1, to minimize availability of active TGF-β1 in the tissue microenvironment, decreases the incidence α-smooth muscle actin-positive cells (presumably myofibroblasts) in the UUO-injured kidney and blocks SMAD2/3 activation (Huang et al., 2006, 2008). The peroxisome proliferator-activated receptor gamma agonist troglitazone similarly reduces development of UUO-induced renal interstitial fibrosis and inflammation through suppression of TGF-β1 expression (Kawai et al., 2009). Collectively, these data are consistent with the concept that TGF-β1 is, indeed, the key driver of fibrosis in UUO either directly by impacting the transcription of disease-relevant genes or indirectly via angiotensin signaling (Ishidoya et al., 1995; Pimentel et al., 1995; Fern et al., 1999; Satoh et al., 2001; Inazaki et al., 2004; Shin et al., 2005). Indeed, angiotensin stimulates the expression of ECM structural elements (e.g., collagen, fibronectin, laminin) as well as inhibitors of ECM degradation including PAI-1 (SERPINE1) through TGF-β1-dependent mechanisms, thus promoting tissue fibrogenesis (Kagami et al., 1994; Wolf, 2006). While global TGF-β1-null mice exhibit no gross abnormalities at birth but die soon thereafter due to wasting associated with severe multifocal inflammation (Yaswen et al., 1996), carefully focused anti-TGF-β therapies, and perhaps targeting disease-critical downstream genes or enhancers of TGF-β1 profibrotic signaling, may be a more prudent and translationally-adaptable therapeutic approach. As one example, small molecule (SK-216) pharmacologic inhibition of the activity of the TGF-β1 target PAI-1 attenuates TGF-β1-induced fibroblast to myofibroblast transition and lung fibrosis (Omori et al., 2016). Varga and Pasche (2009) suggest, moreover, that neutralizing antibodies, pathway antagonists and soluble (i.e., trap) receptors attenuate excessive (e.g., disease-associated) TGF-β bioactivity

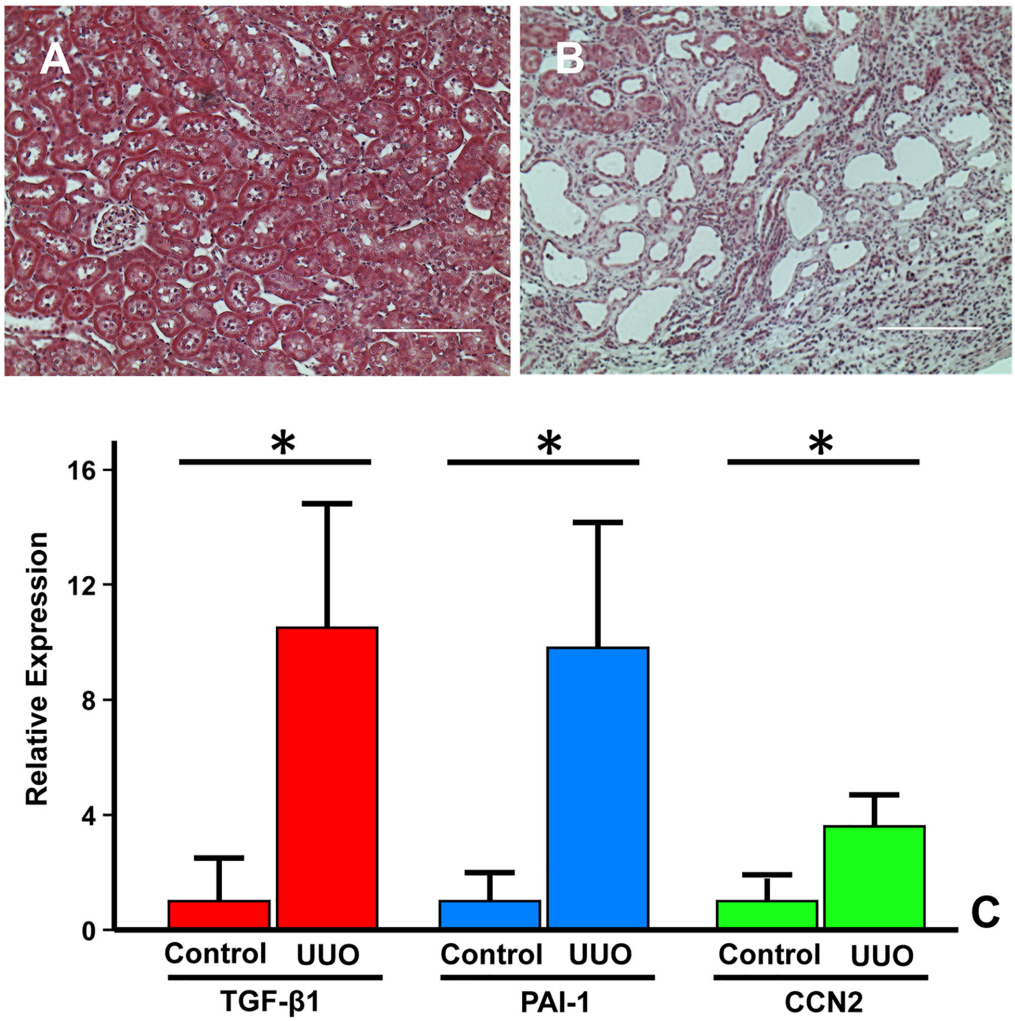

FIGURE 8 | Fibrotic response of the murine kidney to UUO. Compared to the relatively normal histology of the contralateral control or sham-operated kidney **(A)**, a dysmorphic and flattened epithelium with extensive tubular dilation, expanses of denuded basement membrane and accumulation of connective tissue (blue stain) in the expanded interstitial regions is evident in the obstructed kidney **(B)**. **(A,B)**, Trichrome stain. Morphometric analyses of immunohistochemical-stained paraffin-embedded sections of the UUO-injured kidney and the contralateral control 7–14 days post-surgery, revealed significant increases in PAI-1, TGF-β1, and CCN2 in the obstructed kidney **(C)**. Histograms illustrate the mean ± SD staining intensity (ImageJ threshold analysis) for TGF-β1, PAI-1, and CCN2 between the two experimental groups. $^{*}p < 0.05$.

while retaining homeostatic TGF-β signaling functions. Such approaches may avoid the adverse outcomes that result from TGF-β depletion (Yaswen et al., 1996; Yang et al., 2020).

MULTIPLE MODES OF TGF-β1 ACTIVATION

The tissue response to injury is largely dependent on multi-level controls on the persistence of TGF-β isoform expression and activation in the immediate pericellular microenvironment. The transition of TGF-β1 from a latent to bioactive configuration is a critical checkpoint in the fibrogenic response. TGF-β1-3 pro-proteins are comprised of a dimeric growth factor and N-terminal latency-associated peptide (LAP) domains. Disulfide bonding between LAP and the latent TGF-β binding protein (LTBP) occurs within the endoplasmic reticulum (Robertson and Rifkin, 2016). In the Golgi, LAP is cleaved from the pro-protein by the subtilisin-like pro-protein convertase furin prior to extracellular transport of the ternary large latent complex, consisting of TGF-β, LAP and the latent TGF-β binding protein (TGF-β/LAP/LTBP). The 4 LTBP isoforms (LTBP1-4) then interact with different structural elements of the ECM including fibrillin microfibrils and the fibronectin network (Zilberberg et al., 2012; Tsuda, 2018). While the different LTBPs exhibit some preferences for TGF-β isoform recognition, LTBP-1 has a particular affinity for fibronectin and, more specifically, for the extra domain A (EDA) splice variant of fibronectin (FnEDA) (Zilberberg et al., 2012; Tsuda, 2018; Zent and Guo, 2018). FnEDA appears particularly critical in TGF-β1 signaling as interference

with EDA domain function attenuates both LTBP-1 binding and TGF-β1 activation (Klingberg et al., 2018). Latency, however, is strictly dependent on LAP as a LAP mutant that cannot bind the LTBP effectively retains TGF-β1 in an inactive configuration (Robertson and Rifkin, 2016).

Mechanisms underlying release of latent TGF-β1 from the LAP cage include proteases, integrins, other proteins such as thrombospondin-1 and various physicochemical factors both alone and in combination (Robertson and Rifkin, 2016). Several proteases cleave the hinge region in LAP freeing the TGF-β dimer for receptor occupancy, although the physiologic relevance of protease-only liberation is complicated by the considerable redundancy in the various participating enzyme systems. Non-proteolytic as well as protease-requiring mechanisms involving αv subunit integrins (e.g., αvβ1, β3, β5, β6, β8), however, also activate TGF-β1 particularly in the context of a progressively fibrosing, increasingly stiff, renal microenvironment (Hysi and Yuen, 2020). Binding of αv integrins to the LAP N-terminal arginine-glycine-aspartic acid (RGD) motif generates Rho/RhoA-dependent tractional forces with ECM-anchored LTBPs; the resulting distortion of the LAP cage liberates and, thereby, activates the TGF-β1 dimer (Buscemi et al., 2011; Hinz, 2015; Sheppard, 2015; Robertson and Rifkin, 2016; Dong et al., 2017; Nickel et al., 2018; **Figure 9**). Cooperative involvement of both integrins and proteases is an additionally proposed mechanism. One model suggests that tensional strain generated by complex formation between αv integrins and the RGD motif on ECM-tethered LAP predisposes LAP to cleavage by cell surface-proximal proteases (Robertson and Rifkin, 2016). There appears to be, however, significant differences in the type of strain, the activation of latent TGF-β1 and the amplitude of expression of the engaged genes. Compared to steady-state shear strain, oscillatory forces generate significantly greater levels of active TGF-β1 resulting in the increased expression of the profibrotic triad PAI-1, collagen 1A1 and periostin (Kouzbari et al., 2019). Among the αv integrin subtypes, αvβ6 is a major TGF-β1 release trigger and a likely fibrotic effector since renal obstruction in β6-deficient mice is associated with a reduction in TGF-β1 activity and decreases in collagen I, collagen III and PAI-1 expression (Ma et al., 2003). Regardless of the actual pathway, computational modeling suggests that a protease (i.e., plasmin)-dependent bistability mechanism regulates TGF-β1 bioactivity (Li et al., 2017). It appears that TGF-β1 undergoes a bistable switch in response to increasing concentrations of plasmin from a high-level thrombospondin-1-mediated to lower-level predominantly plasmin-dependent mode of activation; both have implications to the development and progression of fibrotic disorders.

αvβ8 also releases TGF-β1 from the LAP:TGF-β1 complex bound to GARP (glycoprotein A repetitious predominant) on the surface of regulatory T cells (Lienart et al., 2018). This mechanism is unique to T-regs; membrane tethered GARP/LAP/TGF-β1 promotes presentation of the LAP RGD sequence to the αvβ8 integrin on adjacent cells. Tensional strain releases and activates the TGF-β1 dimer in much the same way as occurs via ECM-anchored LTBP-1. In addition, recent findings using cryo-electron microscopy to probe LAP:TGF-β complex interactions with the αvβ8 integrin suggests an alternative mode of TGF-β activation that does not necessitate release of dimeric TGF-β from the LAP (Campbell et al., 2020). While the existence of multiple mechanisms of TGF-β1 activation may be cell- and tissue-type dependent, complicating the adaption of a universal therapeutic strategy, pharmacologic inhibition of RGD-binding integrins attenuates renal fibrosis and improves organ function following injury (Basta et al., 2020) and antibody targeting of αvβ6 mitigates bleomycin-induced lung fibrosis (Horan et al., 2008). Integrin-focused therapies, however, are not without controversy. Phase 2 clinical trials of antibody BG00011 (previously known as STX-100), which targets αvβ6 was terminated by Biogen due to safety concerns (Freeberg et al., 2021)[4].

Elevated TGF-β1 levels, coupled with loss of tissue elasticity, further increases FnEDA expression while promoting LTBP-1/FnEDA co-localization, facilitating integrin/LAP engagement and the subsequent creation of tensional strain stimulating the generation of bioactive TGF-β1 (Wynn and Ramalingam, 2012; Chang et al., 2017; Freeberg et al., 2021). Progressive ECM stiffness and a TGF-β1-rich microenvironment promotes myofibroblast differentiation and survival while activating the Hippo pathway mechanosensitive transcriptional co-activators YAP and TAZ (Liu et al., 2015; Dupont, 2016; Jorgenson et al., 2017; Misra and Irvine, 2018; Santos and Lagares, 2018; Totaro et al., 2018). Convergence of YAP/TAZ and TGF-β1 pathways, in the context of recurrent or persistent tissue injury, induces expression of several major profibrotic genes including CCN2, fibronectin and PAI-1 contributing, thereby, to the eventual development of fibrotic disease (Kim et al., 2019; **Figure 7**). These findings suggest a complex mechanism for TGF-β1 involvement over the course of renal fibrosis in which induction of FnEDA is a critical element in a TGF-β/FnEDA/αv integrin positive feed-forward loop. It should be mentioned that there have been some attempts to assess these requirements for TGF-β1 mobilization in a translational context. Systemic injection of a bi-specific antibody with FnEDA binding and TGF-β1 neutralizing domains confirmed both construct accumulation and reduced fibrosis in the injured kidney providing supporting evidence for such a model (McGaraughty et al., 2017). How such a strategy may be adapted for patient treatment, however, remains to be determined.

INVOLVEMENT OF P53 IN TGF-β1-INDUCED RENAL FIBROSIS

Since TGF-β1 signaling is a major driver of UUO-induced renal fibrosis (Richards et al., 2018), clarification of the involved intermediates downstream of the activated TGF-β receptors may have therapeutic implications for patients with UPJ disease. In the canonical pathway, occupancy of a type II receptor (TGF-βRII) by the TGF-β1 dimer drives complex formation with, and subsequent phosphorylation of, the ALK5 type I receptor (TGF-βRI) that, in turn, phosphorylates receptor (R) SMADs (predominately SMAD2/3 in fibrotic disease) at the distal

[4]www.fiercebiotech.com

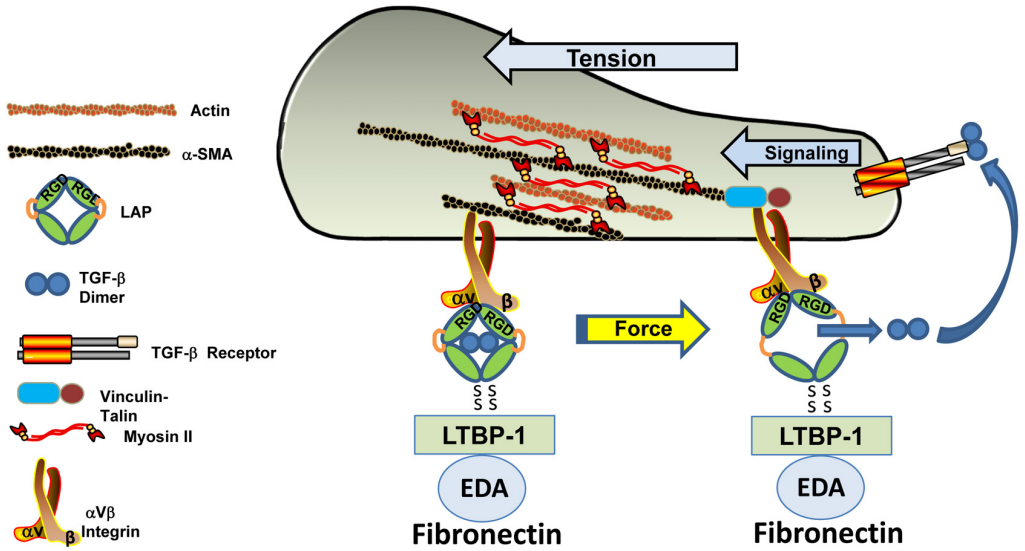

FIGURE 9 | Tension-dependent release of active TGF-β from the LAP cage. The ternary large latent (LTBP/TGF-β/LAP) complex forms a bridge between an αV integrin bound to the RGD site on the latency-associated peptide and LTBP-1 tethered to the fibronectin-rich ECM. Actinomyosin-based contractility generates mechanical tension within this ternary complex inducing a conformational change in the LAP that releases the now-active TGF-β dimer that, in turn, occupies the TGF-βR to initiate downstream signaling.

C-terminal SxS motif ($Ser^{423/425}$ and $Ser^{465,467}$ for SMAD3 and SMAD2, respectively) (Matsuzaki, 2013). While early models suggested that SMAD2 interacts with the SMAD binding domain (SBD) of the SMAD anchor for receptor activation (SARA) followed by SARA:SMAD2 delivery to the TGF-βRI via the C-terminal domain of SARA to facilitate R-SMAD phosphorylation, the actual involvement of SARA in TGF-β signaling is controversial (Rozés-Salvador et al., 2018, 2020). Regardless of the precise mechanism, pR-SMADs complex with the shuttle SMAD4 and translocate to the nucleus to impact transcription of a rather large slate of TGF-β1 responsive genes (Massagué, 2000; Massague, 2012). Identification of differentially expressed genes (DEG), using an unbiased microarray analysis, at two time points post-UUO disclosed 606 upregulated (including 430 annotated) and 485 downregulated (including 251 annotated) genes (Higgins et al., 2003). More than 70 such DEG partitioned to the ECM/cytoskeletal cluster indicative of the breath of targets that may well impact the fibrogenic phenotype. KEGG analysis of the transcriptome of diabetic and non-diabetic mice indicated, in fact, that significant differentially-expressed genes closely associate with the p53 signaling network, as well as the MAPK and TGF-β pathways (Wang et al., 2016).

The growing number of non-canonical (i.e., non-SMAD) elements and their associated pathways in the TGF-β1 network, however, suggests a more significant level of mechanistic diversity in the control of gene expression and the potential existence of an expanding repertoire of regulated sequences (Zhang, 2017). Appropriately recognized as the master regulator of fibrosis (Meng et al., 2016; Lodyga and Hinz, 2020), the TGF-β1 signaling apparatus, including the downstream SMAD effectors, cross-talk with an extensive and highly interactive system that includes the Raf/MEK/ERK, JAK/STAT, Wnt, Notch, Hippo/YAP/TAZ, PI3K/AKT, GSK3/Twist/FOXO, and PKC/Smurf1/RhoA/Rock cascades (Böttinger and Bitzer, 2002; Piersma et al., 2015; Zhang, 2017; Ahmadi et al., 2019; Patel et al., 2019; Finnson et al., 2020; Labibi et al., 2020). The increasing complexity of participating co-factors in the regulation of TGF-β1-responsive genes likely reflects the comparatively low affinity of DNA-SMAD interactions.

One such important co-activator is the tumor-suppressor p53. The involvement of p53 in renal disease was initially defined in a rat model of ischemia-reperfusion injury (Kelly et al., 2003). p53 induction and increased p53 serine 15 phosphorylation is also evident in the kidney following nephrotoxin (e.g., cisplatin, aristolochic acid) administration or UUO, particularly in the dysmorphic epithelium (Zhou et al., 2010; Wei et al., 2007; Samarakoon et al., 2013a,b), and renal allograft rejection (Higgins et al., 2019). Recent studies, furthermore, link tubular epithelial dysfunction in response to both acute (e.g., ischemia-reperfusion, nephrotoxins) and more protracted (UUO) injury to the progression of renal fibrosis via the p53 and JNK pathways with the retention of TGF-β signaling (Yang et al., 2010). p53 is activated in the injured renal epithelium initiating cell cycle arrest at the G_1 and G_2/M checkpoints depending on the participating effectors (e.g., ATM, ATR, CK1, CK2, p21, TGF-β1) and extent of tissue hypoxia (Thomasova and Anders, 2015; Tang et al., 2018, 2019, 2020; Liu et al., 2019). TGF-β1 signaling in the damaged kidney increases p53 levels and phosphorylation, particularly at $p53^{S9/15}$, promoting p53 stabilization and triggering p53-SMAD2/3 interactions resulting in transcription of the growth inhibitor p21 and subsequent p21-dependent G_1 arrest (Higgins et al., 2019). While p21 is a major p53 responsive gene, p53 upregulation in hypoxic tubular cells also suppresses CDK1, cyclin B_1,

and cyclin D_1 expression, potentially increasing residence time in G_2/M. Such interrelationships are complicated, however. Oscillations in p53/p21 transcription impact accumulation of p21 protein and, thereby, cellular arrest and death programs. Single-cell analysis indicates that p21 transcription reflects p53 dynamics although p21 protein levels increase only gradually (Hafner et al., 2020).

Molecular approaches confirmed the involvement of p53 in several models of injury-induced kidney disease. While $p53^{-/-}$ mice largely retain renal architecture and function following cisplatin or aristolochic acid treatment, wild-type animals develop severe renal damage exhibiting all the hallmarks of a maladaptive repair process (Wei et al., 2007; Zhou et al., 2010). siRNA-directed silencing of p53, moreover, mitigates the severity of cisplatin- and ischemic-induced kidney damage (Molitoris et al., 2009). Pharmacologic inhibition of p53 activation with pifithrin-α, delivery of p53 siRNA or genetic deletion of p53 in the proximal tubular epithelium attenuates both prolonged G_2/M residence and the fibrotic response to cisplatin, UUO or ischemic injury (Wei et al., 2007; Yang et al., 2010; Ying et al., 2014; Tang et al., 2018; Higgins et al., 2019; Liu et al., 2019; Molitoris, 2019). Similarly, cisplatin- or bilateral ischemia-induced AKI in streptozotocin-treated mice or genetically susceptible (Akita) diabetic animals is significantly diminished by pifithrin-α, p53 siRNA or proximal tubule-targeted p53 ablation (Peng et al., 2015). There appears to be a timing dependency, however, for maximal efficiency with short term p53 knockdown (i.e., day 14) effective at reducing both the senescence cellular load and the ischemic phenotype; a longer course of p53 siRNA administration did not provide any additional therapeutic benefit (Baisantry et al., 2019).

Assessment of the toxicologic and pharmacokinetic properties of 2′-O-methyl sugar-modified p53 siRNAs indicated preferential localization to, and rapid uptake (peak levels 5–30 min post-inoculation) by, the kidney as well as short residence duration in the proximal tubular epithelium (Thompson et al., 2012). Most encouraging from a potential clinical utility perspective, p53 knockdown was achieved within 3–6 h after intravenous administration of a single bolus of 12 mg/kg of these modified siRNAs to animals with ischemic- hypoperfusion- and cisplatin-induced renal injury (Molitoris et al., 2009) which is well below the dose of 200 mg/kg that corresponded to the no observable adverse effect level (NOAEL) in the rat (Thompson et al., 2012). Intravenously-delivered p53 siRNA, moreover, also mitigates the structural and functional damage to transplanted kidneys upon ischemia/reperfusion injury in two syngeneic rat models (Imamura et al., 2010), consistent with 2′-O-methyl sugar-modified siRNA knockdown of p53 transcripts (Molitoris et al., 2009), suggesting the potential clinical utility of targeting p53 in patients with failing renal allografts.

GENOMIC TARGETS OF TGF-β1/P53 SIGNALING IN OBSTRUCTIVE RENAL DISEASE

TGF-β1 stimulates p53 transcriptional activity largely by serine phosphorylation in the N-terminus transactivation domain

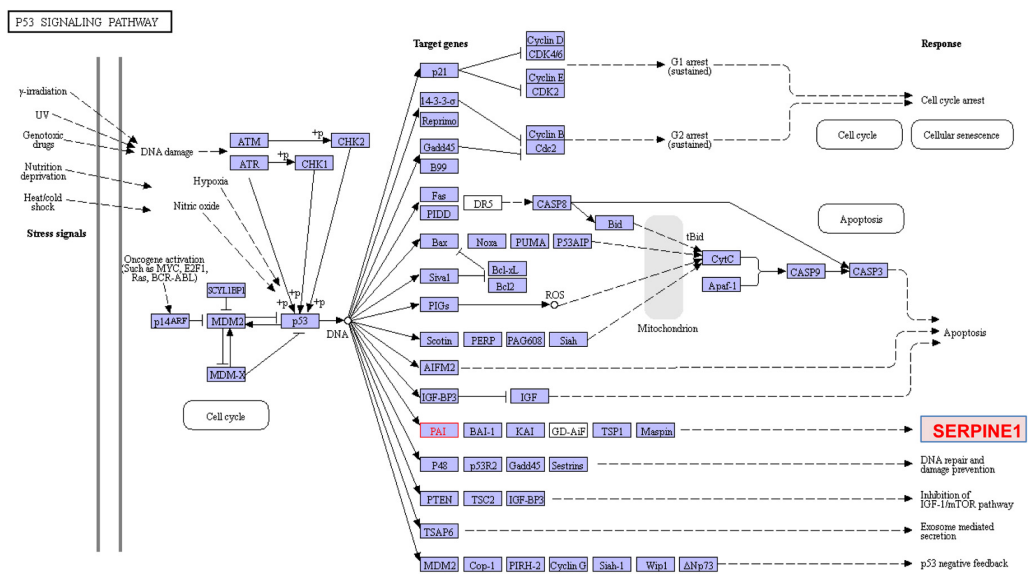

FIGURE 10 | KEGG p53 signaling. p53 is mobilized in response to various signals including DNA damage, oxidative stress and oncogene activation usually by phosphorylation by elements of the ATM/ATR pathway. p-p53 initiates transcription of target genes that result in cell cycle arrest (at both G_1 and G_2/M), acquisition of a senescent phenotype or apoptosis. SERPINE1 (PAI-1) and p21 are prominent members of both the cellular senescence (**Figure 6**) and p53 signaling pathways. TGF-β1 also activates p53 stimulating $p53^{Ser9,15}$ phosphorylation and acetylation, promoting interactions with activated SMADs and subsequent binding of p53/SMAD3 complexes to the PAI-1 promoter in human renal tubular epithelial cells (Overstreet et al., 2014). Consistent with a potential *in vivo* role for p53 and SMADs in TGF-β1-driven renal fibrosis, co-induction of pSMAD2/3, p53, $p53^{Ser15}$ and the target growth arrest/senescence genes SERPINE1 and p21 are evident in the tubular epithelium of the obstructed kidney (e.g., Overstreet et al., 2014; Patel et al., 2019).

and serine/lysine acetylation/methylation in the C-terminal tetramerization and regulatory domains (Maclaine and Hupp, 2009) facilitating interactions between p53 and SMAD2/3 (Piccolo, 2008; Overstreet et al., 2014). Phosphorylated p53 and SMAD2/3 form transcriptionally active multi-protein complexes on the promoter regions of a subset of TGF-β1 target genes (Cordenonsi et al., 2003, 2007; Piccolo, 2008; Overstreet et al., 2014, 2015). Such reprogramming may not be reflected, however, in detectable changes in p53 protein abundance. In this regard, short-term treatment with Nutlin-3, which interferes with the p53-binding hydrophobic pocket in MDM2 functioning thereby as a p53 competitive inhibitor, results in p53-dependent transcription of a large complement of direct target genes without any significant increase in p53 levels, at least in the brief window of Nutlin-3 exposure used (Allen et al., 2014). While these findings suggest that the expression of novel, albeit likely low abundance, genes may be independent of perceived changes in p53 cellular abundance, p53 pulsing (i.e., stimulus-dependent changes in the amplitude, duration and period of p53 levels) impacts TGF-β1-response gene dynamics differently than transcription patterns evident under gradually increasing p53 levels (Porter et al., 2016). Indeed, single cell transcriptome profiling revealed that mitigating p53 pulsing by retaining p53 at high levels by treatment with Nutlin-3 results in the creation of a single, large network of coordinated genes rather than the two discrete subnetworks evident under conditions that allow p53 oscillation (Porter et al., 2016).

The KEGG-defined p53 signaling pathway and the cooperative p53/TGF-β1 genomic cluster, moreover, includes genes involved in cell growth control and ECM remodeling (Dupont et al., 2004; Elston and Inman, 2012; Slattery et al., 2019; **Figure 10**). While the molecular basis for this co-dependency requires clarification, many TGF-β1-responsive sequences possess p53-recognition motifs as well as SMAD-binding elements (Wang et al., 2001; Takebayashi-Suzuki et al., 2003; Allen et al., 2005; Qi et al., 2006). Indeed, p53 participates in the transcription of several renal disease-causative genes including CNN2, collagen I and SERPINE1 (PAI-1) underscoring the complexities of non-canonical pathways in TGF-β1-induced fibrosis (Kodama et al., 2011; Elston and Inman, 2012; Overstreet et al., 2014, 2015). PAI-1 is a member of the "high-confidence" complement of 151 p53 target genes (Chang et al., 2014), a major p53 direct effector hub gene (**Figure 11**) and ranks 131 among 343 of the most prominently identified p53-activated genes (Fischer, 2017). In view of the extensive repertoire of direct (i.e., 943 genes; the p53 cistome) and indirect p53-responsive genes, stringent criteria are required for identification of actual p53 genomic targets (e.g., Ali et al., 2014; Nguyen et al., 2018) (summarized for PAI-1 in **Table 1**). The two 10-bp p53 response elements (p53-REs) in the PAI-1 promoter, which provide a platform for p53 docking in a typical dimer-of-dimers configuration, moreover, are not separated by a nucleotide spacer (Kunz et al., 1995; Riley et al., 2008; Fischer, 2017). Variable p53-RE spacing affects transactivation of target genes. Indeed, *in vivo* analysis disclosed that decamer pairs with no spacers exhibited a strong preference of p53 binding (Nguyen et al., 2018). This has pathophysiologic implications since p53-RE penalty scoring indicates that a high negative impact was attributed to spacers longer than two nucleotides and that p53-REs with long spacer length are not likely to be bound by p53 *in vivo* (Tebaldi et al., 2015).

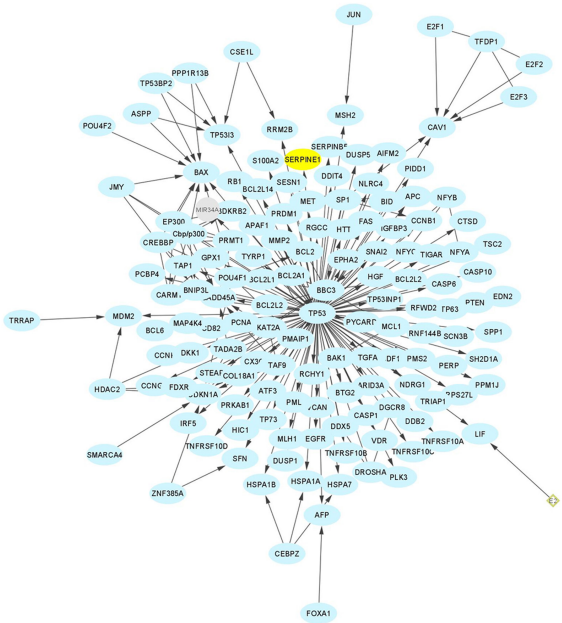

FIGURE 11 | Direct p53 effectors. There are a significant number of p53 target genes that control a broad range of cellular processes, including cell cycle arrest, cell senescence, DNA repair, metabolic adaptation and cell death. Of these, approximately 135 are listed in the Pathway Interaction Database as direct p53 effectors. SERPINE1 (PAI-1) (highlighted in yellow) is among the most prominent since, as a major p53 target gene, it regulates cell proliferation and migration, ECM remodeling, wound healing, invasion and metastasis, senescence and survival (e.g., Higgins et al., 2018, 2019; Tang et al., 2018).

GenBank annotation and computational analysis identified at least one p53 binding motif within 2,000 bp upstream of the transcription start site in approximately 1,100 human genes; transcript mapping added significantly to the number of candidate p53-regulated promoter sequences (Wang et al., 2001). Alignment of the complement of differentially expressed TGF-β1-induced genes (DEGs) with the p53 Target Database further revealed that the majority of those responsive to TGF-β also possesses p53 binding sites and recent studies clarified the transcriptional basis for such TGF-β1/p53 cross-talk (Elston and Inman, 2012; Overstreet et al., 2014; Kawarada et al., 2016; Higgins et al., 2018). Among the impacted set of DEGs, which includes the fibrosis-causative PAI-1 and CNN2 genes, pharmacologic inhibition of p53 function may well have clinical implications in disease management. Indeed, p53 is required for TGF-β1-induced expression of fibronectin, CNN2 and PAI-1, the latter a major profibrotic hub gene and critical factor in the initiation as well as progression of TGF-β1-dependent fibrotic disease (Higgins et al., 2018; Xu et al., 2020).

TGF-β1 impacts p53 function, and thereby the fibrotic program, via an additional (albeit complex) mechanism that involves the serum response factor (SRF) and its co-activators

TABLE 1 | Summary of p53 involvement in expression of the target PAI-1 gene.

1. TGF-β1 stimulates $p53^{ser9/15}$ phosphorylation promoting binding of p-p53/SMAD3 complexes to the PAI-1 promoter in HK-2 human renal tubular epithelial cells (Overstreet et al., 2014).
2. Two canonical p53-binding motifs in the PAI-1 promoter (Kunz et al., 1995; Parra et al., 2001; Riley et al., 2008) meet the >90 p53MH score threshold required for the identification of potential p53-responsive genes (Hoh et al., 2002). Both p53-binding sites (nt −224 to −204) are proximal to the transcription start site.
3. PAI-1 is a member of the "high confidence" cohort of 151 p53 target genes (Chang et al., 2014).
4. PAI-1 ranks 131 among 343 of the most prominent p53-activated genes (Fischer, 2017).
5. PAI-1 is included in the p53 Target Database (Fischer, 2017).
6. PAI-1 is a component in the p53 circuit board (Sullivan et al., 2012).
7. p53 silencing, genetic ablation/subsequent rescue, and pharmacological inhibition confirmed that p53 is required for PAI-1 expression in TGF-β1-stimulated cells (Overstreet et al., 2014).
8. TGF-β1-initiated PAI-1 expression is attenuated in p53 knockdown cells (Cordenonsi et al., 2003).
9. $p53^{-/-}$ fibroblasts are not inducible for increased PAI-1 expression in response to TGF-β1 (Samarakoon et al., 2013a,b).
10. Pretreatment of Mv-1Lu mink lung cells (stably expressing a PAI-1 promoter-luciferase reporter construct) with the p53 inhibitor pifithrin-α effectively suppressed TGF-β1-dependent PAI-1 transcription (Samarakoon et al., 2013a,b).
11. Radiation-induced PAI-1 expression requires p53/SMAD3 cooperativity (Milliat et al., 2008).
12. Mutation of the p53-binding sites in the PAI-1 promoter inhibits γ-radiation-induced PAI-1 transcription and attenuates the dual γ-radiation + TGF-β1 synergy (Hageman et al., 2005).
13. γ-radiation did not induce PAI-1 expression in p53-null cells; p53 expression rescue largely restored the PAI-1 response to γ-radiation + TGF-β1 co-stimulation (Hageman et al., 2005).
14. p53 knockdown mitigates radiation-dependent PAI-1 expression (Szołtysek et al., 2018).
15. Overexpression of the Δ133p53α isoform of p53, which lacks the two transactivation domains, inhibits expression of the p53-inducible genes p21, IGFBP7, and PAI-1 (Fujita, 2019).

including the myocardin-related transcription factors (MRTF-A, MRTF-B) and the ternary complex factors (TCF) (Small, 2012; Gau and Roy, 2018; Onuh and Qiu, 2021). This pathway is likely to be particularly important in the TGF-β1-directed differentiation of interstitially-trafficked vascular pericytes into the pro-fibrotic, highly contractile and α-smooth muscle actin-rich myofibroblast lineage. Indeed, complex formation between SRF and MRTF vs. TCF dictates the particular subset of SRF target genes induced (Onuh and Qiu, 2021). Emergence of the myofibroblastic phenotype appears coupled to joint regulation by the TGF-β1 and MRTF-A/SRF signaling networks which cooperate to promote expression of a distinct set of contractile and profibrotic genes (Olson and Nordheim, 2010; Small, 2012; Velasquez et al., 2013; Gau and Roy, 2018; Werner et al., 2019). Altered actin cytoskeletal dynamics, as a function of growth factor stimulation or a changing mechanical environment, releases MRTF-A from its G-actin cytoplasmic tethers facilitating MRTF-A nuclear translocation and interaction with SRF to trigger transcription of SRF target genes which possess the CArG consensus element (e.g., collagen I, α-smooth muscle actin) many of which are also TGF-β1/p53 responsive genes as well. It was noted in recent reports (Werner et al., 2019; Onuh and Qiu, 2021), in fact, that there appears to be appreciable overlap in the TGF-β1/p53 and Rho/MRTF/SRF genomic signatures (e.g., Esnault et al., 2014) consistent with the role of MRTF-A in myofibroblastic differentiation (Crider et al., 2011). Interestingly, SRF/TCF complexes appear to drive the increased transcription of MDM4 (Pellegrino et al., 2021), a key member of the MDM2/MDMX/MDM4 repertoire of p53 activity regulators. Collectively, these findings indicate that there is significant context-specific cross-talk between the TGF-β1/SMAD3/p53 and Rho/MRTF/TCF/SRF signaling pathways that impact the expression of gene clusters that regulate the adaptive and maladaptive tissue repair outcomes. The differential partnering of SRF with MRTF vs. TCF may well determine, at least in part, the particular subset of SRF-dependent genes engaged and the nature of the wound healing response.

CONCLUSION

PAI-1 negatively regulates the plasmin-dependent pericellular proteolytic cascade effectively limiting ECM degradation and fibrinolytic activity contributing, thereby, to the initiation and/or progression of interstitial fibrosis and progressive renal disease (Ghosh and Vaughan, 2012; Flevaris and Vaughan, 2017; **Figure 3**). PAI-1 deficient mice are, in fact, protected from excessive ECM accumulation in several organ systems including the kidney and PAI-1 decoys attenuate both UUO-initiated and established interstitial fibrosis (Gonzalez et al., 2009). Apart from an impact on ECM turnover, the p53→PAI-1 axis likely drives renal cell stalling in both G_1 and G_2/M (Kelly et al., 2003, 2013; Kortlever et al., 2006; Yang et al., 2010; Overstreet et al., 2014; Moonen et al., 2018; Wu and Prives, 2018; Liu et al., 2019; Oliva-VIlarnau et al., 2020) suggesting an additional mechanism for the repair deficiency. PAI-1 is a prominent member of both the growth arrest/fibrosis genomic cluster in the diabetic rat kidney (Kelly et al., 2013) and the 11-gene urine mRNA signature predictive of human renal allograft fibrosis (Anglicheau et al., 2012). While initially a protective response, when trauma is chronic or sustained, the associated G_2/M arrest contributes to development of kidney disease due to TGF-β1-directed expression of profibrotic factors (Canaud et al., 2019; Koyano et al., 2019). During the initial stages of UUO-induced renal damage or ischemia/reperfusion injury, tubular cells arrest in G_2 due, albeit perhaps partially, by a p21-dependent pathway (Yang et al., 2010; Lovisa et al., 2015; Canaud et al., 2019; Koyano et al., 2019).

Similar to p53 deficiency, PAI-1 knockdown also results in escape from TGF-β1-induced cytostasis in various cell types including those derived from the renal proximal tubular epithelium (Kortlever et al., 2008; Overstreet et al., 2015). MEFs from $PAI\text{-}1^{-/-}$ mice proliferate well beyond the senescence checkpoint while ectopic expression of PAI-1 in p53-null fibroblasts rescues a phenotype displaying the hallmarks of replicative senescence-induced growth inhibition

(Kortlever et al., 2006). PAI-1 expression in response to TGF-β1 is required for a senescence-associated proliferative arrest, moreover, and PAI-1-deficient mouse embryonic fibroblasts (MEFs) or PAI-1 knockdown in wild-type MEFs and human keratinocytes confers resistance to TGF-β1-induced growth arrest (Kortlever et al., 2006, 2008). Indeed, human keratinocytes engineered to overexpress PAI-1 enter into growth arrest while overexpression of PAI-1 alone is sufficient to halt G_2/M transit in proximal tubule cells (Kortlever et al., 2008; Gifford et al., 2021). Cellular PAI-1 "status" has a profound effect on genomic reprogramming as transcript profiling indicates that 1,283 genes are upregulated in PAI-1 knockdown cells while 1,891 are reduced suggesting that PAI-1 negatively and positively impacts gene expression, either directly or indirectly. Among the genes repressed by PAI-1 deficiency are members of the SASP complement, a finding consistent with the involvement of YAP-induced PAI-1 as a major contributor to an oncogene-induced senescent phenotype (Marquard et al., 2020). Since renal repair requires a regenerative phase to replace injured or dying tubular epithelial cells, incomplete injury resolution compromises nephron function and leads to persistent inflammation and increased matrix deposition. The prolonged G_1 and G_2/M arrest in severe AKI, although necessary to insure DNA fidelity and maintain genome integrity, may adversely impact regenerative growth if cell cycle re-entry is excessively delayed while promoting maladaptive fibrotic repair in a TGF-β1-rich environment (Yang et al., 2010; Moonen et al., 2018).

Collectively, it appears that p53 plays an important role in TGF-β-induced proliferative arrest via induction of both p21 and PAI-1 transcription and that loss of p53 or its target gene PAI-1 confers resistance to TGF-β1-mediated growth inhibition. The mechanism remains to be clarified but recombinant PAI-1 induces collagen I and fibronectin expression in renal mesangial cells via a TGF-β1-dependent mechanism and PAI-1 stimulates TGF-β1 promoter activity (Seo et al., 2009). PAI-1 may initiate, perhaps maintain, a pro-fibrogenic "loop" in the context of renal disease (Nicholas et al., 2005; Seo et al., 2009). It is tempting to speculate, therefore, that targeted down-modulation of PAI-1 expression or function may provide multi-level therapeutic opportunities to inhibit the onset and progression of tissue fibrosis.

AUTHOR CONTRIBUTIONS

CH, JT, SH, CG, and AC performed the experiments. RS and PH designed the experiments and analyzed the data. BM, DJ, WZ, DC, RS, and PH provided pathology consultation and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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