

**Mechanisms and Regulation of Transforming Growth Factor Superfamily
Mediated Gene Expression**

by

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DEDICATION

To my loved wife, Yi Feng, and my parents for
their selfless love and supports

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Abstract

Mechanisms and Regulation of Transforming Growth Factor Superfamily Mediated Gene Expression

The TGF- β superfamily, including TGF- β s and BMPs, is critical for normal embryonic development, as well as disease progression, and is tightly regulated both within and out of cells. *In vitro*, TGF- β signaling mediated epithelial-mesenchymal transition (EMT) by activating mesenchymal genes and suppressing epithelial markers. We discovered that Wnt11 was directly regulated by the mediators of TGF- β signaling, Smad proteins. The induction of Wnt11 expression was critical for TGF- β associated activation of mesenchymal marker genes. Instead of modulating Smad proteins or activating canonical/ β -Catenin signaling, Wnt11 controlled mesenchymal gene activation through JNK signaling. Our findings, for the first time, demonstrated the cooperativity among the TGF- β , Wnt11 and JNK signaling pathways in the context of EMT. Both TGF- β and BMP signaling are involved in renal fibrosis, but with opposite functions. TGF- β is a well known pro-fibrogenic factor, while BMP counteracts TGF- β to protect kidney from injuries. In our study, transgenic expression of kielin/chordin-like protein (KCP), an inhibitor of TGF- β and enhancer of BMP7, in renal epithelia attenuated the upregulation of mesenchymal genes in the injured kidney of unilateral ureteral obstruction (UUO) mouse model. These data demonstrated the importance of the balance of TGF- β and BMP signaling in the progression of renal fibrosis and provided a new potential therapeutic target. During kidney development, both BMP7 and Tle4, a

common corepressor, are present in metanepheric mesenchymal cells. However, their relationship is unknown. Here, we found that overexpression of Tle4 not only activated a BMP reporter, but also enhanced and sustained the upregulation of endogenous Id1 gene induced by BMP7. The effect of Tle4 on BMP signaling was through mediating Smad7 protein, for Tle4 repressed Smad7 expression and overexpression of Smad7 totally abolished the activation of the BMP reporter by Tle4. Our study provides a new potential mechanism for the regulation of BMP signaling in the kidney development.

Chapter I

General Introduction

The transforming growth factor β (TGF- β) signaling pathway

TGF- β Signal transduction

Since the purification of its first ligand, TGF- β 1, from human platelets in 1983 (Assoian et al., 1983), much of research has focused on this superfamily and, more than 30 ligands have been discovered in the human genome (Feng and Derynck, 2005; Massague, 2008). According to their sequence similarity and biological effects, the TGF- β superfamily can be divided into two distinct groups, the TGF- β /activin/nodal subfamily and bone morphogenetic proteins (BMPs)/anti-muellerian hormone (AMH)/growth and differentiation factors (GDFs) subfamily. The TGF- β signaling regulates a diverse set of cell processes. For example, TGF- β s caused cell cycle arrest in epithelial and hematopoietic cells and controlled mesenchymal cell proliferation and differentiation, while BMPs were important for the differentiation of osteoblasts and the survival of renal mesenchymal cells (Massague, 1998; Patel and Dressler, 2005; Reddi, 1998). In fact, the TGF- β superfamily plays a key role throughout the whole embryonic development process and is involved in the formation of nearly all organs.

Although there are a number of ligands and several receptors, the general signaling transduction for TGF- β superfamily is relatively simple. In mammals, the binding of TGF- β ligand to its receptor, TGF- β receptor type II, leads to the recruitment and phosphorylation of TGF- β receptor type I (Derynck and Zhang, 2003). The activated TGF β RI is a serine/threonine kinase that transduces the signal through phosphorylating

receptor-activated Smad proteins (R-Smads), which are the main mediators for TGF- β signaling. Commonly, for TGF- β s, the R-Smads are Smad2 and 3, while for BMPs, they are Smad1, 5 and 8. The phosphorylated R-Smads usually form a heteromeric complex with a common partner, Smad4 (Co-Smads), and translocate into the nucleus. Normally, the Smad complex requires other transcriptional factors to activate or repress target gene expression (Itoh et al., 2000; Labbe et al., 2000; Sano et al., 1999). Besides R-Smads and Co-Smads, TGF- β signaling can induce the expression of a third group of Smad proteins, Smad 6 and 7 (Inhibitory Smads, I-Smads), which inhibits TGF- β signaling through competitive receptor binding and blocking the interaction between R-Smads and Co-Smads (Hayashi et al., 1997; Imamura et al., 1997) (Figure 1-1).

Since the TGF- β superfamily is widely involved in embryogenesis and subsequent organogenesis, it interacts with other signaling pathways, such as Wnt and Notch signaling. Also, because the TGF- β superfamily plays a critical role in a variety of biological process, it is highly regulated at different levels, from ligand releasing to mediator activation, and finally to transcriptional complex formation and target gene expression. In the following, we will discuss how TGF- β signaling is regulated and functions synergistically with other signaling pathways in a defined biological context.

Regulation of receptor activation

Despite the diversity of ligands in the TGF- β superfamily, they all share similar sequence and structure features. The active form of TGF- β cytokines is a homodimer of two 12.5 kd polypeptides stabilized by hydrophobic interactions and further joined by a

disulfide bond (Feng and Derynck, 2005; Shi and Massague, 2003). As for TGF- β isoforms (TGF- β 1, 2, 3), its mature form is cleaved from homodimeric proproteins (pro-TGF- β) and interact with its N-terminal peptides, called the latency-associated proteins (LAPs), to form a small latent TGF- β complex (SL-TGF- β). When secreted from cells, the SL-TGF- β complex further interacts with the latent-TGF- β -binding protein (LTBP) through disulfide linkages to form the TGF- β large latent complex (LLC), which may be covalently anchored to the extracellular matrix (ECM) for storage (Annes et al., 2003; Hyttiainen et al., 2004). Whether the ligands from other TGF- β subfamily undergo the same secreting process is not clear.

Based on their structural and functional properties, the TGF- β receptor family is catalogued into two groups: type I receptors and type II receptors. Up to now, 7 type I and 5 type II receptors are dedicated to TGF- β signaling in the human genome (Manning et al., 2002) (Table 1-1). Both types of the receptors are serine/threonine kinases, sharing a similar structure as an N-terminal extra-cellular ligand binding domain, a transmembrane region and a C-terminal serine/threonine kinase domain (Shi and Massague, 2003). Compared to the type II receptor, the type I receptors have an extra domain between the transmembrane region and the kinase domain, termed GS domain (sequence as SGSGSG), which can be phosphorylated by the type II receptors and critical for the signaling activation (Souchelnytskyi et al., 1996; Wrana et al., 1994). As for the interaction between the ligands and receptors, there are two distinct modes represented separately by TGF- β /Activin subfamily and BMP subfamily. TGF- β and Activin showed a high affinity for the type II receptors and the type I receptor was recruited only after the ligand-type II receptor complex was formed (Massague, 1998). In contrast, from the

analysis of binding affinity, BMPs interacted with the type I receptors first, then the type II receptors (Liu et al., 1995). No matter the sequence, the activation of the type I receptors and its interaction with Smad proteins required the phosphorylation of its GS domain by type II receptors (Feng and Derynck, 2005; Massague, 1998; Shi and Massague, 2003).

The regulation of TGF- β receptor activation comprises two aspects: (1) controlling the access of TGF- β ligands to their receptors; (2) controlling the activation of type I receptors. Two classes of molecules with opposing function regulate the access of TGF- β ligands to their receptors (Massague and Chen, 2000; Shi and Massague, 2003). One class consists of a variety of soluble proteins that sequester TGF- β ligands and prevent their binding to the receptors. According to their targets, they can be further divided into three groups: (1) LAP, the small proteoglycan Decorin, the circulating protein α 2-macroglobulin for TGF- β s; (2) Noggin, Chordin/SOG and DAN/Cerberus for BMPs; (3) follistatin for Activins and BMPs. The other class, membrane-anchored proteins, including betaglycan and endoglin may function as accessory receptors to enhance respective TGF- β signaling.

As we discussed earlier, when the three TGF- β subfamily isoforms are secreted from cells, they are trapped by LAP and anchored to ECM by LTBP. The formation of this LLC prevents the mature TGF- β factors from binding to type II TGF- β receptor (Annes et al., 2003). Several factors or physiological condition changes can destroy this LLC and help releasing the active TGF- β ligands. First, a number of proteases, including plasmin and matrix metalloproteinase 2 and 9 (MMP2 and 9) can activate LLC through either proteolytic cleavage of LTBP (Taipale et al., 1994) or LAP (Lyons et al., 1988).

Second, thrombospondin-1 (TSP1) can physically interact with LAP and induce a conformational change to release mature TGF- β ligands (Murphy-Ullrich and Poczatek, 2000). Importantly, TSP1 null mice shared a similar phenotype with the TGF- β 1 null mice (Crawford et al., 1998) and also TSP1 blocking peptides reduced the TGF- β activation in a rat fibrotic renal disease model (Daniel et al., 2004), suggesting that TSP1 is responsible for a significant proportion of TGF- β activation *in vivo*. Third, it has been reported that TGF- β 1 LAP was a ligand for the integrin $\alpha_v\beta_6$, and $\alpha_v\beta_6$ -expressing cells induced spatially restricted activation of TGF- β 1 (Munger et al., 1999). Furthermore, other integrins, such as $\alpha_v\beta_8$ and $\alpha_v\beta_3$ can function as a docking point for MMPs to activate TGF- β signaling (Mu et al., 2002; Rolli et al., 2003). Finally, some physiological changes in the microenvironment can destroy LLC, such as increasing reactive oxygen species (ROS) (Barcellos-Hoff et al., 1994) or decreasing pH (Lyons et al., 1988).

Although the length and structure vary a lot among BMP antagonists, such as Noggin, Chordin/Sog and DAN family, they all share a common cysteine-rich region. For example, Noggin contains a carboxy-terminal cysteine-rich (CR) domain, while Chordin contains four cysteine-rich repeats (Massague and Chen, 2000). The CR domains of the antagonists form homodimers to match the structure of BMP ligand homodimers. The crystal structure of the Noggin-BMP7 complex directly showed that Noggin inhibited BMP7 by blocking the surfaces that were required to interact with the type I and type II BMP receptors (Groppe et al., 2002). Those antagonists are expressed during embryogenesis, and critical for the dorsal-ventral patterning and left-right asymmetry. Because those antagonists are important for the embryonic development, their expression was highly regulated. For example, in chicken, expression of Caronte, which belongs to

the DAN family, was induced by the sonic hedgehog signaling (Rodriguez Esteban et al., 1999) and its diffusion was restricted by Lefty1 (Yokouchi et al., 1999). Once the antagonist-BMP complex was formed, it could be further activated through proteolytic cleavage by secreted metalloproteases, like Tolloid in *Drosophila* and zebrafish (Blader et al., 1997; Marques et al., 1997), Xolloid in *Xenopus* (Piccolo et al., 1997), and BMP1 in human (Takahara et al., 1994). The effects of these metalloproteases on the BMP inactive complexes may be antagonist-dependent, since Xolloid *in vivo* specifically block the anti-BMP action of Chordin, but not Noggin or Follistatin (Blader et al., 1997). Interestingly, although most of the BMP antagonist shared the CR domain, not all proteins containing CR domain counteract BMP. Instead of blocking BMP signaling, the CR domain protein KCP and CV2 enhanced BMP-receptor interactions (Ikeya et al., 2006; Lin et al., 2005). Thus, the interaction between the CR domain proteins and other proteins, such as ECM, may be also important for their function.

Follistatin is a soluble secreted glycoprotein that could repress Activin signaling through direct ligand binding (de Winter et al., 1996). Besides Activin, it could also block BMP signaling through the similar mechanism (Iemura et al., 1998).

Another class of proteins that could facilitate the delivery of TGF- β ligands to the receptors are the membrane-anchored proteins, for which betaglycan is a good example. Betaglycan comprised a large extracellular domain for TGF- β ligand binding, a single-pass transmembrane region, and a short intracellular domain associated with receptor trafficking (Bilandzic and Stenvers, 2011). It binds all three TGF- β isoforms with a preference for TGF- β 2 (Esparza-Lopez et al., 2001), which could partially compensate the relatively low intrinsic affinity of TGF- β 2 for type II TGF- β receptors (Cheifetz and

Massague, 1991). On the other hand, Betaglycan can also bind to inhibin and facilitates its access to Activin receptors, thus blocking Activin from its receptor (Lewis et al., 2000). Recent work has also focused on function of the intracellular domain of Betaglycan. It revealed that the scaffolding protein β -arrestin2 interacted with Betaglycan and mediate its internalization with type II receptor through a clathrin-independent/lipid raft pathway, which repressed TGF- β signaling (Chen et al., 2003). Thus, the eventual outcome of these membrane-anchor proteins on TGF- β signaling may be highly dependent on cellular context.

Besides the interaction control between the TGF- β ligands and their receptors, the signal transduction is also regulated on the type I receptor activation. As we mentioned above, the GS domain in type I TGF- β receptor is important for its activation. It has been reported that the immunophilin FKBP12 bound to this region, capping the type II TGF- β receptor phosphorylation sites and stabilizing the inactive conformation of type I receptor (Huse et al., 1999). The BMP and Activin receptor membrane bound inhibitor protein (BAMBI), a pseudoreceptor with a similar homodimerization interface of the type I receptor, can prevent the formation of receptor complexes, thus blocking BMP and Activin, as well as TGF- β signaling (Onichtchouk et al., 1999).

Regulation of Smad proteins

Smad proteins are the major mediators for the TGF- β signaling. Based on their structure and function, they can be divided into three groups: (1) receptor Smads (R-

Smads, Smad 1, 2, 3, 5 and 8); (2) common Smad (Co-Smad, Smad4); (3) inhibitory Smad (I-Smad, Smad6 and 7).

Generally, Smad1 and its close homologues Smad5 and Smad8 are substrates for the type I BMP receptors and respond to BMP signals, while Smad2 and 3 are the type I TGF- β and Activin receptors and respond to TGF- β and Activin signals (Massague, 1998). In fact, the activation of a particular type of R-Smad is only associated with the specific type I receptors and has no relationship with the ligands. For example, in endothelial cells, TGF- β ligands activated the Activin receptor-like kinase1 (ALK1) and cause the phosphorylation of Smad1, 5 and 8 (Goumans et al., 2002). The Co-Smad is not ligand restricted and does not interact with receptors. It can form a complex with all R-Smad, but sometimes, its existence is dispensable. For example, the ubiquitous nuclear protein Transcriptional Intermediary Factor 1 γ (TIF1 γ) selectively bound phosphorylated Smad2 and 3 in competition with Smad4, so that TIF1 γ and Smad4 mediated different biological effects of TGF- β in human hematopoietic stem/progenitor cells (He et al., 2006). In spite of their different properties, the R-Smads and Co-Smads have similar structure. They all contain two conserved structural domains, the N-terminal Mad-homology 1(MH1) domain and the C-terminal MH2 domain, separated by a more variable linker region (Ross and Hill, 2008; Shi and Massague, 2003). In addition, the R-Smads have a conserved SXS motif at their extreme C-termini, which is the site for receptor-regulated phosphorylation. The MH1 domain of Co-Smads and R-Smads, except for the most common isoform of Smad2, exhibits sequence-specific DNA binding ability (Shi et al., 1998). The MH1 domain is also involved in nuclear import (Xiao et al., 2000) and has an autoinhibitory effect on their MH2 domain (Hata et al., 1997). The MH2 domain

confers the interaction between Smads and receptors (Lo et al., 1998) and is responsible for the formation of homomeric as well as heteromeric Smad complexes (Wu et al., 1997). Both the MH1 and MH2 domain can interact with a variety of transcriptional factors, activators and repressors, which largely increase the diversity of the biological effects of TGF- β signaling (Ross and Hill, 2008). The linker region is relative divergent among Smads, but it contain several phosphorylation sites that are important for the regulation. It also has a PY motif, which can be recognized by Smurf proteins for its ubiquitination (Izzi and Attisano, 2004).

There are two types of I-Smads in vertebrate, Smad6 and Smad7. The MH1 domain of I-Smads is less conserved to that of R-Smads or Co-Smads, however, their MH2 domain shows a similar amino acid sequence to other Smad proteins, but lack the C-terminal sites for receptor-mediated phosphorylation (Imamura et al., 1997; Nakao et al., 1997). Because of the similarity in the MH2 domain, I-Smads compete with R-Smads to interact with either type I receptors or Co-Smads, thus abolishing the transduction of TGF- β and BMP signaling. Generally, it is believed that Smad7 inhibits TGF- β /Activin and BMP signaling, whereas Smad6 works primarily on the BMP signaling (Hata et al., 1998; Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). More recently, another mechanism was found for the regulation of TGF- β signaling by I-Smads. It is reported that Smad7 could work as a connector to link Smurf ubiquitin ligases to the membrane receptors, resulting in the ubiquitination of the receptors and effectively blocking the signaling (Kavsak et al., 2000; Suzuki et al., 2002).

Besides I-Smads, other proteins, such as Smad Anchor for Receptor Activation (SARA) and a cytoplasmic isoform of the promyelocytic leukemia protein (cPML), also

mediate the interaction between R-Smads and type I receptors. SARA is a multidomain protein, containing a Smad-binding domain in the middle and a FYVE phospholipid-binding domain, which is important for the localization of SARA but not its interaction with R-Smads or TGF- β receptors. SARA enhanced the interaction between Smad proteins and activated TGF- β receptors either on the plasma membrane or on the early endosomes. Once the R-Smads were activated by the receptors, its interaction with SARA was weakened and the Smad-SARA-receptor complex was disassociated (Di Guglielmo et al., 2003; Tsukazaki et al., 1998). Meanwhile, SARA is also important for the receptor turnover and prevents its degradation mediated by the Smad7-Smurf complex (Di Guglielmo et al., 2003). The cPML is required for the association between Smad2/3 and SARA, and enhances the accumulation of SARA and TGF- β receptor in the early endosome through direct interaction with Smad2/3 and SARA (Lin et al., 2004). The function of cPML is interfered by a homeodomain protein, TG-interacting factor (TGIF), working in concert with c-Jun (Seo et al., 2006). Some modifications on the R-Smad proteins can also regulate their interaction with receptors. It has been shown that the E3 ligase Itch promoted ubiquitination of Smad2 and facilitated complex formation between the TGF- β receptors and Smad2 proteins (Bai et al., 2004).

After R-Smad proteins are phosphorylated by the type I receptors, they will form a complex with Co-Smads and translocate into the nucleus, where they either activate or repress target gene expression. This translocation process is another regulation point for the TGF- β signaling transduction. The Smad complex can be imported into the nucleus either through importin-dependent mechanism or importin-independent mechanism. First, it has been shown that the MH2 domain of Smad proteins could interact directly with the

FG repeat regions on nucleoporins Nup153 and 214, thus helping transport the Smad complex into the nucleus independent of importin proteins (Xu et al., 2002). In addition, a conserved lysine-rich sequence in the MH1 domain of Smads could interact with importins (Kurisaki et al., 2001). However, the efficiency for the Smad translocation through the importin-dependent mechanism is lower than that through the importin-independent mechanism (Xu et al., 2003). The modifications of R-Smads, especially in the linker region are critical for their translocation and transcriptional ability. It has been reported that the linker region of Smad1 could be phosphorylated by the Erk MAP kinases, which were activated by epidermal growth factor (EGF), fibroblast growth factor (FGF) or stress. Phosphorylation prevented the nuclear accumulation of Smad1 and reduced its transcriptional activity (Kretzschmar et al., 1997; Pera et al., 2003; Sapkota et al., 2007). However, in contrary to Smad1, the phosphorylation of Smad2 and 3 by MAP kinases or upstream MEK kinase enhanced their translocation and gene transactivation (Brown et al., 1999; de Caestecker et al., 1998; Funaba et al., 2002). Thus, the overall outcome of MAPK-related phosphorylation of R-Smad proteins is cellular context-dependent and may be affected by other signaling pathways. Other kinases can also phosphorylate R-Smad proteins. It has been shown that G1 cyclin-dependent kinases Cdk2 and Cdk4 phosphorylated Smad3 at its linker region and negatively regulated the TGF- β mediated block of cell cycle progression (Matsuura et al., 2004). Calcium/Calmodulin-dependent kinase II (CamKII) phosphorylated Smad2, 3 and 4 in the MH1 and linker region, preventing Smad complex formation and nuclear translocation (Wicks et al., 2000).

The degradation of Smad proteins and their dephosphorylation are equally important in regulating the TGF- β pathway and terminate its signaling. Usually, the active Smad proteins are the targets of homologous to E6-AP carboxyl terminus (HECT) domain E3 ligases and undergo the ubiquitin/proteasome mediated degradation pathway. The first HECT domain E3 ligase for Smad proteins was discovered in 1999 and called the HECT-domain Smad ubiquitination regulatory factors (Smurf) E3 ligase, Smurf1 (Zhu et al., 1999), followed by the discovery of another member in this family, Smurf2 in 2001 (Zhang et al., 2001). Smurf proteins interact with the PPXY motif of R-Smad proteins directly through their WW domains and it is believed that Smurf1 is responsible for the degradation of Smad1, while Smurf2 degrades both Smad1 and Smad2 (Zhang et al., 2001; Zhu et al., 1999). Besides Smurf1 and 2, the R-Smads are also degraded through other E3 ligase complex. For example, Smad3 physically interacted with Regulator of Cullins 1 (ROC1) and is degraded by Skp/cullin/F-box E3 ligase (SCF) complex (Fukuchi et al., 2001). Since Smad4 lacks a PPXY motif, its degradation by the HECT domain E3 ligases was mediated by the interaction with R-Smads (Moren et al., 2005). The degradation of Smad4 could also be mediated through SCF complex (Wan et al., 2004). The mechanisms for the degradation of I-Smad proteins were similar to that of R-Smads. Furthermore, Smad7 proteins could function as an adapter to link Smurfs to the TGF- β receptors, thus facilitating the receptor degradation. This process may be further regulated by some other accessory proteins, such as WW domain-containing protein 1 (WWP1) and ubiquitin-specific peptidase 15 (USP15) (Ebisawa et al., 2001; Eichhorn et al., 2012; Komuro et al., 2004). Additional modifications on Smad proteins also affect their degradation. The sumoylation of Smad4 by SUMO1/Ubc9 prolonged its half-life

because this modification competed with ubiquitination (Lin et al., 2003a; Lin et al., 2003b). Smad7 could be acetylated by p300, thus preventing it from Smurf-mediated degradation (Goumans et al., 2002). This effect was counteracted by several class I, II and III histone deacetylases (HDAC) (Kume et al., 2007; Simonsson et al., 2005). Another way to terminate the TGF- β signaling is to remove the active phosphorylation of R-Smads at the C-terminal SXS motif. This could be achieved by pyruvate dehydrogenase phosphatase (PDP) and RNA polymerase II small C-terminal phosphatases (SCPs) for Smad1 (Chen et al., 2006; Knockaert et al., 2006), and Mg²⁺-dependent phosphatase PPM1A for Smad2 and 3 (Lin et al., 2006).

Smad-dependent gene expression

As transcriptional factors, R-Smads and Co-Smads, but not Smad2, have both DNA binding ability (except Smad2) through MH1 domain and transactivation ability through the linker region (de Caestecker et al., 2000; Wang et al., 2005). However, the Smad complex still requires the association with other transcription factors to regulate target genes more specifically and precisely. Both the MH1 and MH2 domains of Smad proteins can mediate the interaction with a long list of Smad interacting proteins, including the basic helix-loop-helix (bHLH) family, like E2F4/5 (Chen et al., 2002) and Max (Grinberg and Kerppola, 2003), basic leucine zipper (bZIP) family, like c-Fos and c-Jun (Zhang et al., 1998), Forkhead family, like FoxO 1, 3 and 4 (Seoane et al., 2004), Runx family, like Runx2 (Zhang et al., 2000), Zinc finger protein family, like Sp1 (Feng et al., 2000) and YY1 (Lee et al., 2004) and mediators of other signaling pathway, like β -

catenin (Zhou et al., 2012) and Notch intracellular domain (NICD) (Itoh et al., 2004). The interaction between Smad proteins and other transcriptional factors not only affects the number of target genes regulated by TGF- β signaling, but also functions as a platform, enabling the crosstalk between TGF- β and other signaling pathways. Furthermore, the Smad proteins also interact with coactivators or corepressors, which are equally important in mediating target gene expression. The coactivators, such as SMIF, Swift and Zeb1, enhanced Smad-dependent gene activation (Bai et al., 2002; Postigo et al., 2003; Shimizu et al., 2001), while the corepressors, such as TGIF, c-Myc and SnoN help Smads repress target genes (Feng et al., 2002; Luo, 2004; Wotton et al., 1999). On the chromatin level, the gene activation mediated by the Smad complex involved the recruitment of histone acetyltransferase CBP/p300 to the promoter sites, either through its direct interaction with Smads or through the coactivators (de Caestecker et al., 2000; Feng et al., 1998; Postigo, 2003). Histone methylation, especially histone 3 lysine 4 (H3K4) trimethylation, may be also involved in Smad-dependent gene activation (Patel et al., 2007; Shimizu et al., 2001). On the other hand, the recruitment of C-terminal-binding protein 1 (CtBP) repressor and histone deacetylases (HDACs) is critical for Smad-mediated gene repression (Akiyoshi et al., 1999; Izutsu et al., 2001; Wotton et al., 1999). Besides regulating the histone modification and chromatin remodeling, Smad proteins could further repress gene expression by sequestering the transcriptional factor from its coactivators. For example, Smad3 could bind with MEF2, blocking its interaction with the coactivator GRIP1, thus inhibiting myogenic differentiation (Liu et al., 2004).

Smad-independent TGF- β signaling transduction

Many other signaling pathways may participate in TGF- β mediated biological responses. First, TGF- β can activate the MAPK pathway. In monkey derived COS7 cells, overexpressed human X-chromosome-linked inhibitor of apoptosis protein (XIAP) linked TAB1 to type I BMP receptor and this interaction may be necessary for the activation of TAB1-associated protein, TAK1, which belongs to the MAP kinase kinase kinase (MAPKKK) family (Yamaguchi et al., 1999). However, evidence for the direct interaction between the endogenous XIAP, TAB-TAK complex and type I BMP receptor, is still missing. Similarly, in mouse mammary epithelial (NMuMG) cells or human fibrosarcoma cells, p38 MAPK or JNK signaling was activated by TGF- β (Hocevar et al., 1999; Yu et al., 2002), but the mechanisms involving this activation were poorly characterized. Besides MAPK cascades, TGF- β could also activated small GTPases, like RhoA and Cdc42, which are important for TGF- β mediated epithelial-mesenchymal transition in epithelial cells and re-organization of stress fibers in human prostate carcinoma cells (Bhowmick et al., 2001; Edlund et al., 2002). However, like the MAPK pathways, little evidence showed that the activation of these small GTPases is through TGF- β receptors, and not through secondary effects. Finally, it has been shown that upon the ligand-dependent activation, type I TGF- β receptor physically bound to B α , a WD-40 repeat subunit of phosphatase 2A (PP2A), which in turn dephosphorylated and inactivate p70(s6k), inducing cell cycle G1 arrest (Griswold-Prenner et al., 1998; Petritsch et al., 2000). This mechanism is partial responsible for the TGF- β induced epithelial cell G1 arrest.

Epithelial-Mesenchymal Transition (EMT)

The epithelial mesenchymal transition (EMT) is a common biological process, which occurs when epithelial cells lose their polarized morphology and tight junctions and acquire mesenchymal properties, such as enhanced migratory capacity, elevated resistance to apoptosis and increased production of extracellular matrix. In fact, EMT occurs throughout development, from embryogenesis to the organ formation, and is involved in a variety of diseases. Based on the functional distinctions, EMT can be further divided into three types: (1) EMT that occurs during normal development processes, such as implantation, embryo formation and organ development. The mesenchymal cells derived from this type of EMT can also undergo a mesenchymal-epithelial transition (MET) to generate secondary epithelia; (2) EMT that is associated with wound healing, tissue regeneration and organ fibrosis. This type of EMT is frequently triggered by ongoing inflammation, however, its real existence *in vivo* remains controversial; (3) EMT that is related to cancer progression, especially the metastasis of carcinoma cells. One prominent characteristic for this type of EMT is its heterogeneity with some cells keeping most of epithelial traits and acquiring some mesenchymal properties, while others shedding all vestiges of their epithelial origin and becoming fully mesenchymal (Kalluri and Weinberg, 2009; Thiery et al., 2009; Zeisberg and Neilson, 2009).

Type I EMT

Type I EMT is involved in several stages during development. First, during mouse gastrulation, epiblast cells at the primitive streak, which is a transient structure forming along the posterior midline of the embryo, undergo the EMT (also known as epiblast-mesoderm transition) and then ingress between the epiblast and visceral endoderm to participate in the formation of either the mesoderm or the definitive endodermal germ layers (Ciruna and Rossant, 2001). Another EMT occurs in neural-crest cells, which is a transient population of cells at the boundary between epidermal and neural territories, to promote migration, thus giving rise to many different derivatives (Thiery and Sleeman, 2006). Subsequently, EMT is involved in the organ formation. For example, EMT is necessary for the regression of the Mullerian duct in male reproductive tracts (Zhan et al., 2006). Also, endothelial cells from the atrioventricular canal undergo EMT to invade the cardiac jelly and form the endocardial cushion, which will later assemble into the atrio-ventricular valvulo-septal complex (Nakajima et al., 2000).

Type II EMT

Type II EMT was thought to occur in the fibrosis of several organs, such as kidney, liver, lung and heart (Iwano et al., 2002; Kim et al., 2006; Zeisberg et al., 2007a; Zeisberg et al., 2007b). Here, we will take renal interstitial fibrosis as an example. Renal interstitial fibrosis is a common pathology in most chronic and progressive kidney diseases and characterized by inflammatory cell infiltration, fibroblast activation and

expansion, extracellular matrix deposition and tubular atrophy (Liu, 2011). The increasing number of fibroblasts may come from various resources. First, they can originate from the local resident interstitial cells (Humphreys et al., 2010; Lin et al., 2008). Another source is the recruitment of circulating fibrocytes, which are a subset of bone marrow-derived, circulating monocytes capable of producing collagen I. However, because specific markers for this group of cells are lacking, their contribution for renal fibrosis remains controversial (Niedermeier et al., 2009; Roufosse et al., 2006). Lastly, fibroblasts may come from epithelial cells through EMT.

The initial evidence supporting EMT involved in renal fibrosis *in vivo* came in 2002 (Iwano et al., 2002). Through a lineage tracing experiment, the authors showed that the epithelial cells expressed the fibroblast marker, fibroblast specific protein 1 (FSP1) and synthesize collagen I. Since then, more and more research tried to demonstrate the existence of EMT in renal fibrosis. These studies can be categorized into two groups. The first group of studies showed the up-regulation of mesenchymal markers in renal epithelial cells in injured kidney, such as α -smooth muscle actin (α SMA) (Yang and Liu, 2001), plasminogen activator inhibitor 1 (PAI1) (He et al., 2010), vimentin (Rastaldi et al., 2002), and Snail1 (Yoshino et al., 2007). The second group revealed that genetic or pharmaceutical manipulation of EMT related transcriptional factors or signaling pathways enhanced or attenuated renal fibrosis. For example, mice lacking Smad3, the key mediator for TGF- β signaling pathway were protected against tubulointerstitial fibrosis by blocking EMT (Sato et al., 2003). A similar experiment showed that systemic administration of recombinant human BMP7 led to repair of the damage in renal tubular epithelial cells through counteract the EMT effects induced by endogenous TGF- β 1

(Zeisberg et al., 2003). Furthermore, ectopic activation of Snail1 in kidney was sufficient to induce renal fibrosis (Boutet et al., 2006). Besides these *in vivo* results, more *in vitro* studies proved the existence of EMT in cell culture (Cheng and Lovett, 2003; Huang et al., 2009; Qi et al., 2005; Slattery et al., 2005).

However, improved lineage tracing results published in recent years argued strongly against the EMT model in renal fibrosis. In one study, the authors separately used the *Six2* promoter to mark all tubular cells derived from the cap mesenchyme and the *Hoxb7* promoter to mark all tubular cells from the ureteral bud to label the tubular cells, but failed to find the fibroblasts in peritubular interstitium with these positive genetic markers (Humphreys et al., 2010). Another study used *Pax8*-rtTA mice to simultaneously induce TGF- β 1 and label all proximal, distal and collecting duct tubular cells (Koesters et al., 2010; Traykova-Brauch et al., 2008). Even under these favorable conditions with overexpressing TGF- β 1, labeled epithelial cells were not found in the interstitial fibrosis region, but instead, underwent the autophagy. If there is EMT in fibrosis, the transiting epithelial cells must migrate through the tubular basement membrane (TBM) into the interstitial region, where they proliferate and deposit ECM. However, up to now, no transmission electron microscopy data are provided to show that even a single cell is crossing the TBM. Furthermore, although the TBM may become collapsed and highly folded, its integrity is preserved (Kriz et al., 2011).

Based on these two contradictory facts, a concept, called “partial EMTs”, was proposed (Kalluri and Weinberg, 2009; Liu, 2011). In this partial EMT, epithelial cells only change one or two phenotypic markers, while maintaining other epithelial characteristics. Although the current results do not support a typical EMT process in renal

fibrosis, the upregulation of mesenchymal markers in epithelial cells may still be biologically important. For example, it has been reported that Snail and Zeb proteins could directly repress the expression of E-Cadherin, which is a critical marker for epithelial cells (Cano et al., 2000; van Grunsvan et al., 2003). This may be significant for the apoptosis or autophagy of epithelial cells, and eventually the tubular atrophy in renal fibrosis. Also, the metalloproteinases, such as MMP2 and MMP9, secreted from epithelial cells could be critical for the remodeling of ECM in interstitium, thus activating quiescent fibroblasts. So the precise function of upregulated mesenchymal markers in epithelial cells and the paracrine effects of those cells on adjacent cells still require further studies.

Type III EMT

The EMT program confers upon cancer epithelial cells the ability to detach from each other and invade adjacent cell layers or migrate to distant locations (Yang and Weinberg, 2008). In fact, EMT was found in nearly all types of carcinoma (Thiery et al., 2009). For example, in breast carcinoma, a mouse lineage tracing study proved that EMT occurred specifically in *Myc*-initiated tumors (Trimboli et al., 2008). The EMT biomarkers, such as *Snail*, are associated with histological grades and the metaplastic subtype of breast carcinoma (Blanco et al., 2002; Lien et al., 2007). Similarly, based on an analysis of 123 primary human hepatocellular carcinoma (HCC) samples, the overexpression of Snail and Twist was correlated with a worse prognosis (Yang et al., 2009). This analysis was supported by the *in vitro* study, showing that overexpression of

Snail in an established HCC cell line HepG2 caused its dedifferentiation into fibroblastoid featured with increasing invasion activity (Miyoshi et al., 2004). Recent studies revealed that EMT not only increases the mobility of transformed cells, but also confers upon them the stemness with enhanced ability to form mammospheres and tumorigenesis (Mani et al., 2008; Santisteban et al., 2009). These results suggested that besides increasing the tumor invasion, EMT may be involved in the initiation of breast carcinoma by increasing the tumor cell pool. Interestingly, when migratory tumor cells settle at distant sites, they no longer exhibit the mesenchymal phenotypes ascribed to metastasizing carcinoma cells (Kalluri and Weinberg, 2009). This observation indicates that the microenvironment surrounding the tumor cells is critical in maintaining their transforming state (Scheel et al., 2011).

Signaling pathways associated with type II EMT

Although EMT can be divided into three types and occur in different situations, its key events at the molecular level are the same, downregulation of epithelial markers, such as E-Cadherin, and upregulation of mesenchymal genes, such as Snail, Zeb, Pai and Twist. The signaling pathways involved in three types of EMT are also similar. So here, we will mainly focus on the signaling pathways associated with type II EMT in the context of renal fibrosis.

TGF- β signaling

TGF- β signaling pathway is the most well studied pathway for EMT. *In vitro* study suggested that TGF- β could activate mesenchymal marker genes, such as α SMA

and Snail, as well as repress epithelial markers, like E-Cadherin and Occuldin in at least two different renal epithelial cell lines, MDCK from dog and HKC8 from human (Medici et al., 2006; Yang and Liu, 2001). Snail1 could further form a complex with Smad3 and 4, which was targeted to the promoters of CAR, a tight-junction protein, and E-cadherin to repress their expression (Vincent et al., 2009). *In vivo*, the severity of renal histopathology has been significantly lessened in experimental glomerular nephritis models by treating animals with antibodies against TGF- β ligands (Border et al., 1990) or the type II TGF- β receptors (Kasuga et al., 2001). Tubular epithelial cells are the main targets for TGF- β in renal fibrosis, since in the unilateral ureteral obstruction (UUO) mouse model, expression of both TGF- β and its type I receptor increased rapidly and specifically in renal tubular epithelia (Yang and Liu, 2001). BMP7 signaling pathway counteracts the effects of TGF- β in inducing EMT. As we mentioned above, systemic administration of recombinant human BMP7 led to repair of severely damaged renal tubular epithelial cells (Zeisberg et al., 2003). *In vitro*, BMP7 not only restored the expression of epithelial markers, repressed by TGF- β in renal epithelial cells, such as E-Cadherin and ZO-1 (Zeisberg et al., 2003), but also caused the mesenchymal-epithelial transition (MET) in adult renal fibroblasts, characterized by cell condensation, decreased motility and increased expressing E-Cadherin (Zeisberg et al., 2005). At the molecular level, BMP7 prevented TGF- β mediated loss of the transcriptional repressor SnoN and limited Smad3 DNA binding without affecting its phosphorylation or stability (Luo et al., 2010).

Canonical Wnt/ β -Catenin signaling

The canonical Wnt signaling pathway functions by preventing the degradation of cytoplasmic β -Catenin by GSK3 β /Axin/APC complex. The accumulated β -Catenin translocates into the nucleus, where it interacts with TCF/LEF proteins to regulate gene expression (MacDonald et al., 2009). In the UUO models *in vivo*, several Wnt ligands were up-regulated, such as Wnt1, Wnt4 and Wnt11 (He et al., 2009; Surendran et al., 2002), and β -Catenin predominantly accumulated in tubular epithelia. Blocking of Wnt signaling by administering its inhibitor, such as Dkk1 and Sfrp4, attenuated the renal injury, reduced the upregulation of mesenchymal genes and maintained the expression of epithelial markers. (He et al., 2009; Surendran et al., 2005). *In vitro*, it has been shown that β -Catenin mediated the activation of mesenchymal genes, such as Pai1, α SMA and Snail1, alone or together with Smad proteins (Hao et al., 2011; He et al., 2010; Zhou et al., 2012). Besides the Wnt ligands, β -Catenin may also accumulate from the collapse of cell-cell contacts during EMT induced by TGF- β . In this case, β -Catenin seems to work downstream of TGF- β signaling to regulate mesenchymal gene expression (Zheng et al., 2009). It is not clear whether Wnt ligands are also up-regulated in the process and facilitate β -Catenin accumulation. However, under most cases, Wnt and TGF- β signaling function cooperatively in mediating EMT and renal fibrosis.

Notch signaling

The notch signaling pathway is comprised of four receptors and two ligands, Delta and Jagged (Jag) (Guo and Wang, 2009). When ligands from the signal-giving cells bind to receptors on the signal-receiving cells, Notch intracellular domain (NICD) will be released from the plasma membrane through a series of proteolytic cleavages on the Notch receptors. NICD will then translocate into nucleus and regulate target gene

expression. In UUO models, the Notch ligand, Jagged1, was up-regulated in the distal tubular epithelial cells in injured kidneys. Along with this, the mediator of Notch signaling, NICD also accumulated in tubular epithelial cells. The mouse results were confirmed by human biopsy with diabetic kidney diseases (Bielez et al., 2010; Morrissey et al., 2002). Pharmaceutical block of Notch signaling by γ -secretase inhibitor ameliorated tubulointerstitial fibrosis, while enhanced expression of NICD driven by the *Pax8* promoter, in tubular epithelial cells caused activation of mesenchymal markers and renal fibrosis (Morrissey et al., 2002). In vitro, it is not clear whether Notch signaling alone can induce EMT in renal epithelial cells. However, Notch signaling could be activated by TGF- β signaling to promote EMT in either rat or human tubular epithelial cells (Bielez et al., 2010; Nyhan et al., 2010). In the type III EMT, Notch signaling promoted EMT by suppressing expression of the microRNA-200 (miR-200) through GATA-binding (Gata) factors in lung adenocarcinoma (Yang et al., 2011) or Zeb1 in pancreatic cancer cells (Brabletz et al., 2011). Whether this mechanism is still applicable to the type II EMT is unknown.

Integrin-associated signaling

Integrins consist of an α -subunit and a β -subunit, which are transmembrane receptors, that binds to the ECM outside the cell and interacts with cytoskeleton inside the cell. Integrins could pass signaling through two pathways. First, since they connect the ECM and cytoskeleton, it could function as a bridge to physically transduce mechanical strength from cells to the microenvironment or in reverse. Second, their β -subunits interact with integrin-linked kinase (ILK), an intracellular serine/threonine protein kinase and activate signaling through phosphorylation cascades. It has been

reported that both $\alpha_v\beta_6$ integrin and ILK were upregulated in renal epithelia in mouse UUO models or human biopsies with glomerulonephritis or other kidney diseases (Hahm et al., 2007; Li et al., 2003). The severity of renal fibrosis was reduced with application of antibody against $\alpha_v\beta_6$ integrin (Hahm et al., 2007). *In vitro*, overexpression of ILK in human kidney proximal epithelial cells suppressed expression of E-Cadherin, and induced fibronectin and MMP2 expression (Li et al., 2003). Normally, the function of $\alpha_v\beta_6$ integrin in mediating EMT or renal fibrosis is associated with TGF- β signaling. First, $\alpha_v\beta_6$ integrin could stimulate TGF- β signaling through facilitating the release of active form of TGF- β ligands from their latent complex. Consistent with this fact, both active TGF- β protein expression and Smad2 phosphorylation were less in UUO kidney with β_6 subunit knockdown (Ma et al., 2003). Second, inhibition of ILK expression by hepatocyte growth factor (HGF) blocked TGF- β induced EMT and attenuate renal fibrosis (Li et al., 2003). Thus, it seems that TGF- β and $\alpha_v\beta_6$ integrins work in a positive feedback mode to regulate EMT and renal fibrosis. In addition, another subunit of integrin, α_3 , was important for the phosphorylation of β -catenin at tyrosine residue 654 to enhance the interaction between β -catenin and Smad2 and initiate EMT in lung fibrosis (Kim et al., 2009).

Platelet derived growth factors (PDGFs) signaling

PDGF is a major mitogenic factor, whose receptors are tyrosine kinases. Upon stimulation, PDGFR transduce the signaling through activating several downstream molecules, such as Stat proteins, phosphatidylinositol-3 kinase (PI3K) and Ras (Floege et al., 2008). It has been shown that PDGF-C was upregulated at sites of interstitial fibrosis in human and rat kidneys (Eitner et al., 2008). Blocking PDGF-C or PDGF-D by

neutralizing antibodies reduced tubulointerstitial damage and fibrosis (Eitner et al., 2008; Ostendorf et al., 2006). The increase in PDGF-C expression was due, in large part, to infiltrating macrophages (Eitner et al., 2008). Although PDGF antibodies help preserve cortical expression of E-Cadherin and reduce expression of vimentin and α -SMA *in vivo*, the direct effects of PDGF signaling on renal epithelial cells are unknown. Some studies have focused on exploring the function of PDGF in type III EMT. It was found that mammalian target of rapamycin (mTor) and nuclear factor- κ B (NF- κ B) were activated upon PDGF-D overexpression in human prostate cancer cell line PC3 cells (Kong et al., 2008). Another study revealed that PDGF treatment promotes β -catenin nuclear translocation through disrupting GSK-3 β /Axin/APC degradation complex by p68 RNA helicase in a Wnt-independent manner (Yang et al., 2006). These studies may provide hints for researches exploring mechanisms of PDGF signaling on renal fibrosis.

Hypoxia associated signaling

Renal hypoxia, instead of being a consequence of renal fibrosis, may serve as an inducer for fibrogenic process (Manotham et al., 2004). Hypoxia inducible factor 1 (HIF1), the stability of which is based on the oxygen concentration, is the main regulator for the cellular responses to hypoxia. It has been reported that hypoxia induces morphological and gene expression profile change in proximal tubular epithelial cells partial through HIF1 (Higgins et al., 2007). Also, HIF1 accumulation was found in tubular epithelial cells in both mouse UUO models and human renal biopsies from patients with chronic kidney disease. HIF1 deletion or lysyl oxidases inhibition attenuated fibrosis in UUO kidneys (Higgins et al., 2007). On the molecular level, the mesenchymal marker genes, Twist and Snail are the direct targets of HIF1 α (Luo et al.,

2011; Yang et al., 2008). HIF1 α mediated activation of mesenchymal genes required the recruitment of histone methyltransferase (HMT) by histone deacetylase 3 (HDAC3) and WDR5 (Wu et al., 2011).

Summary

TGF- β superfamily, which includes TGF- β s and BMPs, is critical for normal development, as well as disease progression, and is tightly regulated both within and out of cells. *In vitro*, TGF- β induced the transition of renal epithelial cells into fibroblast-like mesenchymal cells by activating mesenchymal genes and repressing epithelial markers. Although the existence of this EMT in renal fibrosis is controversial, the upregulation of mesenchymal genes in renal epithelia may be critical for the initiation and progression of renal fibrosis. Considering the obvious and consistent phenotype change, the *in vitro* EMT model is also a good platform to study the mechanisms of TGF- β signaling in target gene activation, and crosstalk among different signaling pathways. Outside the cells, the regulation of TGF- β signaling could be achieved by sequestration or enhanced delivery of ligands to receptors. Inside the cells, TGF- β signaling could be regulated at different stages, such as the activation of R-Smads, the interaction between R-Smad and Smad4, and the access of Smad containing transcriptional complex to target genes.

In this dissertation, we will discuss both mechanisms and regulations of TGF- β superfamily mediated gene expression. In Chapter II, our results demonstrated that TGF- β activated Wnt11 was important for the upregulation of mesenchymal genes in renal epithelial cells. Furthermore, Wnt11 mediated TGF- β induced mesenchymal gene

expression through JNK signaling. In Chapter III, we showed that specific overexpression of KCP, a secreted cysteine rich protein, in renal epithelia attenuated the upregulation of some mesenchymal marker genes in injured kidneys of unilateral ureteric obstruction (UUO) model, by repressing TGF- β signaling and sustaining BMP signaling. In Chapter IV, our study revealed that Tle4 overexpression not only activated a BMP reporter, but also enhanced and sustained the upregulation of endogenous Id1 gene induced by BMP7. Furthermore, the effect of Tle4 on the BMP reporter was mediated by Smad7. Tle4 suppressed Smad7 expression and overexpression of Smad7 totally abolished the activation of Tle4 on the BMP reporter. Taken together, this dissertation dissected the mechanism of TGF- β to activate target gene expression in renal epithelial cells and also studied the regulation of TGF- β and BMP signaling, extracellularly by KCP and intracellularly by Tle4, providing new insights to the mechanisms of TGF- β superfamily action.

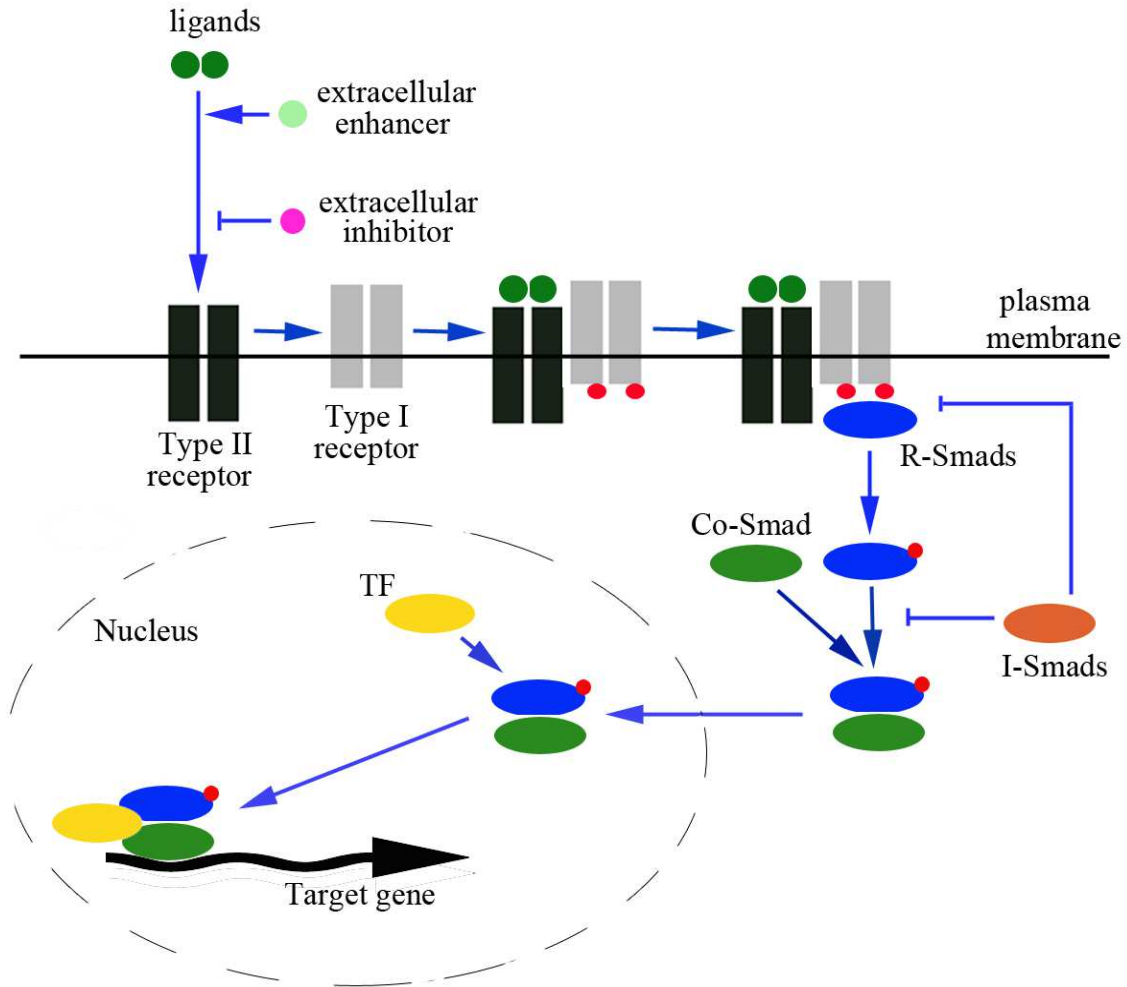


Figure 1-1. **Schematic diagram of TGF- β superfamily signaling from cell membrane to the nucleus.** The arrows indicate signal flow and phosphate groups are represented by red dots. R-Smad, receptor-Smad; Co-Smad, common-Smad; I-Smad, inhibitory-Smad; TF, transcriptional factor.

Table 1-1. Combinational interactions of Type II and Type I TGF- β receptors in vertebrates.

Type II	Type I	R-Smad
T β RII	ALK-5 (T β RI)	Smad2, Smad3
	ALK-1	Smad1, Smad5
	ALK-2	
BMPRII, BMPRIIB	ALK-2 (ActRI)	Smad1, Smad5, Smad8
	ALK-3 (BMPRIA)	
	ALK-6 (BMPRIIB)	
ActRII, ActRIIB	ALK-4 (ActRIB)	Smad2
	AIK-7	
AMHR	ALK-2	Smad1, Smad5
	ALK-3	
	ALK-6	

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Chapter II

Activation of Wnt11 by TGF- β Drives Mesenchymal Gene Expression through Non-Canonical Wnt Signaling in Renal Epithelial Cells

Abstract

Transforming growth factor β (TGF- β) promotes renal interstitial fibrosis *in vivo* and the expression of mesenchymal genes *in vitro*, however most of its direct targets in epithelial cells are still elusive. In a screen for genes directly activated by TGF- β , we found that components of the Wnt signaling pathway, especially Wnt11, were targets of activation by TGF- β and Smad3 in primary renal epithelial cells (PRECs). In gain and loss of function experiments, Wnt11 mediates the actions of TGF- β through enhanced activation of mesenchymal marker genes, such as Zeb1, Snail1, Pai1, and α SMA, without affecting Smad3 phosphorylation. Inhibition of Wnt11 by receptor knockdown or treatment with Wnt inhibitors limited the effects of TGF- β on gene expression. We found no evidence that Wnt11 activated the canonical Wnt signaling pathway in renal epithelial cells, rather the function of Wnt11 was mediated by the c-Jun N-terminal kinase (JNK). Our findings demonstrate cooperativity among the TGF- β , Wnt11 and JNK signaling pathways and suggest new targets for anti-fibrotic therapy in renal tissue.

Introduction

Renal interstitial fibrosis is a common pathology in most chronic and progressive kidney diseases (Liu, 2010). The function of the profibrotic cytokine TGF- β in the

initiation and progression of fibrosis has been intensively studied in the kidney and other tissues (Bottinger and Bitzer, 2002; Yang and Liu, 2001; Zeisberg et al., 2007). In almost all animal models examined, increased renal fibrosis correlates with increased expression of TGF- β ligands. Renal fibrosis is observed upon overexpression of TGF- β or application of recombinant TGF- β in mice, whereas inhibition of the TGF- β pathway can alleviate the severity of progressive renal fibrosis (Border et al., 1990; Kopp et al., 1996; Ledbetter et al., 2000).

In mammals, the binding of TGF- β ligand to its receptor, TGF- β receptor type II, leads to the recruitment and phosphorylation of TGF- β receptor type I (Feng and Derynck, 2005). The activated TGF β RI is a serine/threonine kinase that transduces the signal through phosphorylating receptor-activated Smad2 and Smad3. Phosphorylated Smad2/3 form a heteromeric complex with a common partner, Smad4, and translocate to the nucleus. Normally, the Smad complex requires other transcriptional factors to activate or repress target gene expression (Labbe et al., 2000; Sano et al., 1999). Both Smad2 and Smad3 are activated in TGF- β signaling pathway, but their targets and functions are distinct (Meng et al., 2010; Phanish et al., 2006). Genetic experiments point to a critical role for Smad3 in promoting TGF- β mediated renal fibrosis. Despite the evidence pointing to TGF- β as a profibrotic agent, its gene targets and detailed mechanisms that promote fibrosis are still not well characterized.

Renal tubular epithelial cells are target cells for TGF- β in kidney fibrosis (Yang and Liu, 2001). In the unilateral ureteral obstruction (UUO) mouse model, expression of both TGF- β and its type I receptor increased rapidly and specifically in renal tubular epithelia. *In vitro*, epithelial cells treated with TGF- β lost expression of epithelial markers

and assume a more mesenchymal phenotype. This epithelial-to-mesenchymal transition (EMT) was thought to occur in animal models of interstitial fibrosis and was an attractive model to explain the increased number of fibroblasts and the loss of epithelial tubular integrity (Iwano et al., 2002). In chapter I, we have thoroughly discussed different types of EMT and the controversy of the existence of type II EMT in renal fibrosis. Regardless of whether EMT occurs *in vivo*, the direct impact of TGF- β on the renal epithelial cells appears critical for initiation and progression of fibrosis. Meanwhile, the *in vitro* cell studies were a good model to study the function of different signaling pathways in cell fate determination, and provide valuable insights into *in vivo* disease models.

The Wnt signaling pathways have also been linked to TGF- β and to EMT during normal development and diseases. There are 19 Wnt ligands in the mouse and human genomes. These different Wnt ligands can signal through the canonical, β -catenin dependent pathway, or the non-canonical, β -catenin independent pathway. In the canonical pathway, activated Wnt signaling prevents the degradation of β -catenin by the GSK3 β /Axin/APC complex. The accumulating β -catenin then interacts with TCF/LEF proteins to regulate gene expression (MacDonald et al., 2009). One branch of the non-canonical Wnt pathway involves the calcium influx and further activation of Ca²⁺/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC) (Kuhl et al., 2000; Veeman et al., 2003). Another branch of the non-canonical pathway transduces its signal by activating the c-Jun N-terminal kinase (JNK) pathway either through small GTPase or other mechanisms (Veeman et al., 2003).

Although the Wnt signaling pathways were shown to function in EMT *in vitro* and in fibrosis *in vivo* (He et al., 2009; Scheel et al., 2011), the relationships with the

profibrotic cytokine TGF- β are not well defined. A limited number of studies addressing the cross-talk of TGF- β and Wnt signaling pathway converged on the β -catenin, as TGF- β could stabilize β -catenin by inhibiting its GSK3 β -dependent degradation through p38 MAPK and Akt (Hwang et al., 2009; Liu, 2010; Masszi et al., 2004). Also β -catenin could physically interact with Smad proteins to regulate target gene expression (Kim et al., 2009; Zhang et al., 2010; Zhou et al., 2012b). Yet, little is known about the function of non-canonical Wnt signaling pathway in fibrosis and its relation to TGF- β .

In this chapter, we defined the targets of TGF- β in renal epithelial cells *in vitro* by global gene expression analysis. We showed that components of the Wnt signaling pathways were activated by TGF- β . Among these, the non-canonical signaling protein Wnt11 was directly regulated by TGF- β through Smad3 in both primary and immortalized renal epithelial cells. Wnt11 enhanced the effects of TGF- β and was necessary for maximal activation of mesenchymal gene such as Zeb1, Snail1, Pai1 and the myofibroblast marker α SMA. Wnt11 did not enhance P-Smad3 nor activate the canonical Wnt signaling pathway, rather it appeared to increase mesenchymal gene expression through the non-canonical JNK pathway. These results pointed to a critical role for non-canonical Wnt signaling in TGF- β mediated fibrosis and suggested that autocrine and paracrine mechanisms could mediate TGF- β dependent effects in epithelial cells and adjacent cells.

Materials and Methods

Animals

C57BL/6 mice were kept according to NIH guidelines. Animal use was approved by the University Committee on Use and Care of Animals at the University of Michigan.

Primary and immortalized renal epithelial cells

Primary renal epithelial cells were isolated from the cortex of 5-6 week old female mice. Briefly, the medulla was manually removed and cortex was digested by liberase DH (Roche) in Dulbecco's modified Eagle's medium (DMEM, Lonza). The tissue fragments were sieved through a 212 μm pore size mesh. After three washes with cold DMED, cells were expanded in UltraMDCK serum free medium (Roche) supplied with 0.5X Insulin-Transferring-Ethanolamine-Selenium (ITES, Lonza), 60 $\mu\text{g/L}$ Epidermal Growth Factor (EGF, R&D systems), 10^{-9}M triiodothyronine and 1X antibiotic antimycotic (Gibco). Cells were split and frozen in Fetal Bovine Serum (FBS, Gibco) with 10% dimethyl sulfoxide. Recombinant human TGF- β 1 and Wnt11 were from R&D systems.

To inhibit translation, cycloheximide (5 $\mu\text{g/mL}$, Sigma) was added half an hour before TGF- β treatment (10 ng/mL) for the indicated times. To inhibit Smad3 phosphorylation, Specific Inhibitor of Smad3 (SIS3, Sigma) was added into the medium at the concentration of 5 μM 1 hour before 10 ng/ml TGF- β 1 treatment for 24 hours. To inhibit JNK signaling, 20 μM SP600125 (Sigma) or 10 μM JNK inhibitor III (EMD) was added into the medium 1 hour before 10 ng/ml TGF- β 1 treatment for 24 hours. To inhibit

Wnt signaling, Sfrp1 (R&D systems) was added at 0.5 $\mu\text{g}/\text{mL}$ together with 10 ng/mL TGF- β 1 for 24 hours

Immortalized renal epithelial cells (TKPTS) were a kind gift from Dr. Bello-Reuss. Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco) with 2% FBS, 1X ITES and Penicillin Streptomycin (Gibco). UltraMDCK serum free medium was used when serum starvation was necessary.

To overexpress Smad3 or Wnt11, TKPTS cells were cultured on 6 well plate in UltraMDCK serum free medium and transfected with 3 μg DNA of Smad3 or Wnt11 expressing vector or SHS (sonicated herring sperm) DNA control using Fugene6 (Roche) as per manufacturer's instruction. TGF- β 1 at the indicated concentrations was added into the medium 24 hours after transfection and cells were cultured for an additional 24 hours.

Microarrays expression analysis

PRECs were grown on 100 mm dishes until confluency reached 80%. Cycloheximide (5 $\mu\text{g}/\text{mL}$) was added half an hour before TGF- β 1 treatment (10 ng/ml) for 4 hours. RNA was extracted using the TRIzol RNA isolation system (Invitrogen). All samples were done in triplicate. Gene expression microarray analysis was done by the University of Michigan Comprehensive Cancer Center (UMCCC) Affymetrix and Microarray Core Facility. Briefly, The FL-Ovation cDNA Biotin Module V2 kit (NuGEN Technologies, San Carlos, CA) was used to produce biotin-labeled cRNA, which was then fragmented and hybridized to a Mouse 430 2.0 Affymetrix GeneChip 3 expression arrays (Affymetrix, Santa Clara, CA). Array hybridization, washes, staining, and scanning procedures were carried out according to standard Affymetrix protocols. Expression data

were normalized by the robust multiarray average (RMA) method and fitted to weighted linear models in R, using the affy and limma packages of Bioconductor, respectively (Irizarry et al., 2006; Smyth, 2004). Only probe sets with a variance over all samples superior to 0.1, a p-value inferior or equal to 0.05 after adjustment for multiplicity using the false discovery rate (Benjamini and Hochberg, 1995), and a minimum 2-fold difference in expression were selected for the analysis.

Western blot analysis

Cells were directly lysed in 2X SDS buffer (4% sodium dodecyl sulfate, 20% glycerol, 0.2M dithiothreitol, 125 mM Tris, pH 6.8) and boiled at 94°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes and immunoblotted with antibodies as indicated. Rabbit anti-phosphorylated Smad3, rabbit anti-Wnt11 and rabbit anti-phosphorylated CamKII are from Abcam. Rabbit anti-Smad2/3, rabbit anti-phosphorylated Smad2, rabbit anti-phosphorylated JNK, rabbit anti-phosphorylated c-Jun and rabbit anti-c-Jun are from Cell Signaling. Mouse anti-SMA, mouse anti-flag and mouse anti β -tubulin are from Sigma-Aldrich. Mouse anti-active β -Catenin is from Millipore. Mouse anti β -Catenin is from BD transduction Lab. Mouse anti-N-Cadherin is from Upstate. HRP-linked secondary antibodies and ECL reagent are from GE healthcare.

RNA reverse-transcription and real-time PCR

2-3 μ g total RNA was reverse-transcribed into complementary DNA with SuperScript First-Strand Kit (Invitrogen). The cDNA products were diluted 5 times and amplified with the iTaq Sybr green master mix (Bio-Rad) in a Prism 7500 (Applied

Biosystems). Primers pairs for PCR are as follows: Wnt11 5'-GGGCCAAGTTTTCCGATGCT, 5'-TTCGTGGCTGACAGGTAGCG; ZEB1 5'-TCAAGTACAAACACCACCTG, 5'-TGGCGAGGAACACTGAGA; PAI1 5'-ACATGTTT TAGTGCAACCCTG, 5'-GGTCTATAACCATCTCCGTG; Snai1 5'-GGAAGCCCAACTATAGCGA, 5'-AGCGAGGTCAGCTCTACG; Fzd7 5'-GAAGCTGGAGAAGCTGATGG, 5'-ATCTCTCGCCCCAAACTCT; Axin2 5'-TGAGCTGGTTGTCACCTACT, 5'-CACTGTCTCGTCGTCCCA; Wisp1 5'-GCCAGAGCAGGAAAGTCG, 5'-TACTTGGGTCGGTAGGTGC; GAPDH 5'-ACCACAGTCCATGCCATCAC, 5'-TCCACCACCCTGTTGCTGTA.

shRNA mediated Gene knocking-down

TKPTS cells were seeded on the 6 well plate 1 day before transfection. Cells were transfected with 2 µg DNA of Wnt11 shRNA lentivirus vector 54666, 53302 and a scrambled shRNA lentivirus vector (Open Biosystems) using Fugene6. For Smad3 or Fizzled7 knockdown, TKPTS cells were infected with lentivirus expressing Smad3 shRNA 54904 or Fzd7 64762 (Open Biosystems) in the presence of 8 µg/mL polybrene and kept overnight. Puromycin (Sigma-Aldrich) was added into the medium at 10 µg/ml and kept in culture medium for constitutive selection. Survival cells were cultured, expanded and frozen for further experiments.

Packed Wnt11 53302 shRNA lentivirus was used to knockdown in PRECs. Cells were seeded on 100 mm dishes for 24 hours. Lentivirus was added with 8 µg/mL polybrene and kept overnight. Puromycin was added for selection for 10 days before experiments.

Luciferase assays

TKPTS cells were seeded on 12 well plates. 3TP-lux reporter vector was transfected (1 µg/well) together with SHS or Smad3 expressing vector (2 µg/well) into the cells in triplicate 24 hours later. TOPFLASH or FOPFLASH reporter vector was transfected (1 µg/well) together with SHS or Wnt11 expressing vector (2 µg/well). Cells were lysed 48 hours after the transfection with dual luciferase assay kit (Promega) and results were read. In assays requiring TGF-β1 or LiCl treatment, TGF-β1 (10ng/ml) or LiCl (20 mM) was administrated 24 hours after the transfection and kept for another 24 hours.

Cellular fraction extraction

PRECs were treated with Wnt11 (500 ng/mL) or LiCl (20 mM) for 24 hours. To extract cytoplasm fraction, cells were washed with PBS once, scraped off and lysed in cell lysis buffer (5mM PIPES at pH 8.0, 85mM KCl, 0.5%NP40 and 1X protease inhibitors) on ice for 10 min. Then, the cells were spun at 3000 rpm for 5 min and supernatant was collected as cytoplasmic fraction. To extract nucleus fraction, cells were scraped off, resuspended in 5mL nuclear buffer 1 (10mM Tris at pH 8.0, 10mM NaCl, 3mM MgCl₂, 0.5mM DTT, 0.1% Triton X-100, 0.1M sucrose and 1X protease inhibitors) and dumped 20 times with a loose fitting (type A) dounce homogenizer. Then, 5 mL nuclear buffer 2 (10mM Tris at pH 8.0, 10mM NaCl, 3mM MgCl₂, 0.5mM DTT, 0.1% Triton X-100, 0.25M sucrose and 1X protease inhibitors) was added and mixed. At last, 2.5 mL nuclear buffer 3 (10mM Tris at pH 8.0, 5mM MgCl₂, 0.5mM DTT, 0.33M sucrose

and 1X protease inhibitor) was added from the bottom. The whole lysate was spun at 2000 rpm for 5 min at 4°C. The pellet was collected as nuclear fraction.

Immunofluorescence

PRECs were seeded on 4 well chamber slide (Thermo Fisher) over night and treated with 10 ng/mL TGF- β 1 or 0.5 μ g/mL Wnt11 for 24 hours or 1 μ M Ionomycin (Fisher) for 1 hour. Cells were washed with PBS once, fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.5% Triton X-100. PBS containing 0.1% Tween20 was used to wash slides after the incubation with primary and secondary antibodies. Mouse anti β -Catenin is from BD transduction Lab. Rabbit anti-CamKII is from Abcam. Goat anti-rabbit IgG-TRITC and Goat anti-mouse IgG-FITC are from Sigma-Aldrich. 4',6-diamidino-2-phenylindole dihydrochloride (Dapi) is from Sigma-Aldrich.

Results

Primary renal epithelial cells respond to TGF- β in a dose and time dependent manner

The effects of TGF- β on various cells in culture have been studied in detail (Scheel et al., 2011; Yang and Liu, 2001; Zeisberg et al., 2007). To confirm that primary renal epithelial cells (PRECs) from the adult kidneys respond to TGF- β , we isolated cells from adult mouse kidneys and cultured them in a defined serum-free medium.

Once the digested renal tubules were placed on the culture dishes, they would stick to plates. After 4-5 days of culture, renal epithelial cells spread out from the tubules and formed islands with clear boundaries. Other cell types, such as podocytes and blood cells, cannot grow in this conditional medium and were lost after the first passage (Fig 2-1A).

When PRECs were exposed to TGF- β 1, cells detached from each other and migrate out of the islands (Fig 2-1B). Mesenchymal markers were activated in those cells, although the kinetics of activation varied among different markers. The genes encoding α SMA, Zeb1, Pai1 and Snail1 were more sensitive to TGF- β 1 with a response in 24 hours, while the up-regulation of N-cadherin became obvious by 48 hours (Fig. 2-1D, E). A significant decrease of E-cadherin in protein level was not observed until 72 hours (Fig. 2-1C). The effects of TGF- β at a low dose (0.5ng/ml) were limited. When the dose of TGF- β 1 was increased to 2 ng/ml or 10ng/ml, its ability to change cell morphology and gene expression profile was significantly increased. These data indicated that the primary isolated cells showed a consistent and dose dependent response to TGF- β and activate genes associated with a more mesenchymal phenotype.

TGF- β 1 induced a global gene expression change in PRECs

Once we had established the effects of TGF- β 1 on PRECs in vitro, we sought to determine more precisely what genes are directly under the control of TGF- β 1. Thus, we derived PRECs from adult (5-6 weeks) mouse kidneys and cultured them in serum free media for 2 passages before splitting into replicates for our screening strategy. Cells were then cultured with cycloheximide (CHX) to inhibit further protein synthesis so only mRNAs of direct TGF- β targets would be activated. Subsequently, TGF- β 1 was added and RNA prepared after 4 hours incubation. Total RNA from PRECs treated with TGF- β 1 and cycloheximide was compared to RNA from cells cultured with cycloheximide alone using Affymetrix Mouse 430 2.0 cDNA microarrays. Three independent samples for each group were hybridized and statistically significant changes noted.

Overall, 454 Affymetrix probe sets, representing at least 318 annotated unique genes, showed significantly altered RNA expression levels of at least 2 fold or greater. Of these, 248 genes exhibited increased expression levels, whereas 70 genes showed a decrease in RNA levels, 4 hours post TGF- β 1 treatment (Table 2-1). In terms of molecular functions, 127 genes were assigned to functional groups involved in DNA binding or transcription regulation. More than 21 kinases showed changed expression levels, whereas 19 receptor binding proteins were altered. Given the effects of TGF- β on cell migration and dissociation, it was also noted that 12 GTPase regulatory proteins showed changed expression levels. Among those genes, 10 genes were associated with Wnt signaling pathway (Table 2-2). These include 4 Wnt ligands (Wnt1, Wnt9a, Wnt10a and Wnt11), 4 transcriptional factors (Tle1, Nfatc2, Nfatc3 and Sox17), 1 secreted protein (Wisp1) and 1 cyclin protein (Ccdn2). For further study, we would focus on Wnt11 because previous work suggested the cross-talk between Wnt11 and TGF- β (He et al., 2009; Schiro et al., 2011).

Activation of Wnt11 by TGF- β 1 was Smad3-dependent

First, we used qRT-PCR to insure that the change in Wnt11 mRNA levels was accurate in Affymetrix datasets. Up-regulation of Wnt11 in the presence of cycloheximide was confirmed in PRECs and also in an immortalized renal epithelial cell line TKPTS that expresses SV40 large T antigen (Ernest and Bello-Reuss, 1995). While Wnt11 up-regulation could be seen as early as 2 hours after TGF- β addition in PRECs, up-regulation in TKPTS cells was a bit slower but still robust (Fig 2-2 A, B).

Smad2 and Smad3 are the most critical mediators in TGF- β signaling pathway. Although both Smad2 and Smad3 are phosphorylated after TGF- β stimulation, their functions are not necessarily similar. In recent studies utilizing TGF- β mediated animal models of fibrosis, Smad2 activation was protective while Smad3 functions as an enhancer of the disease phenotype (Meng et al., 2010). Thus, we asked whether Wnt11 up-regulation was mediated by Smad3. In PRECs, the induction of Wnt11 mRNA was TGF- β dose-dependent, in a range from 2 ng/ml to 10 ng/ml (Fig. 2-2C). The fold change of Wnt11 mRNA level correlated with the amount of phosphorylated Smad3 (Fig. 2-2G). A specific Smad3 inhibitor, SIS3, which blocked its phosphorylation upon TGF- β treatment (Jinnin et al., 2006), abolished the activation of Wnt11 by TGF- β (Fig. 2-2D, H). In TKPTS, Wnt11 mRNA level was up-regulated by transient transfection of Flag-tagged Smad3 vector (Fig. 2-2E). This activation was due to phosphorylation of transfected Smad3 without TGF- β and was further enhanced with TGF- β treatment (Fig. 2-2I). Next, we used an shRNA lentivirus to specifically knockdown Smad3 in TKPTS. After puromycin selection, Smad3 protein level was significantly knocked down, while Smad2 protein level remained the same (Fig 2-2J). Both the basal and activated Wnt11 expression levels were reduced in the Smad3 knockdown group, consistent with the decreased basal and TGF- β activated P-Smad3 in this group (Fig. 2-2F). We also checked the P-Smad2 level under different conditions mentioned above. Neither the Smad3 inhibitor in PRECs nor the modulation of Smad3 proteins in TKPTS affected the response of Smad2 to TGF- β (Fig 2-2H, I, J). Considering the Wnt11 expression change in those conditions, our results suggested that TGF- β up-regulated Wnt11 in renal epithelial cells mainly through Smad3 proteins.

Wnt11 enhances activation of TGF- β dependent mesenchymal markers

In epithelial cell culture, TGF- β activates a program of mesenchymal marker gene expression associated with the transition of epithelial cells to a more mesenchymal phenotypes. We thus tested whether Wnt11 induction helped mediate this TGF- β dependent EMT *in vitro*. In addition to the myofibroblast marker α SMA, we examined cells for activation of the mesenchymal markers Zeb1, Pai1, and Snail1. Recombinant Wnt11 by itself did not activate any mesenchymal marker gene. At a high dose of TGF- β (10 ng/ml) all of the mesenchymal markers were activated by 24h. However, at lower dose of TGF- β (2ng/ml), recombinant Wnt11 significantly enhanced the expression of α SMA and other mesenchymal marker genes (Fig. 2-3A, B). Using a Wnt11 expression plasmid, a similar effect was observed in TKPTS, as both Pai1 and Snail1 expression increased significantly upon TGF- β treatment and Wnt11 expression (Fig. 2-3C).

Block of Wnt11 signaling abolishes the up-regulation of mesenchymal genes induced by TGF- β

Since endogenous Wnt11 is activated by TGF- β , a better test for Wnt11's contribution to EMT *in vitro* is to knockdown endogenous Wnt11 and test for TGF- β activation of target genes. Thus, we used shRNA lentivirus to produce two stable cell lines, 53302 and 54666, derived from the immortalized renal cell line TKPTS. In cell line 53302, basal Wnt11 expression was reduced by approximately 60% compared to a scrambled control line and Wnt11 was not activated by TGF- β (Fig 2-4A). The up-regulation of Pai1 was abolished in these Wnt11 knockdown cells, while up-regulation of Zeb1 and Snail1 were also reduced (Fig. 2-4C). In the 54666 cell line, basal Wnt11

expression was reduced by approximately 40%. However, these cells up-regulated Wnt11 about two folds in response to TGF- β 1, though this was still significantly less than the scrambled control cells (Fig. 2-4B). Nevertheless, the up-regulation of EMT marker genes was also attenuated in the 54666 Wnt11 knockdown cells (Fig. 2-4D). Moreover, administration of Wnt11 recombinant protein totally rescued the TGF- β dependent up-regulation of Zeb1 and partially rescued the PAI1 and Snail1 in the 53302 cells (Fig. 2-4E). We also used the Wnt11 specific 53302 shRNA lentivirus for knockdown in PRECs. Although the basal Wnt11 expression was not affected, its up-regulation upon TGF- β treatment was largely reduced, as were the response of EMT marker genes and the expression of α SMA (Fig. F, G, L).

We also determined if Frizzled7 (Fzd7), a well-characterized Wnt11 receptor (Djiane et al., 2000; Yamanaka and Nishida, 2007), was important for mediating the TGF- β effects in EMT. Although Fzd7 was expressed in TKPTS, its expression was not affected by TGF- β treatment. Fzd7 shRNA lentivirus knocked down approximately 60% of its expression (Fig. 2-4H). Similar to Wnt11 knockdown, the activation of mesenchymal marker genes, especially Zeb1 and Pai1, in response to TGF- β was reduced in the Fzd7 knockdown cell lines (Fig. 2-4I). Lastly, we inhibited Wnt signaling by addition of the secreted frizzled related protein Sfrp1, which sequesters Wnt away from its receptors. Similar to Fzd7 knockdowns, Sfrp1 addition significantly inhibited the expression of mesenchymal genes after TGF- β addition (Fig. 2-4K). Taken together, these data indicate that Wnt11 is an important TGF- β target for promoting the activation of mesenchymal marker genes in renal epithelial cell lines.

Wnt11 does not affect Smad3 phosphorylation or its transactivation ability upon TGF- β

treatment

Our data suggests that Wnt11 is an important autocrine mediator of the TGF- β response. To rule out that Wnt11 functions by modulating the levels of P-Smad3, we performed additional assays. First, Wnt11 knockdown did not by itself affect the ability of TGF- β to phosphorylate Smad3 in TKPTS (Fig. 2-5A). This was confirmed using a Wnt11 expression plasmid in TKPTS. If anything, Wnt11 overexpression reduced P-Smad3 slightly (Fig. 2-5B). We also tested the effects of Wnt11 on the 3TP-Lux reporter plasmid that responds to TGF- β . Overexpression of Wnt11 did not activate 3TP-lux by itself and Wnt11 knockdown did not affect the ability of TGF- β to activate 3TP-Lux (Fig. 2-5C, D). Thus, the effects of Wnt11 on TGF- β activation of mesenchymal markers are not mediated through alterations of P-Smad3 levels.

Wnt11 does not activate canonical Wnt/ β -Catenin signaling pathway

Wnt11 could be functioning through the canonical Wnt/ β -catenin pathway, either as a target or as an activator thereof during development process or in cancer cells (Cha et al., 2009; Dwyer et al., 2010; Zhou et al., 2007). Thus, we checked whether Wnt11 activated canonical signaling pathway by western blotting, gene expression, and reporter assays in our system. In PRECs, administration of Wnt11 recombinant protein failed to increase neither the active form of β -catenin, nor the β -catenin level in the nuclear fraction (Fig. 2-6A, B). To our notice, the basal nuclear β -catenin level was relative high in PRECs. However, this was consistent with a previous report that there were more nuclear β -catenin proteins in renal epithelial cells than other cell types, such as adult mesenchymal stem cells (Jian et al., 2006). Two target genes for canonical Wnt signaling pathway,

Axin2 and Wisp1, were also unresponsive to Wnt11 (Fig. 2C). In TKPTS, Wnt11 did not activate the TOPFLASH reporter for canonical Wnt signaling pathway (Fig. 2D). Conversely, Wnt11 overexpression slightly reduced the basal expression level of TOPFLASH, consistent with previous studies that show non-canonical Wnt signaling pathway could inhibit canonical Wnt signaling (Abdul-Ghani et al., 2011). These data showed that Wnt11 was not activating canonical Wnt signaling in renal epithelial cell cultures. Furthermore, there was little evidence that TGF- β activated canonical Wnt signaling when using TOPFLASH reporters or by assaying levels of Axin expression after 4h in the microarray based screens.

Both TGF- β and Wnt11 activates non-canonical/JNK Wnt signaling, but not non-canonical/CamKII-associated Wnt signaling

Since Wnt11 did not activate canonical/ β -Catenin Wnt signaling, we then asked whether the non-canonical Wnt signaling pathway was activated in TGF- β mediated EMT. The cJun-N-terminal Kinase (JNK) signaling pathway is one branch of non-canonical Wnt signaling. We found that phosphorylation of cJun and total cJun was elevated after TGF- β administration (Fig. 2-7A). The calcium-dependent signaling pathway is a second branch, in which calcium influx will eventually cause the activation and autophosphorylation of Ca²⁺/calmodulin dependent protein kinase (CaMKII) (Barria et al., 1997; Kuhl et al., 2000). To our surprise, the basal phosphorylation level of CaMKII in PRECs was high even without TGF- β treatment. After TGF- β administration, the amount of phosphorylated CaMKII increased only slightly (Fig. 2-7A). Recombinant Wnt11 administration in PRECs or overexpression in TKPTS by itself could activate the JNK signaling pathway as evidenced by P-cJun levels (Fig. 2-7B). Under normal conditions, phosphorylated CamKII

was present on the plasma membrane and throughout cytoplasm. In the stimulation of ionomycin, which enhanced Ca^{2+} influx by releasing stored intracellular Ca^{2+} (Morgan and Jacob, 1994), phosphorylated CamKII translocated into the nucleus in a number of PRECs. However, such translocation was seldom found in TGF- β or Wnt11 treated PRECs (Fig. 2-8). To test the role of JNK signaling more directly, we used two specific JNK inhibitors, SP600125 and JNK inhibitor III, to block JNK signaling in PRECs prior to TGF- β administration. The JNK inhibitor III was a cell-permeable 37-mer peptide by fusing human c-Jun δ domain (amino acids 33-57) sequence with that of HIV-TAT protein transduction domain (amino acids 47-57) via a γ -aminobutyric acid spacer. It has been shown to specifically disrupt c-Jun/JNK complex formation and the subsequent phosphorylation and activation of c-Jun by JNK and could be used as a complement inhibitor for SP600125 (Holzberg et al., 2003; Wang et al., 2008). Both the inhibitors blocked the up-regulation of mesenchymal genes induced by TGF- β without affecting P-Smad3 levels (Fig. 2-7C, D). However, despite their common blockade effects on c-Jun phosphorylation, SP600125 decreased the total c-Jun, while JNK inhibitor III slightly increased it. This may reflect the different characteristics of the two inhibitors. Similarly, in TKPTS, both the inhibitors reduced the up-regulation of mesenchymal genes induced by Wnt11 overexpression (Fig. 2-7E). A more precise examination of Wnt11 expression upon the TGF- β treatment showed that Wnt11 was induced as early as 1 hour after TGF- β 1 administration, which was just the time point that the amount of phosphorylated c-Jun began to elevate (Fig. 2-7F, G). The addition of cycloheximide abolished c-Jun phosphorylation, suggesting that the activation of JNK signaling pathway upon TGF- β treatment required new protein synthesis. Also, Wnt11 knockdown suppressed TGF- β

induced c-Jun phosphorylation, which is consistent with a role for Wnt11 in activating the JNK pathway (Fig. 2-7H). Taken these together, our results demonstrated that up-regulation of Wnt11 by TGF- β drives expression of mesenchymal genes through the non-canonical, JNK signaling pathway.

Discussion

Despite the critical role of TGF- β in renal fibrosis, many of its target genes and biological effects in renal epithelial cells remain poorly characterized. We systematically analyzed the effects of TGF- β on transcription of direct target genes using renal epithelial cells and translation inhibition. From the microarray result, we found that more than 300 genes changed their RNA expression levels of at least 2 fold. In the presence of cycloheximide, not all mesenchymal markers, such as *Pai1* and *Zeb1*, were up-regulated by TGF- β , suggesting that these genes are not direct targets of Smad protein dependent transcription. This was consistent with previous studies indicating that some mesenchymal markers, such as *Pai1*, were not directly regulated by Smad proteins, but the targets of other transcriptional factors induced by TGF- β signaling (He et al., 2010). Similarly, E-cadherin was also not on the list. This is not only because that the suppression of E-cadherin required new protein synthesis, such as *Snail1* (Vincent et al., 2009), but also due to a short TGF- β treatment time (Yang and Liu, 2001).

Among the genes activated by TGF- β were many associated with the Wnt signaling pathway. Recent evidence implied that Wnt signaling, especially the canonical Wnt/ β -catenin pathway, was involved in EMT and TGF- β mediated fibrosis (Akhmetshina et al., 2012; He et al., 2009; Surendran et al., 2005; Zhou et al., 2012a). In our studies,

Wnt11 showed the greatest response to TGF- β both in microarray and real-time PCR assays and was thus studied further. Prior studies also suggested that Wnt11 was directly regulated by β -Catenin (Dwyer et al., 2010; Zhou et al., 2007). In our study, LiCl treatment could activate Wnt11 expression in PRECs (data not shown). However, TGF- β did not activate the expression of Axin2, a typical β -Catenin target, in our microarray studies, indicating that at least in the early stage, the upregulation of Wnt11 by TGF- β was β -Catenin independent. In the Smad3 knockdown TKPTS cells, the Smad2 phosphorylation upon TGF- β treatment was not affected, but Wnt11 up-regulation was largely reduced, suggesting that Smad2 alone is not sufficient to activate Wnt11. In Smad3 overexpressed TKPTS, the transfected Smad3 was phosphorylated and activated Wnt11 expression, without affecting Smad2 phosphorylation status. Taken this together, our data clearly shows that Wnt11 activation is through a Smad3 dependent mechanism. Since Wnt11 was critical for the upregulation of mesenchymal genes upon TGF- β 1 treatment, our results supported the different roles of Smad2 and Smad3 in mediating EMT and renal fibrosis (Meng et al., 2010). Considering the protecting effects of Smad2 in renal fibrosis, it is interesting to test whether Smad2 overexpression will inhibit Wnt11 upregulation by TGF- β . Another issue concerning the direct regulation of Wnt11 by Smad3 proteins, is to find the Smad binding element(s) (SBE) in the promoter region of Wnt11. There are 6 transcripts for Wnt11 in mouse genome, sharing two promoter regions. Representatively, Wnt11-001 (ENSMUST00000067495) has 7 exons, while Wnt11-201 (ESMUST00000168655) starts roughly from the exon3 of Wnt11-001 with 5 exons. Both promoter regions have been cloned previously and found to be regulated by β -Catenin (Dwyer et al., 2010; Zhou et al., 2007). In our study, we also cloned a 2kb genome DNA

fragment from these two promoter regions and inserted them into a luciferase reporter vector. However, both Wnt11 promoter reporters failed to respond to TGF- β 1 or Smad3 overexpression in either TKPTS or 293 cells (data not shown). Thus, finding real SBE in Wnt11 promoter regions, a chromatin immunoprecipitation (ChIP) assay with Smad3 antibody would be necessary. Furthermore, it has been reported that Smad proteins also bound to enhancers to regulate target gene expression (Tone et al., 2008). Thus, a conserved region between the exon4 and exon5 (in case of Wnt11-201) is another candidate for the ChIP assay.

Wnt11 has multiple functions in regulating cell properties, such as proliferation, migration and differentiation. However its precise function in different cell types was context dependent and sometimes contradictory. For example, Wnt11 enhanced tight and gap junction formation in a quail mesodermal cell line QCE6 to promote its differentiation to cardiomyocytes (Eisenberg et al., 1997). In contrast, Wnt11 conditional medium induced E-cadherin internalization in a rat intestinal epithelial cell line IEC6, thus increasing its proliferation and migration (Ouko et al., 2004). These data suggested that Wnt11 effects were highly dependent on the cellular context. Although it was shown that Wnt11 was involved in EMT during dorsal fin development in *Xenopus* (Garriock and Krieg, 2007), its detailed function and mechanisms of action were not well characterized. Here, we reported that Wnt11 participated in TGF- β mediated EMT in both primary and immortalized renal epithelial cells, and was necessary for the up-regulation of mesenchymal gene expression induced by TGF- β . Although both Wnt11 administration in PRECs and transfection in TKPTS could enhance TGF- β effects, its treatment alone in two cell types was different. In PRECs, Wnt11 alone did not activate mesenchymal

markers; however, in TKPTS, Wnt11 up-regulated some of the mesenchymal genes. This could result from subtle differences of cell characteristics between primary and immortalized cells and Wnt11 delivery methods. Recombinant protein administration is more like a paracrine model, whereas expressing vector transfection may be more autocrine. Compared to the Wnt11 recombinant protein, the autocrine Wnt11 in TKPTS may interact with other secreted ligands, thus acquiring additional properties. For example, the secreted *Xenopus* Wnt11 physically interacts with Wnt5a and the complexes has more canonical Wnt signaling activity than secreted Wnt11 or Wnt5a acting alone (Cha et al., 2009).

Whether Wnt11 activates the canonical Wnt signaling pathway is also controversial. For example, Cha SW et al (Cha et al., 2009) showed that Wnt11/5a complex could enhance canonical Wnt11 signaling through accumulating cytosolic β -catenin in *Xenopus* oocytes, mouse L cells and human embryonic stem cells. Other reports show that Wnt11 had no effect, and even down-regulated β -catenin signaling (Anton et al., 2007; Maye et al., 2004). To date, most studies are consistent with Wnt11 activating the non-canonical Wnt signaling pathways through the JNK or CamKII kinases (Flaherty et al., 2008; Westfall et al., 2003; Zhou et al., 2007). In our renal epithelial cells, Wnt11 alone did not activate canonical Wnt signaling nor was the CamKII protein activated by TGF- β or Wnt11 directly. In fact, CamKII reportedly can inactivate Smad/TGF- β signaling through blocking the accumulation of nuclear Smad proteins (Wicks et al., 2000). Clearly this was not the case given the robust TGF- β responses observed. However, we did measure activation of the JNK signaling pathway by TGF- β and Wnt11. The abolishment of c-Jun phosphorylation induced by TGF- β in the presence of cycloheximide

indicated that the activation of JNK signaling was a secondary effect for TGF- β , which required new protein synthesis. Activation of JNK was critical for mediating the Wnt11/TGF- β response because inhibition of JNK signaling could decrease up-regulation of the mesenchymal genes induced by Wnt11. Although JNK signaling was activated by Wnt11 alone in PRECs, this was not sufficient to induce expression of mesenchymal genes. The JNK inhibitor only attenuated the effects of TGF- β , but did not entirely block the activation of mesenchymal genes.

Furthermore, by activating families of secreted signaling molecules, TGF- β acting on epithelial cells could impact the environment and the adjacent cells in the renal interstitium. Nowadays, more and more evidence pointed to pericytes for the increasing number of fibroblasts in renal fibrosis (Humphreys et al., 2010; Lin et al., 2008). In this model, pericytes detached from the vasculature, migrate into interstitium, proliferate and differentiate into myofibroblasts. However, the signaling pathways controlling this series of biological process are largely unknown. It has been reported that Wnt11 was upregulated during renal fibrosis (He et al., 2009), also it promoted cell survival, migration and proliferation in different types of cells (Garriock and Krieg, 2007; Matthews et al., 2008; Ouko et al., 2004; Uysal-Onganer et al., 2010). Thus, it is worth exploring the function of Wnt11 in pericyte activation during renal injury.

Taken together, our results showed that TGF- β activates multiple signaling pathways in renal epithelial cells through enhanced expression of secreted signaling proteins and transcription factors. Of these, Wnt11 activation by TGF- β enhances the overall effects attributed to TGF- β on epithelial cells, such as expression of genes associated with more mesenchymal cells. A recent study on EMT in breast tumor

suggested the importance of TGF- β , canonical and non-canonical Wnt signaling pathways in maintaining the mesenchymal state, indicating an interactive communication model between different signaling pathways in a biological process (Scheel et al., 2011). Our findings suggested a transregulation model, whereby TGF- β directly activates signaling factors and effectors necessary for the phenotyping changes observed.

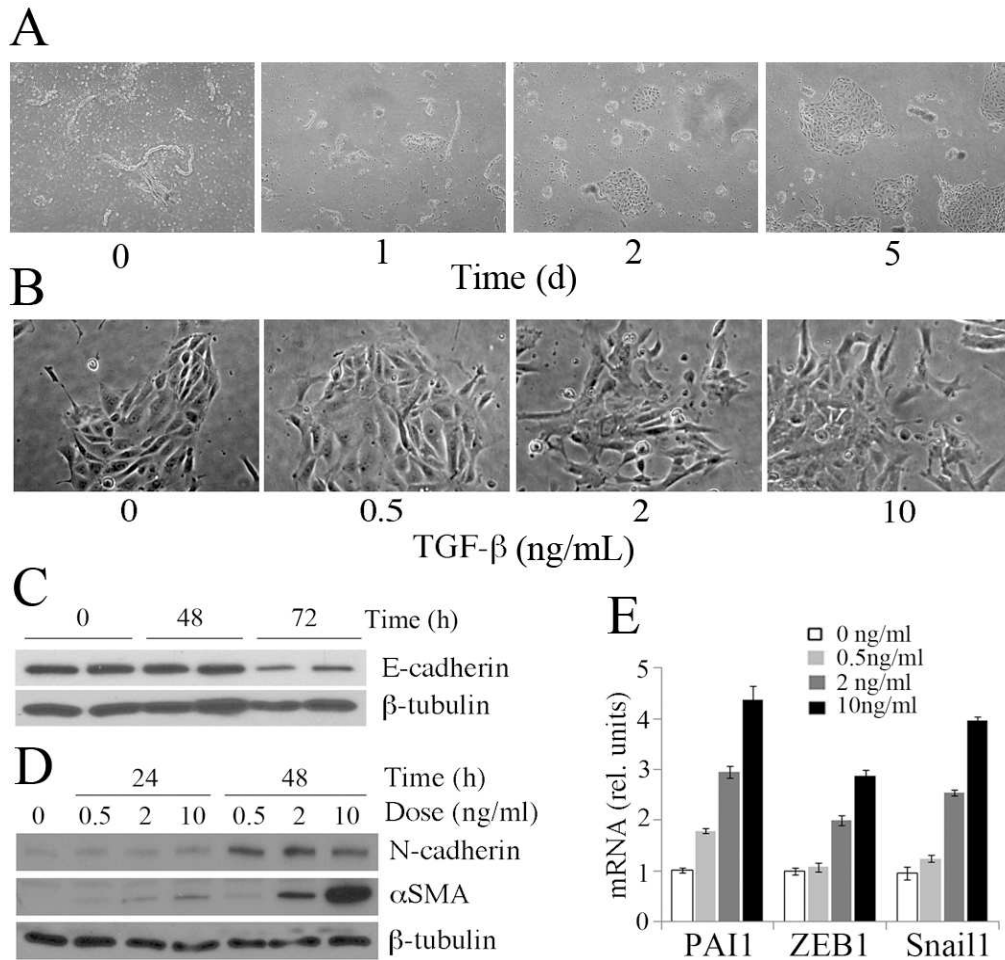


Figure 2-1. Effects of TGF- β on primary renal epithelial cells. A) Phase contrast micrographs of morphology change of PRECs after they were isolated from mouse cortex. Indicated times were the days after cells were isolated. B) Phase contrast micrographs of PRECs treated with increasing concentrations of TGF- β for 24 h. C) Western blot of cells treated for 0, 48, 72 h with 10 ng/ml of TGF- β and probed for E-cadherin. D) Time and dose dependence of α SMA and N-cadherin expression with increasing amounts of TGF- β . E) qRT-PCR of mesenchymal markers after 24h TGF- β treatment at the indicated doses. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

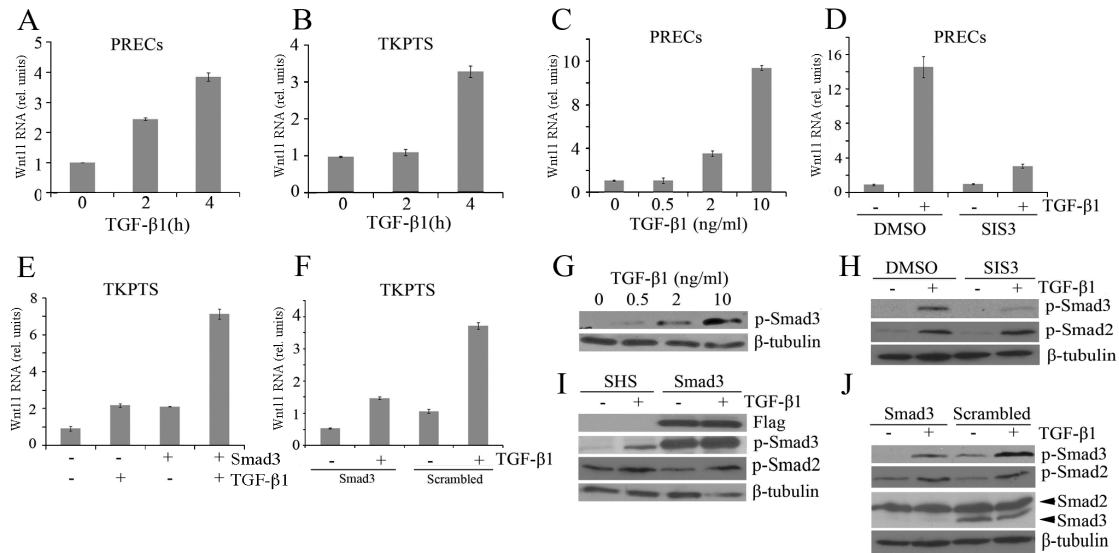


Figure 2-2. Wnt11 are direct targets of TGF-β signaling. A) Wnt11 RNA levels in PRECs cells cultured with TGF-β for the indicated time in hours in the presence of cycloheximide. B) Wnt11 RNA levels in TKPTS cells cultured with TGF-β for the indicated time in hours in the presence of cycloheximide. C) Wnt11 RNA levels after 24h with varying doses of TGF-β as indicated. D) Wnt11 RNA activation in response to TGF-β in the presence or absence of the Smad3 inhibitor SIS3. E) Wnt11 RNA levels measured after Smad3 transfection and/or TGF-β treatment. F) Wnt11 RNA activation in response to TGF-β in the presence or absence of Smad3 shRNAs or scrambled controls. G) Western blot of P-Smad3 in response to increasing doses of TGF-β. H) Western blots of P-Smad3 and P-Smad2 after inhibition by SIS3 and treatment with TGF-β. I) Western blot for P-Smad3 and P-Smad2 after Smad3 transfection and TGF-β treatment. J) Western blot of P-Smad3, P-Smad2 and total Smad2/3 after culture with Smad3 shRNAs and TGF-β treatment. It is noted that Smad3 proteins were shown nearly totally gone in Smad2/3 panel, while it is still detectable in the P-Smad3 panel. This resulted from different expose time and affinity for these two antibodies. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

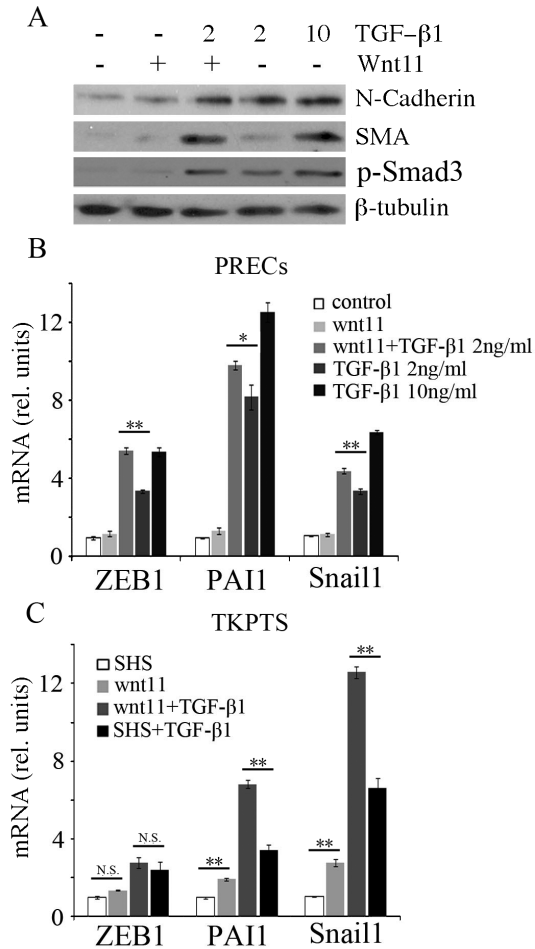


Figure 2-3. Wnt11 increases TGF- β dependent activation of mesenchymal genes. A) Western blots for N-cadherin, α SMA, P-Samd3, and β -tubulin from cells treated with recombinant Wnt11 and different doses of TGF- β as indicated. B) Quantitative RT-PCR for RNAs from the genes indicated under similar conditions as in A. C) Quantitative R-PCR for mesenchymal genes after transfection with Wnt11 expression plasmids and/or TGF- β addition. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean. (* $p < 0.05$; ** $p < 0.01$, n.s. not significant, students-t-test for independent variables)

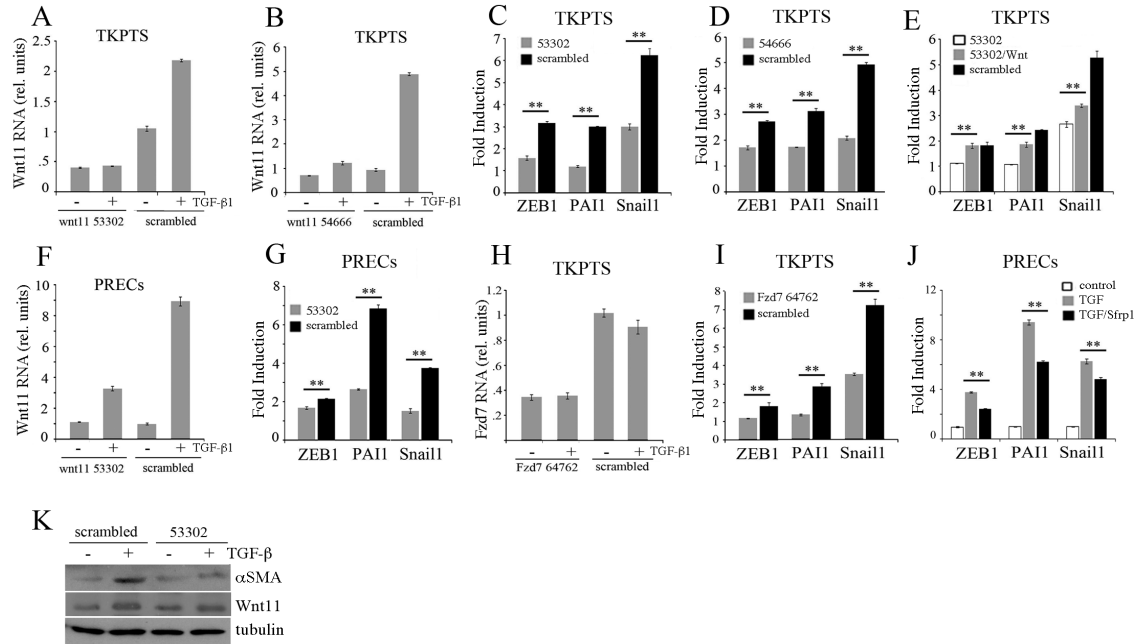


Figure 2-4. Wnt11 is necessary for the TGF-β dependent activation of mesenchymal genes. A) Wnt11 RNA levels after culture with shRNA #53302 or a scrambled control in TKPTS cells with or without TGF-β. B) Similar experiment as in A but using the Wnt11 shRNA #54666. C) The TGF-β induction of RNAs for the indicated genes in the presence or absence of shRNA #53302. Relative amount of RNA is compared before or after TGF-β addition and expressed as fold induction. D) Similar experiment as in C but using the Wnt11 shRNA #54666. E) The induction fold change of indicated genes by TGF-β is measured in cells cultured with shRNA 53302, with shRNA 53302 and recombinant Wnt11, or with scrambled shRNA. Note that recombinant Wnt11 increases the induction of mesenchymal genes in the presence of 53302. F) Wnt11 shRNA 53302 in PRECs reduces TGF-β dependent Wnt11 RNA induction. G) In PRECs, Wnt11 shRNA 53302 reduces the TGF-β mediated fold induction of mesenchymal marker genes. H) In TKPTS, inhibition of Fzd7 RNA by shRNA #64762 is independent of TGF-β. I) Fzd7 shRNA #64762 inhibits the TGF-β mediated induction of mesenchymal marker genes. J) The Wnt secreted inhibitor Sfrp1 reduces the TGF-β mediated induction of mesenchymal marker genes in PRECs. K) Western blots for αSMA and Wnt11. Cells show that inhibition of Wnt11 by shRNAs reduces the accumulation of αSMA in response to TGF-β in PRECs. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean. (*p < 0.05; **p < 0.01, students-t-test for independent variables)

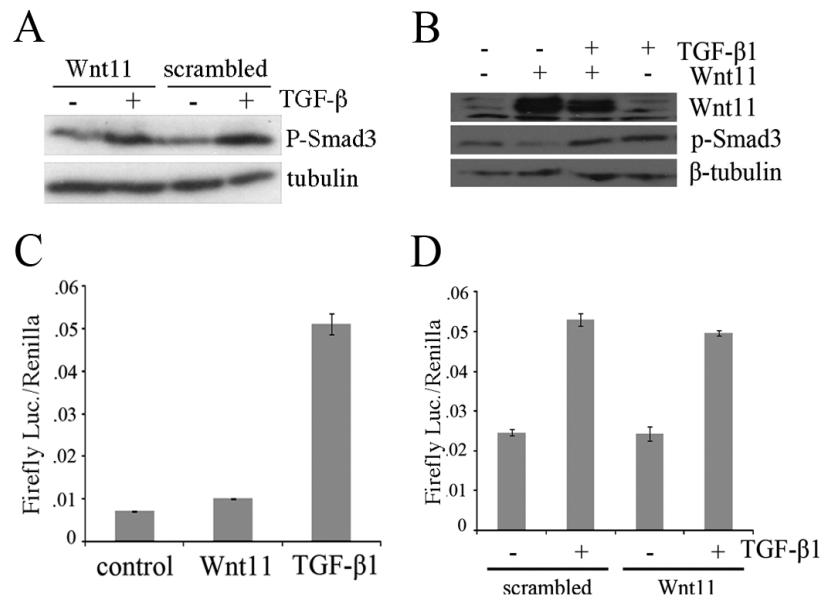


Figure 2-5. **Wnt11 does not modulate Smad proteins.** A) Western blot of P-Smad3 from cells cultured with Wnt11 shRNAs and/or TGF- β as indicated in TKPTS. B) Western blots from cell overexpressing Wnt11 and treated with TGF- β show no affect of Wnt11 on P-Smad3 levels. C) The P-Smad2/3 reporter 3TP-Luc was assayed after co-transfected with Wnt11 or treatment with TGF- β . Note that Wnt11 does not increase 3TP-dependent luciferase. D) Wnt11 shRNA knockdown does not affect the ability of TGF- β to activate 3TP-luc. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

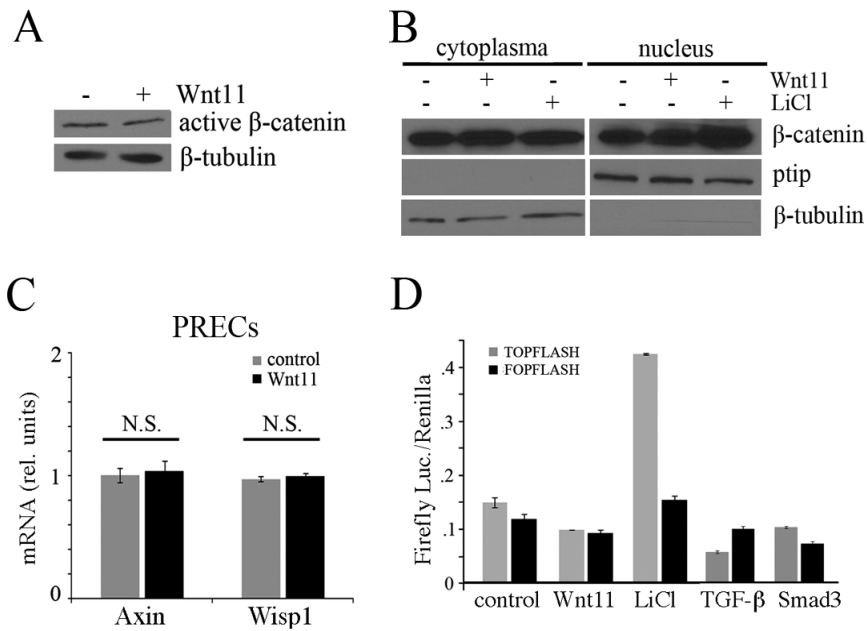


Figure 2-6. **Wnt11 does not mediate β -catenin dependent gene activation.** A) Western blot using antibodies against activated β -catenin show no effects of Wnt11 on activated β -catenin accumulation. B) Western blot using antibody against total β -catenin for cytoplasmal and nuclear fraction of PRECs under Wnt11 or LiCl treatment for 24h. β -tubulin and ptip was used as the loading control of cytoplasmal and nuclear fraction respectively. C) Wnt11 does not activate Wisp1 or Axin, two known β -catenin target genes, as assayed by qRT-PCR in renal epithelial cells. D) Cells were transfected with the β -catenin reporter TOPFLASH and treated with Wnt11, TGF- β , or LiCl, or co-transfected with Smad3. Only the known GSK3 kinase inhibitor LiCl activated the TOPFLASH reporter. FOPFLASH is used as non-specific control plasmid. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean. (n.s. not significant, students-t-test for independent variables)

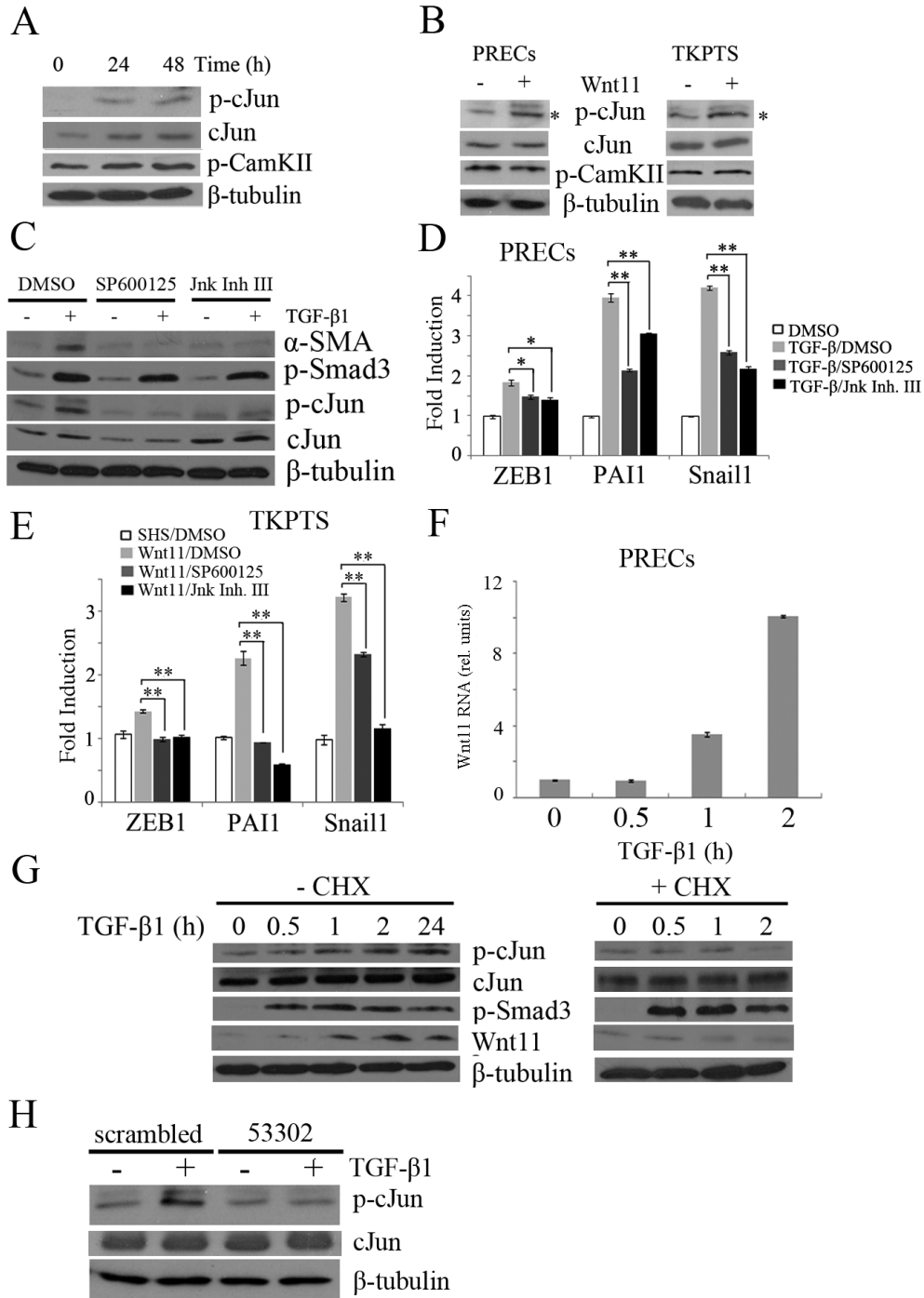


Figure 2-7. Activation of JNK signaling by TGF- β /Wnt11 activates mesenchymal marker genes. A) Western blot for P(S63)-cJun, total cJun and P-CamKII after treatment of renal epithelial cells for 24 or 48 hours with TGF- β shows increased P-cJun and total cJun but not P-CamKII. B) Addition of recombinant Wnt11 in PRECs and Wnt11 overexpression in TKPTS increases levels of P-cJun, but not P-CamKII. C) Western blots of cell lysates show that inhibition of the cJun kinase (JNK) by SP600125 or JNK inhibitor III reduces expression of α SMA in response to TGF- β . Note there is no effect on P-Smad3 levels. D) qRT-PCR of mesenchymal marker genes treated with TGF- β , with or without the JNK inhibitors, show reduced expression of all mesenchymal markers tested upon JNK inhibition in PRECs. E) Activation of mesenchymal marker gene expression in response to Wnt11 is reduced upon JNK inhibition in TKPTS, as determined by qRT-PCR. F) Wnt11 RNA levels in PRECs cells cultured with TGF- β for the indicated times in hours. G) Western blots for P-cJun, total cJun, Wnt11 and P-Smad3 with TGF- β treatment for indicated times absence or in the presence of cycloheximide (CHX). CHX abolished the elevated P-cJun and Wnt11 upon TGF- β treatment. H) Western blots for P-cJun show reduced levels upon TGF- β treatment when cells are cultured with shRNA 53302 against Wnt11. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean. (*p < 0.05; **p < 0.01, students-t-test for independent variables)

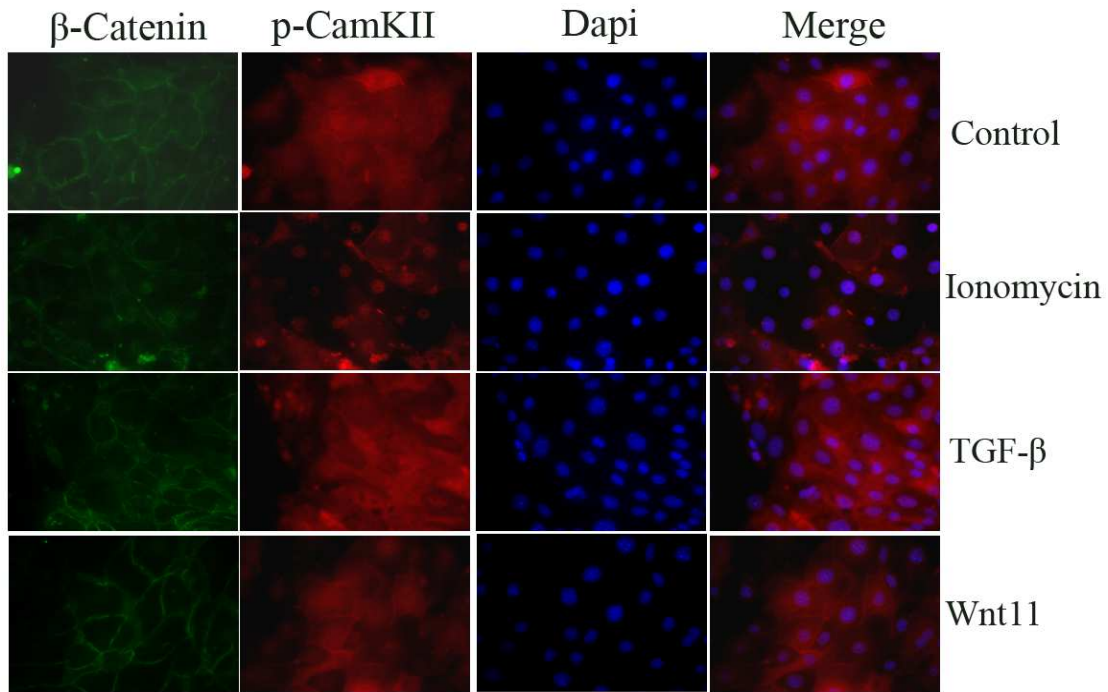


Figure 2-8. **Neither TGF- β nor Wnt11 induced translocation of p-CamKII in PRECs.** Immunofluorescence for p-CamKII (red) and β -Catenin (green) shows that treatment of TGF- β or Wnt11 for 24 hours does not induce the translocation of p-CamKII into nucleus. Ionomycin administration for 1 hour is utilized as the positive control for the activation of CamKII. β -Catenin was stained to define the cell boundary.

Table 2-1. Select genes regulated by TGF- β in renal epithelial cells

<u>Gene</u>	<u>Fold Change*</u>	<u>TGF</u>	<u>S.D.</u>	<u>Control</u>	<u>S.D.</u>	<u>Gene</u>	<u>Fold Change*</u>	<u>TGF</u>	<u>S.D.</u>	<u>Control</u>	<u>S.D.</u>
Growth factors						Chromatin					
Fgf18	2.51	8.03	0.09	5.56	0.09	Dnmt3a	1.51	8.21	0.06	6.71	0.04
Pdgfa	1.72	10.78	0.08	8.99	0.29	Smardc3	1.34	7.66	0.07	6.32	0.23
Vegfa	1.6	10.85	0.08	9.21	0.10	Jmjd4	1.05	8.27	0.10	7.18	0.07
Pdgfb	1.29	11.10	0.22	9.76	0.34	Kinases					
Ngf	1.17	12.20	0.04	11.03	0.11	Sphk1	1.76	8.75	0.04	6.96	0.23
Transcription						Itpkb	1.37	9.49	0.07	8.09	0.13
Ebf2	2.41	7.30	0.20	4.95	0.31	Map2k3	1.06	11.29	0.14	10.20	0.24
Foxq1	2.34	7.42	0.09	5.02	0.34	Pip5k1a	-1.13	9.84	0.22	11.06	0.10
Tle1	1.6	7.94	0.05	6.36	0.06	Rapgef2	-1.08	10.59	0.08	11.72	0.09
Sox7	1.57	6.68	0.14	5.05	0.11	Cytokines					
Hey1	1.53	8.11	0.02	6.60	0.12	Il11	2.68	12.11	0.04	9.41	0.13
Hoxa11	1.51	6.99	0.05	5.49	0.22	Tnfrsf11b	2.46	9.34	0.11	6.82	0.60
Sox10	1.29	8.05	0.21	6.63	0.16	Soes3	2.26	10.30	0.17	8.03	0.20
Mkx	1.18	6.27	0.05	5.07	0.28	Cxcr4	1.68	5.99	0.07	4.34	0.16
Ppargc1b	1.17	8.10	0.06	6.92	0.03	Cd40	1.55	11.14	0.01	9.56	0.17
Hoxa7	1.08	10.11	0.03	9.01	0.09	Cxcl11	1.45	8.03	0.09	6.63	0.15
Sox17	-2.27	4.53	0.15	6.88	0.45	Il6ra	1.41	7.39	0.34	6.15	0.13
Wnt Signaling						Csfl	1.16	10.73	0.06	9.56	0.24
Lrp8	1.95	8.28	0.10	6.35	0.11	Tnfrsf1b	1.09	7.50	0.07	6.44	0.12
Wnt9a	1.83	12.25	0.03	10.43	0.13	Adhesion					
Wnt11	1.82	8.95	0.22	7.00	0.25	Ankrd44	1.85	10.20	0.03	8.33	0.02
Wisp1	1.32	11.63	0.01	10.28	0.19	Dok1	1.59	10.63	0.07	9.04	0.12
Wnt10a	1.22	10.62	0.03	9.38	0.11	Plekhh1	1.47	8.84	0.06	7.37	0.05
Wnt1	1.04	5.73	0.33	4.72	0.12	Adams16	1.43	6.97	0.18	5.63	0.03
Actin Dynamics						Rhof	1.43	8.87	0.05	7.45	0.10
Ankrd34b	3.63	7.56	0.19	3.94	0.26	Mmp2	1.37	7.13	0.10	5.71	0.20
Whrn	3.59	8.39	0.03	4.81	0.12	Snai3	1.25	8.27	0.10	7.08	0.01
Bnc1	3.06	8.87	0.10	5.80	0.17	Pcdh7	1.25	9.36	0.03	8.08	0.11
Efna1	1.51	9.48	0.09	7.99	0.18	Snai1	1.05	6.55	0.11	5.46	0.16
Ephb2	1.39	9.72	0.08	8.27	0.10	Vcam1	-1.13	8.04	0.11	9.21	0.15
Sema4c	1	6.24	0.02	5.22	0.22	Rnd1	-1.16	8.46	0.14	9.66	0.07
Slit2	-1.1	4.12	0.26	5.37	0.09						
Epha4	-1.22	4.22	0.30	5.22	0.19						
Tgf-β Family											
Nog	1.28	8.06	0.21	6.70	0.10						
Acvr1	1.21	10.17	0.04	8.94	0.18						
Bmp4	1	7.03	0.04	6.04	0.09						
Id2	-1.17	6.58	0.28	7.79	0.17						

* log2 scale.

Table 2-2. Wnt associated genes regulated by TGF- β in renal epithelial cells

Symbol	t-statistic	p-value	Fold Change*	CHX**+tgf 1	CHX+tgf 2	CHX+tgf 3	CHX 1	CHX 2	CHX 3
ligands									
Wnt1	7.17	0	1.04	5.96978	5.35596	5.86236	4.78411	4.7823	4.57939
Wnt9a	18.9	0	1.83	12.2283	12.2435	12.286	10.2989	10.4309	10.5492
Wnt11	12.95	0	1.82	9.18986	8.88873	8.77053	7.01167	7.23811	6.7428
transcriptional factors									
Nfatc2	24.26	0	2.38	10.7068	10.7878	10.7703	8.21197	8.44523	8.44996
Tle1	17.47	0	1.6	7.92575	7.89095	7.99194	6.31082	6.34465	6.41562
Nfatc3	10.58	0	1.07	7.31412	7.70672	7.57189	6.52037	6.53303	6.46613
Sox17	-12.2	0	-2.27	4.39058	4.69217	4.49479	7.24982	6.38376	7.00049
secreted proteins									
Wisp1	12.3	0	1.32	11.6179	11.632	11.6419	10.0624	10.4308	10.3462
cyclin proteins									
Ccnd2	-11.25	0	-1.05	8.12537	8.19922	8.31058	9.34725	9.29905	9.32236
* log ₂ scale									
**CHX, cycloheximide									

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Chapter III

Attenuated upregulation of mesenchymal marker genes in unilateral ureteral obstruction (UUO) mouse model with kielin/chordin-like protein (KCP) overexpression

Abstract

Renal interstitial fibrosis is a common pathology in most chronic and progressive kidney diseases. Two main branches of TGF- β superfamily are the direct mediators of fibrosis. TGF- β promoted renal fibrosis, while BMP7 is a protector and counteracts TGF- β s. Recently, our lab discovered a novel cysteine-rich secreted protein, kielin/chordin-like protein (KCP), which is an enhancer for BMP signaling, as well as an inhibitor for TGF- β signaling. Here, in the unilateral ureteral obstruction (UUO) mouse model, we showed that specific overexpression of KCP in renal epithelia significantly attenuated the upregulation of mesenchymal marker genes in the injured kidney. Our study demonstrated the importance of the balance of TGF- β and BMP signaling in the progression of renal fibrosis and provided a new potential therapeutic target for its treatment by the application of KCP.

Introduction

In the United States, approximately 13% of the adult population suffers some degree of chronic kidney disease (CKD) whose prevalence continues to increase (Coresh et al., 2007). Clinically, chronic kidney disease is determined by persistent albuminuria

and decreased estimated glomerula filtration rate (GFR). Pathologically, the CKD, especially at its end stage, is usually accompanied by severe renal interstitial fibrosis (Liu, 2010). Inflammation, such as the infiltration of macrophages to the injured kidney, plays a crucial role in the initiation of renal fibrogenesis (Ricardo et al., 2008), which is followed by the proliferation and activation of fibroblast and enhanced deposition of extracellular matrix (ECM). The increasing number of fibroblasts, myofibroblasts and excess ECM replace the kidney parenchyma, destroy the normal renal tubular architecture, and eventually lead to the end stage renal disease (Liu, 2011).

Among the most well studied signaling pathways in renal fibrotic disease are those of the transforming growth factor β (TGF- β) superfamily, the most relevant of which are the TGF- β s and BMPs. As we discussed in the Chapter I and II, the general signaling transduction pathway for TGF- β s and BMPs is similar (Massague, 1998). The binding of the ligand to its type II receptor, leads to the recruitment and phosphorylation of type I receptor. The activated type I receptor is a serine/threonine kinase that transduces the signal through phosphorylating receptor Smad proteins (R-Smads), which form a heteromeric complex with a common partner, Smad4, translocate to the nucleus and mediate target gene expression. For TGF- β s, the R-Smads are Smad2 and 3, while for BMPs, they are Smad1, 5 and 8. A consensus exists that TGF- β s are pro-fibrogenic cytokines, and BMPs counteract the effects of TGF- β s. *In vivo* studies showed that mice genetically overexpressing TGF- β 1 (Kopp et al., 1996) or treated with recombinant TGF- β 2 (Ledbetter et al., 2000), suffered renal interstitial fibrosis and tubular atrophy over time. Conversely, the severity of renal hisopathology was reduced in experimental glomerular nephritis models by treating animals with a human antibody against rat TGF-

β type II receptor (Kasuga et al., 2001). Immunohistochemical staining revealed that both TGF- β 1 ligand and its type I receptor were robustly upregulated in proximal tubular epithelial cells of the injured kidney (Yang and Liu, 2001). *In vitro*, BMP7 reversed the TGF- β 1 induced epithelial mesenchymal transition. This was supported by the *in vivo* study showing that systemic administration of recombinant human BMP7 led to the repair of severely damaged renal tubular epithelial cells and the reversal of chronic renal injury (Zeisberg et al., 2003). Furthermore, BMP7 induced mesenchymal to epithelial transition (MET) in adult renal fibroblasts (Zeisberg et al., 2005).

In Chapter I, we have thoroughly discussed the regulation of TGF- β signaling both outside and inside of cells. Out of cells, a group of secreted factors, containing the cysteine rich (CR) domains, mediates TGF- β signaling transduction through blocking or facilitating the binding between ligands and receptors. For example, the crystal structure of the Noggin-BMP7 complex directly showed that Noggin inhibited BMP7 by blocking the surfaces that were required to interact with the type I and type II BMP receptors (Groppe et al., 2002). On the other hand, the secreted CR domain protein, connective tissue growth factor (CTGF), not only blocked BMP signaling, but also enhanced TGF- β 1 signaling through direct interaction with TGF- β 1 ligands (Abreu et al., 2002).

Recently, our lab identified kielin/chordin-like protein 1 (KCP1) as a secreted protein with 18 cysteine-rich domains that is expressed in the developing kidney at both early and late stages (Lin et al., 2005). Unlike Noggin or CTGF, KCP1 enhances BMP signaling and inhibited TGF- β 1 signaling (Lin et al., 2005; Lin et al., 2006). The KCP homozygous mutant mice showed no gross developmental abnormalities, but exhibited

enhanced susceptibility to developing renal interstitial fibrosis in both UUO chronic kidney disease model and folic acid acute renal injury model (Lin et al., 2005).

In this chapter, we tried to address whether overexpression of the secreted KCP protein in transgenic mice will alter the balance between the TGF- β and BMP signaling pathway and change the progress of renal fibrosis. Transgenic mice were engineered to express KCP protein specifically in renal proximal tubule cells and subjected to unilateral ureteral obstruction. While KCP expression by itself had few measurable deleterious effects, KCP transgenic mice showed decreased upregulation of mesenchymal genes in injured kidney over time, suggesting a more resistance to interstitial fibrosis. Our studies clearly pointed to the renal protective functions for KCP, and provide a potential target for the treatment of renal fibrosis.

Materials and Methods

Animals

Mice were kept according to NIH guidelines. Animal use was approved by the University Committee on Use and Care of Animals at the University of Michigan. The KCP mice were generated by Dr. Dressler. Pepck promoter was used to drive a myc-epitope tagged form of the KCP protein that also included a human Igk light chain signal peptide to enhance secretion.

For the induction of renal fibrosis, the UUO model was utilized. Mice were anesthetized with intraperitoneal injection of ketamine and xylozine. Through a midline abdominal incision, the right ureter was exposed and tied off at the mid-ureteral level with fine suture materials (4-0 silk) to induce a complete obstruction. Mice were allowed

to recover from anesthesia and were kept with *ad libitum* supply of food and water until the indicated time of sacrifice (0, 7, 14 and 28 days). Both obstructed and contralateral kidneys were harvested for RNA and protein. The UUO model was generated by Abdul Soofi.

RNA extraction

The kidney tissue was added into TRizol reagent (Invitrogen) and homogenized with Polytron Homogenizer. The mixture was centrifuged and supernatant was collected for further isolation following the manufacturer's instructions.

RNA reverse-transcription and real-time PCR

2-3 µg total RNA was reverse-transcribed into complementary DNA with SuperScript First-Strand Kit (Invitrogen). The cDNA products were diluted 5 times and amplified with the iTaq Sybr green master mix (Bio-Rad) in a Prism 7500 (Applied Biosystems). Primers pairs for PCR are as follows: Wnt11 5'-GGGCCAAGTTTTCCGATGCT, 5'-TTCGTGGCTGACAGGTAGCG; ZEB1 5'-TCAAGTACAAACACCACCTG, 5'-TGGCGAGGAACACTGAGA; PAI1 5'-ACATGTTTAGTGCAACCCTG, 5'-GGTCTATAACCATCTCCGTG; Snai1 5'-GGAAGCCCAACTATAGCGA, 5'-AGCGAGGTCAGCTCTACG; GAPDH 5'-ACCACAGTCCATGCCATCAC, 5'-TCCACCACCCTGTTGCTGTA.

Results

Generation of KCP transgenic mice

Since the Pepck promoter was reported to be very active in renal proximal tubular epithelial cells (Short et al., 1992), it was used to drive the expression of transgenic KCP gene (Fig. 3-1A). The transgenic KCP protein was myc-epitope tagged and included a human IgK light chain signal peptide to enhance its secretion. Founder animals were mated to wild-types and subsequent F1 generations genotyped for the transgene. The expression of transgenic KCP varied among littermates, but immunohistochemistry results showed that the exogenous KCP protein were most located in proximal tubules (Fig. 3-1B and C). The strongest expressing strain was used for renal injury studies.

KCP overexpression attenuated the upregulation of mesenchymal genes in UUO model

In UUO disease model, the normal renal tubular architecture was destroyed and replaced by increasing number of fibroblasts and myofibroblasts. Thus an upregulation of mesenchymal marker genes, such as *Pai1*, *Snail1* and *Zeb1*, in the injured kidney was observed. Furthermore, the expression of these mesenchymal markers increased over time, indicating the progress of the injury. In KCP transgenic mice, the basal expression of mesenchymal genes were not much affected. However their upregulation trends, along with the disease progression, were significantly attenuated (Fig. 3-2B). The effect of KCP on mesenchymal gene expression was through enhancing BMP signaling and inhibiting TGF- β signaling, as indicated by the more phosphorylated Smad1, 5 and 8 proteins and less phosphorylated Smad2 and 3 in transgenic mice (Fig. 3-2A). In Chapter II, we demonstrated that *Wnt11* was a direct target of TGF- β signaling. Here, we found that

Wnt11 expression was also reduced by KCP overexpression, further demonstrating the inhibitory effect of KCP on TGF- β signaling (Fig. 3-2B). These taken together, our data suggested that KCP attenuated mesenchymal gene upregulation in injured kidney through enhancing BMP signaling and blocking TGF- β signaling.

Discussion

Although BMPs and TGF- β s belong to the same superfamily, their functions in renal fibrosis are very different. While TGF- β induced EMT *in vitro*, and correlated with increased fibroblast proliferation and ECM deposition to promote fibrosis *in vivo* (Iwano et al., 2002; Poncelet and Schnaper, 2001; Strutz et al., 2001), BMP7 mediated MET *in vitro*, and suppressed inflammation and improved renal recovery *in vivo* (Hruska et al., 2000; Vukicevic et al., 1998; Zeisberg et al., 2005). Over the years, drugs have been designed to repress TGF- β signaling or enhance BMP7 signaling for the treatment of chronic kidney diseases. Our study suggested that KCP may be a better candidate, because it can modulate TGF- β and BMP7 at the same time. Furthermore, KCP was not widely expressed in adult tissues (Lin et al., 2005), suggesting that drug targeting at KCP might cause less side effects.

Besides the upregulation of mesenchymal marker genes, histology and immunostaining showed less Collagen IV deposition and α SMA expression in KCP mice compared to wildtype control at 7 and 14 days after UUO (data not shown). These further demonstrated the protective role of KCP. The quantitative PCR was based on the whole renal tissue, so we did not know whether the attenuated mesenchymal gene expression was due to less proliferation of fibroblasts or reduced EMT effect. However, since the

transgenic KCP was overexpressed specifically in proximal tubular epithelia, our results indicated the important role of those epithelial cells in promoting renal fibrosis either through an autocrine or paracrine manner.

In the normal adult kidney, the BMP7 signaling is active, but its function is not well characterized. The main target cells for the BMP7 signaling in the kidney are epithelial cells, especially proximal tubular epithelial cells (Bosukonda et al., 2000). The function of BMP7 signaling in those epithelial cells may be to maintain the expression of E-cadherin (Zeisberg et al., 2005). It has been reported that BMP signaling was also involved in maintaining the pluripotency of stem cells (Varga and Wrana, 2005). During renal development, not all metanephric mesenchyme cells aggregate and become epithelia. Some cells maintain their mesenchymal properties and remain as interstitial stromal cells (Dressler, 2006). Thus, BMP7 signaling may be important for the survival of these interstitial stromal cells. During renal fibrosis, the decreasing BMP7 signaling may contribute to the differentiation of these stromal cells into fibroblasts. Meanwhile, the destruction of the stem cell pool may also reduce the recovery ability of the kidney from acute renal diseases. This may be one of the reasons why rats receiving BMP7 injection had better recovery from ischemic acute renal failure (Vukicevic et al., 1998).

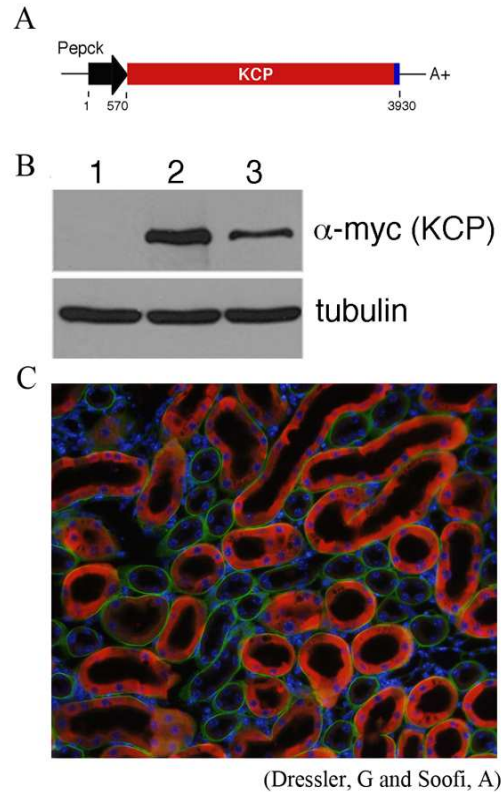


Figure 3-1. **Expression of KCP in the transgenic mice.** A) Schematic shows the expression of transgenic KCP is under the control of pepck promoter. B) Western blot for myc from wildtype (#1) or transgenic kidneys (#2 and #3) indicates the transgenic expression of KCP proteins. C) Immunofluorescence for myc (red) and laminin (green) shows that exogenous KCP is specifically expressed in proximal tubular epithelia. Laminin is used to define the boundary of renal tubules.

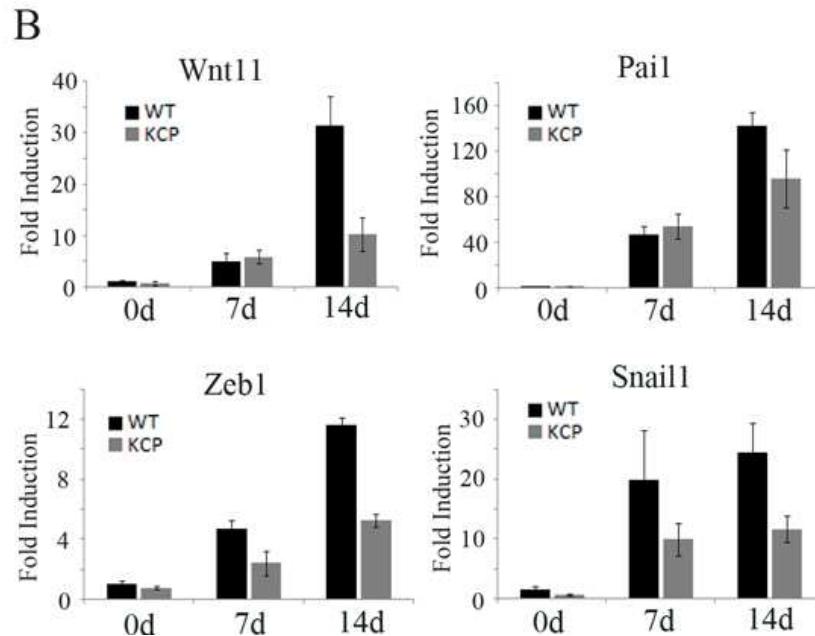
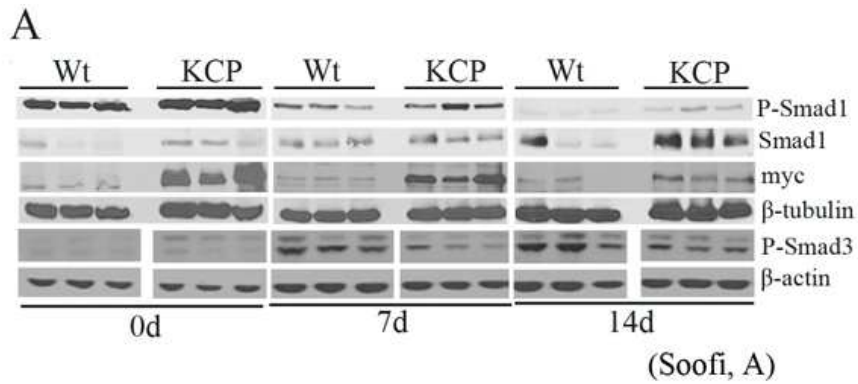


Figure 3-2. KCP overexpression attenuated upregulation of mesenchymal genes in injured kidney. A) Western blot for P-Smad1, P-Smad3 and myc from three independent normal, 7-day or 14-day UUO kidneys of wildtype or KCP transgenic mice. The expression of KCP decreased with the progression of the disease. The amount of P-Smad1 was higher in KCP mice than the wildtype, while the amount of P-Smad3 was lower. β -tubulin was the loading control for P-Smad1, Smad1 and myc. β -actin was the loading control for P-Smad3. B) Gene expression levels were assayed by qRT-PCR for the indicated genes in control (n=3), 7-day (n=3), and 14-day UUO kidney (n=3) RNA isolates. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

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Chapter IV

Tle4 enhances BMP7 mediated gene expression

Abstract

Groucho proteins and their mammalian homologues, Transducin-Like Enhancer of split (Tle) proteins are common corepressors, and are critical for normal development processes. Bone morphogenetic protein 7(BMP7) signaling belongs to transforming growth factor- β (TGF- β) superfamily and plays an important role in controlling kidney development. However, the regulation of BMP7 signaling, especially within cells, is largely unknown. Here, our results showed that overexpression of Tle4 robustly activated the expression of a BMP reporter, as well as enhancing and sustaining the upregulation of endogenous Id1 gene induced by BMP7. BMP7 administration did not affect the endogenous level of Tle4. Tle4 activated the BMP reporter through mediating Smad proteins, as Tle4 repressed the expression of Smad7, an inhibitory Smad protein, and overexpression of Smad7 totally abolished the effect of Tle4 on the induction of the BMP reporter. Our study provided a new mechanism for the regulation of BMP signaling, which may be important for the kidney and neural development, since Tle4 and BMP7 are co-expressed in these developing tissues.

Introduction

The human genome is carried by 23 pairs of chromosomes, containing 20,000-25,000 protein-coding genes (2004). The proper expression of these genes is critical for normal cellular physiological processes, such as proliferation, differentiation and death, thus requiring elegant and tight regulation. Two large families of proteins control gene

expression, the activators and the repressors. As their name suggested, activators help the recruitment of RNA polymerase II to the gene promoter and increase target gene expression, while repressors condense the chromatin structure, exclude activators and transcription machinery, and reduce or even silence gene expression.

Groucho proteins and their evolutionary conserved mammalian Transducin-Like Enhancer of split (Tle) homologues were the first identified metazoan corepressors (Cinnamon and Paroush, 2008). Their structure encompasses five domains, the Trp-Asp-repeat (WDR) domain at the N-terminal, followed by Ser-Pro-rich (SP) domain, a CcN domain, and Gly-Pro-rich (GP) domain, with a Gln-rich (Q) domain at the C-terminal (Buscarlet and Stifani, 2007). The WDR and Q domain are highly conserved and essential for the interaction with other DNA-binding proteins to mediate gene repression (Fisher and Caudy, 1998; Jennings et al., 2006). The SP domain could be phosphorylated by MAPK, which negatively regulates Gro/Tle repression ability (Hasson et al., 2005). The CcN domain has the nuclear localization signals (Buscarlet and Stifani, 2007). The Gro/Tle family represses gene expression through multiple mechanisms. First, it interacts with TFIIE or other transcriptional factors to prevent the assembling of transcription machinery or activator complexes (Buscarlet and Stifani, 2007). Second, Grg3 bound to nucleosomal arrays to promote condensation into higher-order chromatin to block the access of other transcriptional factors (Sekiya and Zaret, 2007). Third, they recruit histone deacetylase or other histone modification complexes to repress target gene expression (Patel et al., 2012; Yochum and Ayer, 2001). Through a series of knockdown and overexpression experiments, Gro/Tle proteins play important roles in embryogenesis,

body patterning and organogenesis (Dasen et al., 2001; Wang et al., 2004; Zamparini et al., 2006).

Bone morphogenetic protein (BMP) belongs to the transforming growth factor (TGF)- β superfamily (Massague, 1998; Patel and Dressler, 2005). In mammals, the binding of BMP ligands to their receptor, BMP type II receptor, leads to the recruitment and phosphorylation of BMP type I receptor (BMPRI). The activated BMPRI is a serine/threonine kinase that transduces the signal through phosphorylating receptor-activated Smad1, 5 and 8. Phosphorylated Smad1, 5 and 8 form a heteromeric complex with a common partner, Smad4, and translocate to the nucleus to regulate target gene expression. The inhibitory Smads (I-Smads, Smad6 and 7) shared a common sequence with R-Smads and competed with them to bind to type I receptor or Smad4, thus blocking signaling transduction.

BMP7 was known to be critical for the normal kidney development. It has been reported that the BMP7 null mice died shortly after birth because of severe renal dysfunction (Luo et al., 1995). *In vitro*, BMP7 promoted the survival of metanephric mesenchymal cells, as well as their differentiation to the diverse epithelial cells types of the nephron (Dressler, 2006; Dudley et al., 1999; Vukicevic et al., 1996). This was consistent with the *in vivo* studies, showing that BMP7 null mice had much fewer glomeruli and nephrons than the wildtype mice. (Dudley et al., 1995; Luo et al., 1995). Besides these genetic data, the BMP type I receptors, as well as their responsive Smads (Smad1, 5 and 8) were expressed in the nephrogenic zone (Martinez et al., 2001; Vrljicak et al., 2004).

Although BMP signaling is important, its regulation during normal kidney development is largely unknown. The best studied mediator is Gremlin1, an extracellular antagonist for BMP signaling, which inhibited the local BMP signaling and facilitating ureteric bud outgrowth and branching (Michos et al., 2007). In addition, the I-Smads were also detected in mesenchymal cells in the nephrogenic zone and at ureteric bud tips (Vrljicak et al., 2004). Recently, we found that Tle4 was expressed in s-shaped bodies, a structure derived from metanephric mesenchyme, as well as periphery mesenchyme (Cai et al., 2003). Tle4 has been reported to participate in the regulation of several signaling pathways, such as Wnt, Notch and EGF signaling (Cinnamon and Paroush, 2008). However, the reports of its involvement in TGF- β signaling are limited. The only data is from *Drosophila*, showing that brinker, a downstream effector of the decapentaplegic (dpp)/TGF- β signaling, recruited groucho and CtBP to suppress specific target genes (Hasson et al., 2001). Recently, Sekiya, T *et al* showed that the recruitment of Grg3 to chromatin created a closed, poorly accessible domain spanning three to four nucleosomes (Sekiya and Zaret, 2007). This long range repression model may be also involved in the regulation of signaling pathways and help explain the various target gene profiles of a certain signaling pathway in different cell types. In this chapter, we reported that, instead of repressing the effects of BMP7 on its reporter vector, Tle4 overexpression strongly activated BMP7 reporter. Similarly, Tle4 also enhanced and sustained the activation of endogenous Id1 gene induced by BMP7. The effects of Tle4 on BMP7 signaling was not through modulating the phosphorylation status of Smad1, 5 and 8, but repressing Smad7 expression. Our study, for the first time, showed the function of Tle4 in BMP signaling.

Materials and Methods

Reporter Molecular Construction

The forward and reverse strands of BRE fragment with BamHI site at the 5'-end were synthesized by Invitrogen. 10 μ M of each strand was added to 5X ligase buffer (Invitrogen), heated to 95°C for 1 min and then cooled down to room temperature for annealing. pRS4-EGFP reporter was digested by BamHI (NEB) for 3 hours and purified by QIAquick gel extraction kit (Qiagen). The BRE fragment was ligated with pRS4-EGFP reporter with the ligation kit (Invitrogen) at 16°C overnight. The ligation product was transformed into competent DH5 α E.coli (Invitrogen) followed the manufacture instruction and cultured on LB (EMD) plates containing 50 μ g/mL ampicillin (Roche). Plasmid DNA was mini-prepared with QIAprep Spin MiniPrep Kit (Qiagen) and examined by PstI digestion (NEB). The positive clones were sent to UM sequencing core to check the insertion orientation. To cut off the Pax2 binding sites from Pax2 and BMP double reporter vector, the vector was digested by HindIII and EcoRV (NEB) overnight, blunted by DNA polymerase I, Large (klenow) Fragment (NEB) and re-ligated with the ligation kit. BRE fragment sense: 5'-GATCCGCGGCGCCAGCCTGACAGCCCGT CCTGGCGTCTAACGGTCTGAGCTAGCG-3'; reverse: 5'-GATCCGCTAGCTCA GACCGTTAGACGCCAGGACGGGCTGTCAGGCTGGCGCCGCG-3'.

Cell Culture

293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 10% Fetal Bovine Serum (FBS), and Penicillin Streptomycin (PS, Gibco).

Immortalized renal epithelial cells (TKPTS) were a kind gift from Dr. Bello-Reuss. Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco) with 2% FBS, 1X Insulin-Transferring-Ethanolamine-Selenium (ITES, Lonza) and PS. UltraMDCK serum free medium (Lonza) was used when serum starvation was necessary.

To test the effect of Pax2, Tle4 or BMP7 on reporter vectors, 293 cells were culture on 6 well plates with low serum medium (LSM, DMEM+0.5% FBS+1X Insulin-Transferring-Selenium (ITS, Gibco)) and transfected with 0.5 μ g reporter vectors and 0.5~1 μ g Pax2, Tle4 expressing vector or SHS (sonicated herring sperm) DNA as control, using Fugene6 (Roche). Cells were harvest 48 hours after transfection for analysis. To test the effect of Smad7 on BMP reporters, cells were transfected with 0.5 μ g pRS4-BRE4+-EGFP reporter vector, 0.5 μ g Tle4 expressing vector, and 0.5 μ g Smad7 expressing vector or SHS DNA control. 100ng/mL BMP7 (R&D systems) was added 24 hour after transfection for another 24 hours. For 1 hour pulse experiment, transfected cells were treated with 100ng/mL BMP7 for 1h, and then washed with PBS once and cultured in new LSM for another 23 hours.

To collect the conditional medium, 293 cells were cultured on 100 mm dishes and transfected with 5 μ g of GFP or Tl4 expressing vector, using Fugene6. 48 hours after transfection, culture medium from each plate was collected and centrifuged at 4000 rpm for 30 min at 4°C. The supernatant was aliquot and preserved in -80°C.

Western blot analysis

Cells were directly lysed in 2X SDS buffer (4% sodium dodecyl sulfate, 20% glycerol, 0.2M dithiothreitol, 125 mM Tris, pH 6.8) and boiled at 94°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes and immunoblotted with antibodies as indicated. Rabbit anti-phosphorylated Smad1/5/8 is from Cell Signaling. Mouse anti-flag and mouse anti β -tubulin are from Sigma-Aldrich. Mouse anti-EGFP, mouse anti-Smad1 and rabbit anti-Tle4 are from Santa Cruz Biotech. Rabbit anti-Pax2 is self-made. HRP-linked secondary antibodies and ECL reagent are from GE healthcare.

RNA reverse-transcription and real-time PCR

Total RNA was extracted from 293 cells with different treatment using TRIzol RNA isolation system (Invitrogen). 2-3 μ g total RNA was reverse-transcribed into complementary DNA with SuperScript First-Strand Kit (Invitrogen). The cDNA products were diluted 5 times and amplified with the iTaq Sybr green master mix (Bio-Rad) in a Prism 7500 (Applied Biosystems). Primers pairs for PCR are as follows:

Id1 5'-CTGCCTGCCCTGCTGGAC-3', 5'-TCTCGCCGTTGAGGGTGC-3';
Tle4 5'-TACCCCTACTCCACGAAC-3', 5'-TCTCCGTTTCATTCCAGCA-3'; Smad4
5'-CACTACGAACGAGTTGTATCAC-3', 5'-CCTTCAGTGGACAACGATG-3';
Smad7 5'-ATCACCTTAGCCGACTCTG-3, 5'-CAGTAGAGCCTCCCCACTC-3';
L32 5'-CAGGGTTCGTAGAAGATTCAAGGG-3',
5'-CTGGAGGAAACATTGTGAGCGATC-3'.

Luciferase assays

293 cells were seeded on 12 well plates and cultured in LSM. BRE-luc reporter vector was transfected (1 µg/well) together with SHS or Tle4 expressing vector (1 µg/well) into the cells in triplicate. Medium containing 100ng/mL BMP7, or GFP or Tle4 conditional medium was added 24 hours after transfection and kept for another 24 hours. Cells were lysed with dual luciferase assay kit (Promega) and results were read.

shRNA mediated Gene knocking-down

Packed Smad4 37196 or 37199 shRNA lentivirus was used to knockdown in PRECs. Cells were seeded on 6 well plates for 24 hours. Lentivirus was added with 8 µg/mL polybrene and kept overnight. Puromycin was added and kept for consistent selection. For the BMP reporter test in Smad4 knockdown cells, cells were seeded on 12 well plate and cultured for 24 hours. 1.5 µg of DNA, containing 0.5 µg of pRS4-BRE4+-EGFP reporter and 1µg of Tle4 expressing vector or SHS DNA control was transfected using Fugene6. 48 hours later, cells were lysed in 2XSDS loading buffer and analyzed by western blotting.

Results

Molecular Construction of Pax2 and BMP7 double reporter vector

Considering the long range repression effect of Gro/Tle proteins, to study the function of Tle4 in BMP signaling, we decided to make a new BMP reporter system, which could recruit Tle4 near the BMP response element (BRE). Because Tle4 has no DNA binding domain, we choose to use Pax2 as the “bridge” factor, since it has been well documented that Tle4 bound to Pax2, 5 and 8 through the conserved octapeptide

(Eberhard et al., 2000; Linderson et al., 2004; Patel et al., 2012). First, we synthesized a 50 bp BRE fragment flanked with a BamHI site. It was defined from Id1 promoter, containing two Smad binding element (SBE) and GC rich region (Korchynskiy and ten Dijke, 2002) (Fig. 4-1A). Then, we inserted this DNA fragment into a Pax2 reporter vector (Patel et al., 2007) at the BamHI site between the Pax2 binding sites and TK promoter. Because the molecular construction was based on a single restriction enzyme, multiple BRE fragments could be inserted into one vector at different orientations (Fig. 4-1B). Here, for convenience, we defined the vector as “+”, when SBE is upstream of GC rich region, and “-” when SBE is downstream of GC rich region. Usually, we got the double reporter vectors with 4 or 6 copies of BRE fragments. No matter how many copies the reporter vectors had, they all responded to BMP7 treatment or Pax2 overexpression when transiently transfected into 293 cells (Fig. 4-1C, D).

Tle4 activated transiently transfected BMP7 reporter independent of Pax2

As previous studies stated, Tle4 abolished the transactivation ability of Pax2 through inhibiting its phosphorylation by JNK signaling and recruiting other corepressors (Cai et al., 2003; Hasson et al., 2001; Patel et al., 2012; Yao et al., 2001). However, to our surprise, Pax2 and Tle4 co-transfection or Tle4 transfection alone strongly activated the Pax2 and BMP7 double reporter (Fig. 4-2A). Since Tle4 alone could activate the reporter, we doubted whether Pax2 binding sites were necessary. Deletion of the Pax2 binding sites from either EGFP or firefly luciferase reporter did not affect its activation by Tle4 (Fig. 4-2B, C). Furthermore, the condition medium collected from Tle4 transfected cells failed to activate the BMP7 reporter, suggesting that this activation was not in a paracrine manner. Both the basal EGFP expression level and the strength of its

activation by Tle4 were dependent on the copy numbers of BRE fragments and the amount of Tle4 proteins, but regardless of the orientation of the inserted BRE fragments. The Tle4 dependent BMP7 reporter activation could be achieved in both human derived 293 cells and mouse derived TKPTS, indicating that it may utilize a universal mechanism (Fig. 4-2D, E).

Tle4 enhanced and sustained BMP7 mediated endogenous Id1 expression

Since the response elements of the BRE reporter vector were isolated from Id1 gene, we then asked whether Tle4 could activate endogenous Id1 gene. Unfortunately, overexpression of Tle4 only slightly increased Id1 expression (usually around 1.5 fold) (Fig. 4-3A). However, the presence of Tle4 significantly enhanced the effect of BMP7 in activating Id1 gene (Fig. 4-3B). Similarly, overexpression of Tle4 also enhanced the induction of genome integrated pRS4-BRE4+-EGFP reporter in at least 2 independent clones upon BMP7 treatment (Fig. 4-3C). It has been shown that BMP7 signaling was required for sustained Id1 mRNA expression in pulmonary artery smooth muscle cells (Yu et al., 2008a). Next we checked whether Tle4 was able to prolong Id1 upregulation induced by BMP7 in 293 cells. After cells were treated with BMP7 for 1 hour and then cultured with new fresh medium for another 24 hours, the expression of Id1 returned to the normal level. However, in the presence of Tle4, the expression level of Id1 remained at the activated state for another 24 hours, after the 1h exposure of BMP7 (Fig. 4-3D). Taken these together, although Tle4 alone did not alter Id1 expression much, it enhanced and sustained BMP7 induced endogenous Id1 expression.

BMP7 did not affect endogenous Tle4 expression

Considering the robust induction of Id1 expression by BMP7, we then asked whether Tle4 is required for the BMP7 signaling. To our surprised, the endogenous Tle4 protein level was low in 293 cells. Although, BMP7 may slightly increase the transcription of Tle4 over time, there was no detectable increasing of Tle4 protein upon BMP7 treatment (Fig. 4-4A, B). So, these data suggested that BMP7 might not require high level of Tle4 to induce target gene expression in these cells.

Tle4 activate BMP reporter vector through mediating Smad proteins

Next, we addressed the potential mechanisms utilized by Tle4 proteins to upregulate BMP reporter vector. Since the Smad proteins are the main mediators for BMP signaling, we first check whether Tle4 affect the phosphorylation of R-Smads. From Figure 4-5A, we could see that overexpression of Tle4 neither induced the phosphorylation of Smad1, 5 and 8, nor increased their response to BMP7 signaling. In the BMP7 one hour pulse experiment, Tle4 also failed to maintain the phosphorylated status of Smad1, 5 and 8 after BMP7 ligands were withdrawn. However, in these experiments, we found that there existed a basal activity of BMP signaling, indicated by consistent low level of phosphorylated Smad1, 5 and 8. Considering the general function of Gro/Tle family as a corepressor, we doubted whether overexpression of Tle4 blocked the expression of BMP inhibitor(s). Thus Tle4 mediated activation of BMP reporter may be due to increase the efficiency of basal active Smads on the reporter. Because R-Smad proteins usually interacted with Smad4 to regulate gene expression (Massague, 1998), we used shRNA to knockdown the expression of Smad4. Two different shRNA lentivirus, 37196 and 37199 knocked Smad4 down by 70% separately (Fig 4-5B). In Smd4 knockdown cells, the response of BMP reporter to Tle4 was reduced, although it was still

strong. Also, Smad4 knockdown slightly reduced the basal level of phosphorylated Smad1, 5 and 8, but the total Smad1 amount was not affected (Fig 4-5C). Thus, the Smad4 activity was at least partially responsible for the Tle4 mediated BMP reporter activation. Smad7 was a common inhibitor for TGF- β and BMP signaling (Hayashi et al., 1997; Nakao et al., 1997). We found that Tle4 inhibited endogenous Smad7 expression (Fig. 4-5D). More importantly, overexpression of Smad7 reduced the basal expression level of BMP reporter vector and totally abolished the activation effect of Tle4 (Fig. 4-5E). Taken this together, we concluded that Tle4 did not affect phosphorylation of Smad1, 5 and 8, but activated the BMP reporter vector through enhancing basal BMP signaling pathway by inhibiting Smad7 expression.

Discussion

BMP signaling pathway is important during normal development and diseases. Gro/Tle family proteins are common corepressors, which are involved in various signaling pathways. For example, Tle proteins competed with β -catenin to interact with Tcf/Lef, thus interfering canonical Wnt signaling (Daniels and Weis, 2005). However, the functions of Gro/Tle proteins in TGF- β signaling are largely unknown. Up to now, it was only reported that Dpp, the TGF- β homolog in *Drosophila*, induced the expression of Brinker, which recruited Groucho and CtBP to repress other Dpp target genes, thus confining the function zone of Dpp signaling (Hasson et al., 2001; Zhang et al., 2001). In this chapter, we discuss the potential function of Tle4 to enhance BMP signaling by suppressing Smad7 expression. However, some major points are still worth discussing further.

First, in our Smad4 knockdown experiment, the endogenous Smad4 expression was knocked down by 70%, but Tle4 still strongly activated the BMP reporter, even though its effect was reduced. Also, there still was a detectable basal level of phosphorylated Smad1, 5 and 8. Since previous studies showed that Smad4 was dispensable for TGF- β signaling (Descargues et al., 2008; He et al., 2006), to further address the question whether Tle4 mediated induction of BMP reporter was through enhancing the basal BMP activity, two more experiments are needed: (1) checking the effect of Tle4 on the BMP reporter in the presence of BMP inhibitors, such as Noggin, a secreted protein preventing BMP ligand-receptor interaction, or dorsomorphin, a small molecule blocking BMP receptors (Yu et al., 2008b); (2) check the effect of Tle4 on modified BMP reporter without Smad binding element.

Second, Smad7 inhibits BMP signaling through interfering the binding of R-Smads to Smad4 or type I receptor (Hayashi et al., 1997). Our data suggested that Tle4 activated BMP reporter by inhibiting Smad7 expression. More experiments are required to support this idea. First, a workable Smad7 antibody is needed to show the decreased protein level of Smad7 upon Tle4 overexpression. Then, we could check whether Smad7 knockdown by lentivirus would mimic Tle4 overexpression to activate BMP reporter. Since Tle4 robustly activated the BMP reporter and Smad7 strongly inhibited this effect, if Tle4 overexpression does not greatly reduce Smad7 at protein level, a second blocking point may exist. Tle4 may prevent Smad7 from interfering with the complex formation between R-Smads and Smad4. Thus, a co-immunoprecipitation (co-IP) experiment is needed to check the interaction between R-Smads and Smad4 in the presence of Tle4 or not. Furthermore, the balance between acetylation and ubiquitination was important to

control the stability of Smad7 (Gronroos et al., 2002; Simonsson et al., 2005). Since Tle4 was able to interact histone deacetylase 1(HDAC1) (Choi et al., 1999), it may also influence Smad7 degradation .

Third, although Tle4 strongly activated BMP reporter, its effect on the expression of endogenous Id1 gene was limited. This results from the different properties between transiently transfected vector and endogenous genes: (1) the regulation for reporter vectors was much simpler than real genes. In fact, besides Smad proteins, Id1 promoter contains binding sites for several other transcriptional factors, such as YY1, Sp1 and ATF3 (Kang et al., 2003; Korchynskiy and ten Dijke, 2002). It is hard to imagine that Tle4 could control all of these factors at the same time on its own; (2) the expression of endogenous genes is usually affected by the chromatin structure, while transiently transfected vectors are more accessible to activators. This idea was supported by the stable transfected cell line with BMP reporters, whose response to Tle4 overexpression is more like endogenous Id1 genes than transiently transfected reporters. Two potential mechanisms may lead to the Tle4 mediated enhanced or sustained expression of endogenous Id1 induced by BMP7. First, just like the situation of BMP reporter vector, the effect of Tle4 on Id1 gene is through repressing Smad7, thus the enhanced and sustained Id1 expression is dependent on R-Smads. To demonstrate this idea, an experiment to check the Tle4 effect on BMP7 induced Id1 gene expression in the presence of Smad7 overexpression is necessary. We have already tried to check the combination effect of Tle4 overexpression and BMP7 administration on Smad7 expression. However, the results were not consistent among repeats. This may result from the technique problem. Since we cannot guarantee the transfection efficiency is 100%,

the Smad7 expression change we saw in RT-PCR may be the net effect of BMP7 mediated upregulation in some of cells and Tle4 mediated downregulation in other cells. The variants of transfection efficiency over repeats may directly influence the results. So, the plan to overexpress Smad7 should be a better choice, because it is easier to guarantee the co-transfection of Smad7 and Tle4. Also, a chromatin immunoprecipitation (ChIP) assay with Smad4 or R-Smads at Id1 promoter will provide more evidence to prove whether the enhanced and sustained Id1 expression is dependent on R-Smads. Although, it is widely believed that Gro/Tle family proteins are corepressors, a recent study showed that Tle3 was also present at the promoters of activated genes (Villanueva et al., 2011). So it is possible that Tle4 may function as an adaptor and also directly mediate Id1 gene expression. Because Tle4 itself has no DNA binding domain, it requires other transcriptional factors and a more accessible chromatin environment, this can explain why Tle4 alone only slightly increased Id1 expression. To demonstrate this idea, a ChIP assay with Tle4 at Id1 promoter is necessary.

Finally, because the endogenous Tle4 level is low in 293 cells, it may not be a good tool to address the biological importance of Tle4 in BMP signaling. The metanephric mesenchymal cells and its derived epithelia in s-shape body have relative high Tle4 expression (Cai et al., 2003), they may be a better tool for further studies. We could try to isolate those cells from E15.5 embryos, knockdown endogenous Tle4 expression and test its response to BMP7 for proliferation and cell survival (Dudley et al., 1999).

In summary, studying the regulation of BMP7 signaling is critical for us to completely understand its function in renal development. The present data showed that

Tle4 play an important role in regulating BMP7 mediated Id1 expression. As shown in Figure 4-6, Tle4 may enhance BMP effects through modulating Smad7. This could be achieved by downregulating Smad7 expression, preventing it from blocking the complex formation of R-Smad and Smad4, or influencing its stability. A more aggressive hypothesis is that Tle4, instead of acting like a corepressor, can directly mediate gene activation. Although more experiments are required to prove these potential mechanisms, our work will provide a new regulatory pathway for BMP signaling.

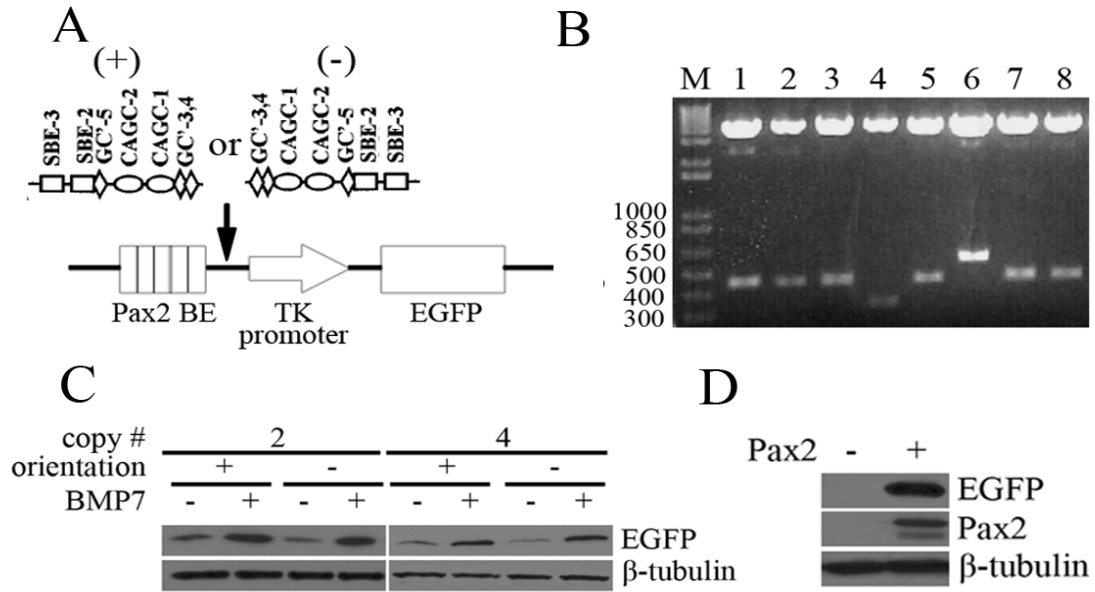


Figure 4-1. **Molecular Construction of Pax2 and BMP7 double reporter vector.** A) Schematic of molecular construction of the double reporter vector. BMP response fragment was inserted into the BamHI site between Pax2 binding element (Pax2 BE) and TK promoter. Insertion with SBE upstream of GC rich region was defined as (+), and reversed insertion was defined as (-). B) DNA agarose electrophoresis showed the restriction endonuclease digestion of double reporter vector with different insertion copies. Vectors were cut by PstI and for every insertion copy, there would be a 50 bp shift in the electrophoresis. Clone #4 was the negative control for empty vector. C) Western blots for EGFP showed the response of double reporter with different insertion copies and orientation to BMP7 treatment for 24 hours. D) Western blots for EGFP showed the response of double reporter with 2 insertion copies and (+) direction to Pax2 overexpression.

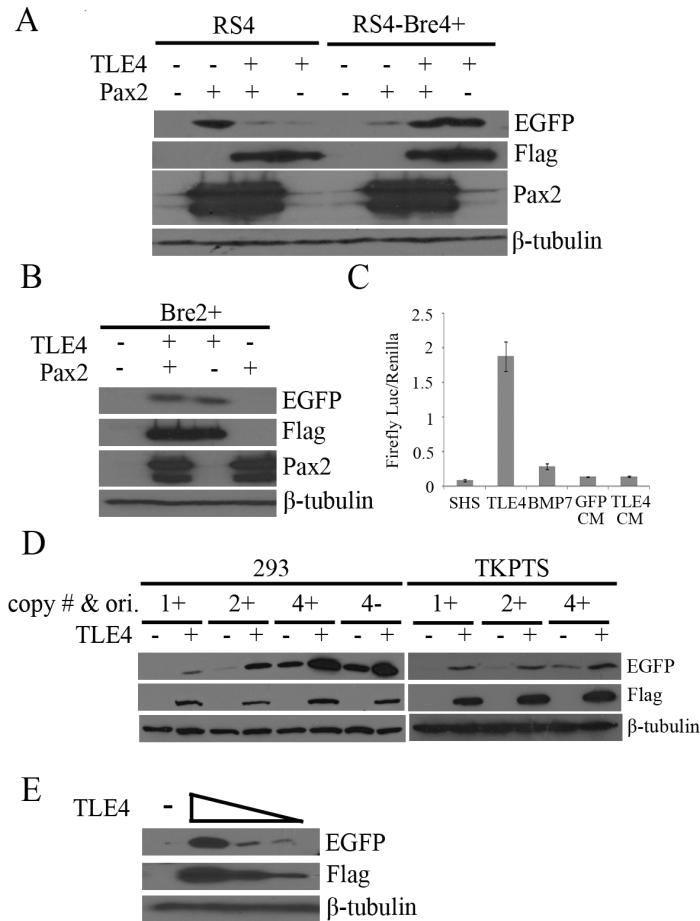


Figure 4-2. Tle4 overexpression activated transiently transfected BMP reporter. A) Western blots showed the response of cells transiently transfected with Pax2 reporter (RS4) or double reporter with four insertion copies in (+) direction to overexpression of Pax2 and Tle4. B) The same experiment as in A) but with BMP reporter without Pax2 binding sites. C) Luciferase assay was used to measure the response of BMP reporter to Tle4 overexpression or conditional medium (CM) collected from cells overexpressing Tle4. BMP7 treatment was used as positive control and CM collected from cells overexpressing GFP was used as negative control. D) Western blots showed the response of cells transiently transfected with the double reporter of different copy numbers or orientation to Tle4 overexpression in 293 cells and TKPTS, an immortalized renal epithelial cell line. E) Western blots showed the response of cells transiently transfected with BRE4+ double reporter to increasing doses of overexpressed Tle4. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

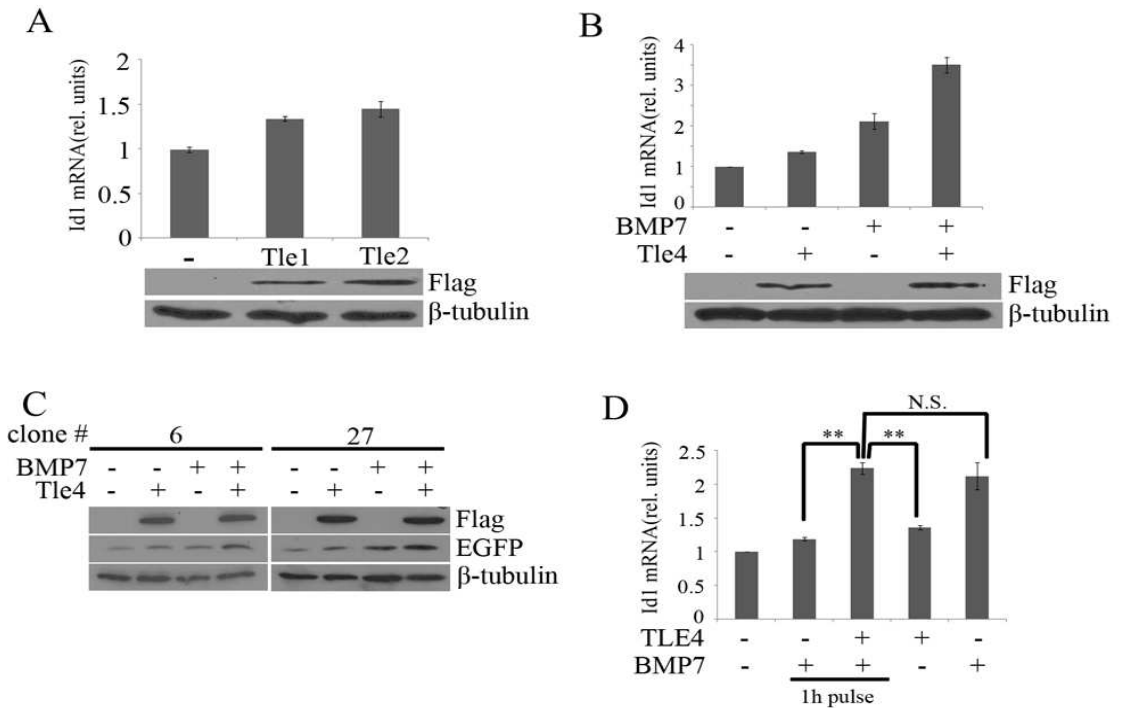


Figure 4-3. Tle4 enhanced and sustained BMP7 mediated endogenous Id1 expression.

A) upper: the response of endogenous Id1 gene to different amount of overexpressed Tle4 proteins was measured by qRT-PCR; lower: increasing amount of Tle4 expressing vector was transiently transfected into 293 cells to achieve different amount of Tle4 expression level, as indicated by western blots. B) upper: the response of endogenous Id1 gene to Tle4 overexpression and/or BMP7 treatment was measured by qRT-PCR; lower: equal amount of Tle4 was expressed with or without BMP7 treatment, as indicated by western blots. C) Western blots showed the response of BRE4+ double reporter stable transfected cell lines (#6 and #27) to Tle4 overexpression and/or BMP7 treatment. D) Id1 expression was measured by qRT-PCR upon Tle4 overexpression, BMP7 treatment for 24 hours, or BMP7 1 hour pulse in the presence or absence of Tle4 overexpression. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

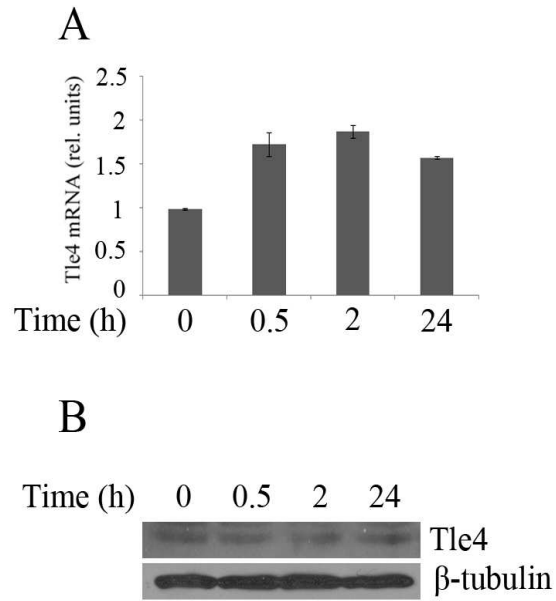


Figure 4-4. **BMP7 did not affect endogenous Tle4 expression.** A) qRT-PCR showed Tle4 RNA levels in 293 cells cultured with BMP7 for the indicated time in hours. B) Western blot showed Tle4 protein levels in 293 cells cultured with BMP7 for the indicated time in hours. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

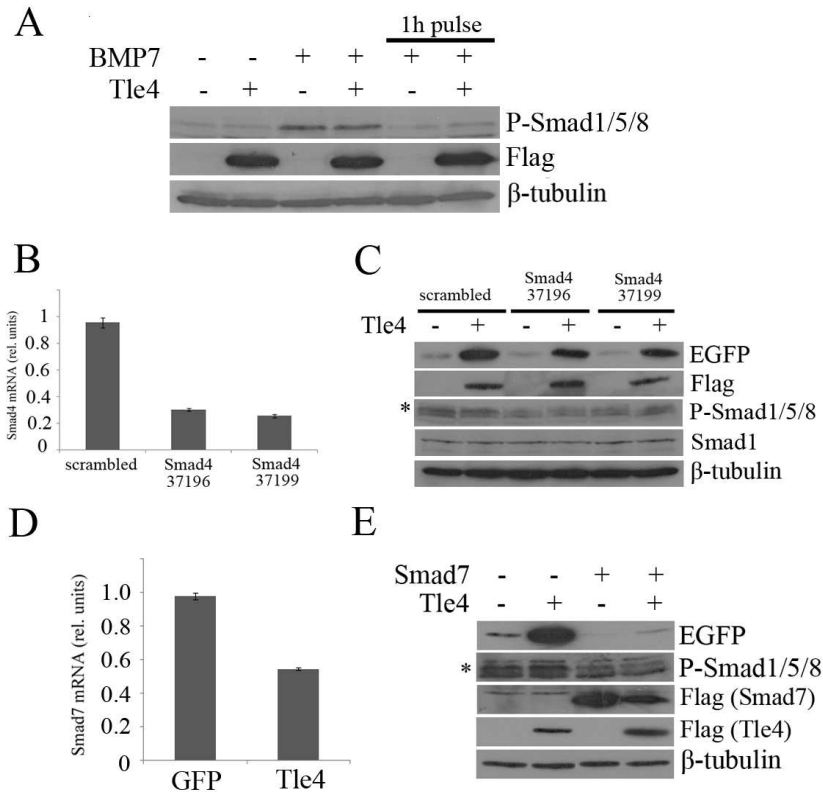


Figure 4-5. Tle4 activate BMP reporter vector through mediating Smad proteins. A) Western blots showed the P-Smad1/5/8 level upon Tle overexpression and/or BMP7 treatment for 24 hours or BMP7 1 hour pulse in the presence or absence of Tle4 overexpression. B) qRT-PCR showed Smad4 RNA levels after culture with shRNA #37196, #37199 or a scrambled control. C) Western blots showed the response of BRE4+ double reporter to Tle4 overexpression in Smad4 knockdown cells by shRNA #37196 and #37199 or scrambled control cells. P-Smad1/5/8 and total Smad1 were also probed. D) qRT-PCR was used to measure the endogenous Smad7 RNA level upon Tle4 overexpression. E) Western blots showed the response of BRE4+ double reporter to Tle4 overexpression and/or Smad7 overexpression. P-Smad1/5/8 was also probed. All samples were done in triplicate with error bars representing one standard deviation (SD) from the mean.

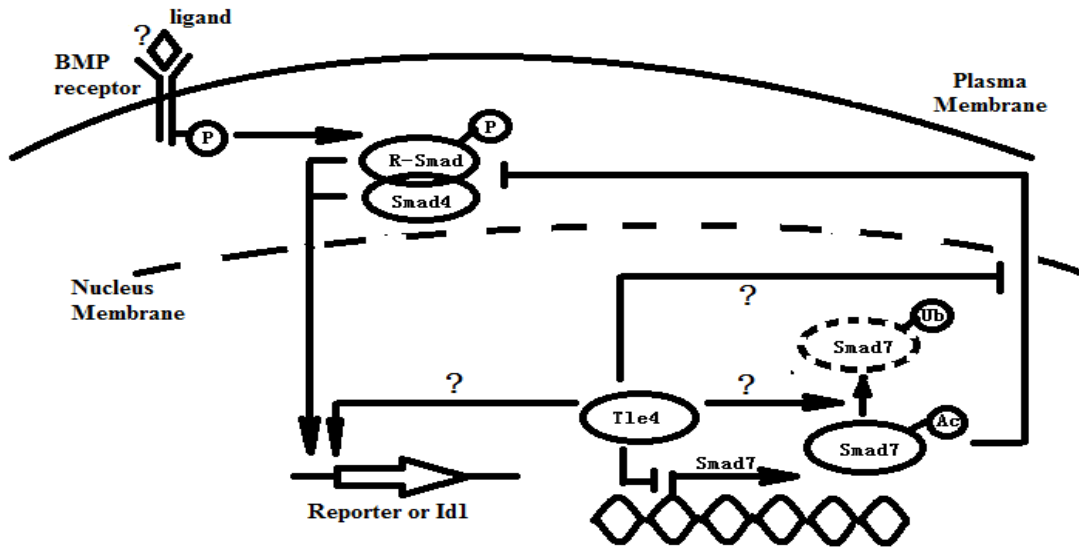


Figure 4-6. **Schematic diagram showing the regulation of Tle4 in BMP7 signaling.** Arrows indicates the promotion, “ \perp ” means the inhibition and “?” means uncertain. Ac, acetylation; Ub, ubiquitination.

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Chapter V

Conclusion

Since the discovery of TGF- β 1 in 1983, more than 30 different TGF- β superfamily ligands have been found in the human genome. Accumulated evidence shows that the TGF- β superfamily is critical for early embryogenesis, as well as the formation of nearly all organs. The TGF- β superfamily is also widely involved in various diseases, such as organ fibrosis and tumor metastasis. Considering its crucial role in development and diseases, it is important to carefully dissect the TGF- β superfamily signaling pathway to understand the mechanisms that it utilizes to regulate target gene expression both outside and inside of cells. In this thesis, we discovered that TGF- β activated JNK signaling through inducing Wnt11 expression and Wnt11 was necessary to upregulate mesenchymal marker genes in renal epithelial cells. These results not only revealed the direct targets of the TGF- β signaling pathway, but also, for the first time, integrated TGF- β , Wnt and JNK signaling within the context of the epithelial-mesenchymal transition.

In the UUO models, the overexpression of the KCP protein, a secreted TGF- β inhibitor, reduced the upregulation of Wnt11 in the injured kidney. Furthermore, the overexpressed KCP proteins disturbed the balance of TGF- β and BMP signaling during renal fibrosis and attenuated the upregulation of mesenchymal genes. This suggested that the extracellular regulation of TGF- β and BMP signaling pathways is critical for their physiological functions during disease progression. Besides the extracellular mediators, the BMP signaling is also regulated within the cells. As shown in my thesis, Tle4 can

enhance and sustain endogenous Id1 gene expression, probably through repressing inhibitory Smad7. This modification of BMP signaling by Tle4 is important for at least two reasons. First, some cellular physiological effects of BMP signaling are dependent on the activation of Id genes. For example, Ids are part of the machinery that mediates the regulation of hair cell and satellite cell differentiation exerted by BMPs (Kamaid et al., 2010; Ono et al., 2011). Second, since Tle4 amplifies the BMP signaling within cells, its specific expression pattern can mimic the effects of BMP gradients, thus causing various responses of different types of cells to the same BMP signaling. Taken together, my thesis systematically studied the mechanisms and regulations of TGF- β and BMP signaling in mediating target gene expression both *in vitro* and *in vivo*, thus deepening our understanding of TGF- β superfamily (Fig. 5-1). In the following paragraphs, I will further discuss the implication of my work by chapters.

TGF- β signaling is well characterized for its pro-fibrogenic effects in kidney diseases (Liu, 2010). *In vitro*, TGF- β promotes the transition of epithelial cells to fibroblasts-like cells by downregulating epithelial markers, such as E-cadherin, and activating mesenchymal genes, such as Snail1, Pai1 and Zeb1 (Yang and Liu, 2001). Although the existence of EMT *in vivo* is controversial (Kriz et al., 2011), enforced expression of mesenchymal genes, such as Snail1, in epithelial cells induced renal fibrosis in mice (Boutet et al., 2006). In human, a drastic accumulation of Snail1 was seen in the nuclei from tubular epithelial cells in kidney samples with multiple myeloma cast nephropathy, a disease characterized by a rapid progression toward fibrosis. However, such accumulation of Snail1 was not found in kidney samples with an idiopathic nephritic syndrome, a syndrome unassociated with renal fibrosis (Hertig et al.,

2011). These data suggest that the upregulation of mesenchymal genes in epithelial cells can be a major factor in the initiation and progression of renal fibrosis.

In the first part of this thesis, we found that Wnt11, a ligand belonged to Wnt signaling family, enhanced TGF- β mediated mesenchymal gene activation in renal epithelial cells. Furthermore, Wnt11 was directly regulated by Smad3 proteins, but not Smad2. This was consistent with the previous results showing that Smad2 functioned as a protector against Smad3 in renal fibrosis (Meng et al., 2010). In fact, although Smad2 and 3 share similar structure and are both activated by TGF- β s, their biological functions are not the same. Compared to the early embryonic lethality of Smad2 mutants (Waldrip et al., 1998), the phenotypes of Smad3 mutants were much less severe and could survive for 1-8 months after birth (Yang et al., 1999). Detailed studies showed the different target gene profiles of Smad2 and 3 in human and rat epithelial cell line (Chung et al., 2010; Phanish et al., 2006). Recently, Smad4 was shown to be important for the progression of renal fibrosis, as well as for Smad3 mediated Collagen I expression (Meng et al., 2012). Since both Smad2 and 3 can interact with Smad4, but Smad2 lacks the DNA binding domain, it is possible that Smad2 counteracts Smad3 by competing for the Smad4 interaction. Thus, it will be interesting to test whether Smad3 proteins interact with Smad4 strongly in the absence of Smad2 and whether a modulated Smad2 protein with DNA binding domain may facilitate Collagen I expression upon TGF- β treatment. With respect to the Wnt11, because it was regulated only by Smad3, but not Smad2, it may serve as a biomarker to distinguish the Smad2 and Smad3 mediated pathways.

The modulation of Wnt11 significantly affected TGF- β mediated upregulation of mesenchymal genes without influencing Smad proteins, suggesting that once activated,

Wnt11 functioned independently of TGF- β signaling. However, the sustained activation of Wnt11 during EMT still required TGF- β signaling, since a selective inhibitor of TGF- β type I receptor, SB431542 strongly blocked the Wnt11 expression, even after Wnt11 has already been activated by TGF- β 1 (data not shown). This further demonstrated the central status of TGF- β signaling in driving EMT.

It has been reported that TGF- β could stimulate JNK signaling, though the mechanisms were unknown (Mao et al., 2011; Shin et al., 2011). Now, our data demonstrated that the activation of JNK signaling in TGF- β mediated EMT was at least partially through Wnt11. Instead of activating canonical/ β -catenin signaling, Wnt11 mediated the expression of mesenchymal genes through non-canonical/JNK signaling. This discovery also broadened the understanding of the crosstalk between TGF- β and Wnt signaling. Previously, the limited number of studies addressing the crosstalk of TGF- β and Wnt signaling pathways converged on β -catenin, as TGF- β could stabilize β -catenin by inhibiting its GSK3 β -dependent degradation through p38 MAPK and Akt (Hwang et al., 2009; Liu, 2010; Masszi et al., 2004). Also β -catenin could physically interact with Smad proteins to regulate target gene expression (Kim et al., 2009; Zhang et al., 2010; Zhou et al., 2012). Our data is the first direct evidence showing the crosstalk between TGF- β and non-canonical Wnt signaling. Indeed, we found no evidence that canonical Wnt pathway was activated by TGF- β .

The studies on the regulation of TGF- β and BMP signaling are equally important, as these regulations control the strength of the signaling and specify the final cellular responses. KCP is a secreted protein containing 18 CR domains. It possesses a dual role in enhancing BMP signaling and inhibiting TGF- β signaling (Lin et al., 2005; Lin et al.,

2006). In the second part of the thesis, we examined whether modulating the balance of TGF- β and BMP7 signaling by overexpressing KCP in transgenic mice affected the progression of kidney injury in UUO models. We found that the upregulation of mesenchymal marker genes was attenuated by KCP overexpression in the UUO mouse model. This is consistent with the retarded progression of renal fibrosis in KCP transgenic mice, as demonstrated by less α -SMA and Collagen V region (data not shown). What is more important, we found that upregulation of Wnt11 was also reduced in KCP transgenic mice. This further demonstrated that Wnt11 was a target of TGF- β signaling and was closely associated with renal fibrosis. Although our *in vitro* cell model indicated that Wnt11 promoted EMT, the current mouse model cannot distinguish whether Wnt11 is only a biomarker for renal fibrosis or a mediator for this process. Since the Wnt11 mutant mice died by 2 days postpartum, because of abnormal heart development (Majumdar et al., 2003), Wnt11 conditional knockout mice are needed to further study its function during renal fibrosis. In this case, Pepck promoter can be used to drive the expression of Cre recombinase, since it is very active in adult renal proximal tubular epithelia (Short et al., 1992). An available alternate plan for Wnt11 conditional knockout mice is to use Fzd4^{-/-} mice. Fzd4^{-/-} mice were viable and showed similar renal hypoplasia as Wnt11^{-/-} mice (Ye et al., 2011), suggesting that Wnt11 may function through Fzd4. However, the potential problem for using this Fzd4^{-/-} mouse model is the receptor redundancy, since it has been reported that Wnt11 can also transduce its signaling through Fzd7 or Fzd8 (Yamanaka and Nishida, 2007; Ye et al., 2011), both of which are expressed during renal fibrosis (He et al., 2009).

Under normal condition, most BMP type II receptors are localized in epithelial cells (Bosukonda et al., 2000), as are TGF- β type I receptors in the UUO model (Yang and Liu, 2001). These data suggested that renal epithelial cells are the main participants in responding to TGF- β /BMP signals. At early time (7 days), the differences of activation of *Wnt11*, *Pai1* and *Snail1*, between KCP and wildtype mice were not significant, indicating that the effects of epithelial cells in initiating renal fibrosis may be limited. However, by 14 days, the upregulation of all the examined mesenchymal genes and myofibroblast markers, was reduced in KCP transgenic mice, indicating that epithelial cells play a crucial role in promoting fibrosis. One defect for our current model is that the expression of transgenic KCP proteins decreased along with progression of renal fibrosis. This may result from the increased apoptosis of epithelial cells or the silencing of *Pepck* promoter during the injury. To better address the function of KCP in renal fibrosis, a new KCP overexpression model may be generated. In this model, KCP gene may be knocked in at *Rosa26R* site with a 5' upstream stop signal flanked by loxp sites. If this mouse was bred to the mouse with Cre expression driven by *Pepck* promoter, KCP will also overexpress in all renal proximal tubular cells. The advantage for this model is that we may avoid the silence of *Pepck* promoter and maintain KCP expression during renal fibrosis.

Considering the secreted property of the KCP protein, it may affect neighboring cells as well. Previous results revealed that BMP7 could induce adult renal fibroblasts to differentiate to epithelial cells (Zeisberg et al., 2005). Thus, the overexpressed KCP may enhance the BMP signaling to limit the number of fibroblasts during renal fibrosis. Another potential target for BMP signaling is interstitial stromal cells, which are

quiescent undifferentiated mesenchymal cells (Dressler, 2006). Since BMP signaling is important to maintain the pluripotency of stem cells (Varga and Wrana, 2005), during renal fibrosis, the decreasing BMP7 signaling may contribute to the differentiation of these stromal cells into fibroblasts. Meanwhile, the destruction of the stem cell pool may also reduce the recovery ability of the kidneys from the injuries. Thus, it will be interesting to test whether KCP overexpression can improve the renal recovery in acute renal diseases, such as acute tubular necrosis (ATN) model induced by injection of folic acid (Lin et al., 2005).

Besides the extracellular regulation, BMP signaling is also modulated within cells. In the third part of the thesis, we discussed how BMP signaling is regulated by Gro/Tle proteins. Gro/Tle family proteins are common corepressors. Up to now, the only reported relationship between TGF- β signaling and Tle proteins was that Dpp, the TGF- β homolog in *Drosophila*, induced the expression of Brinker, which recruited Groucho and CtBP to repress other Dpp target genes, thus confining the functional zone of Dpp signaling (Hasson et al., 2001; Zhang et al., 2001). Since Gro/Tle proteins could mediate a long range suppression via compacting chromatin (Sekiya and Zaret, 2007), our original idea was to test whether the recruitment of Tle4 to DNA can influence the nearby BMP response elements. Thus, we constructed the Pax2 and BMP7 double reporter vector, using Pas2 as the bridge factor to recruit Tle4. Much to our surprise, overexpression of Tle4 strongly activated BMP reporter independent of Pax2, as well as enhancing and sustaining the BMP7 effect on endogenous Id1 gene in 293 cells. The effects of Tle4 on BMP7 signaling may be mediated through Smad7 proteins, since Tle4 suppressed Smad7 transcription, while Smad7 overexpression completely abolished the effects of Tle4 on

BMP reporter. However, Tle4 only suppressed Smad7 transcription by ~50%, but its effect on the activation of BMP reporter was robust. This suggested that Tle4 may also influence Smad7 at the protein level. The balance between acetylation and ubiquitination was important to control the stability of Smad7 (Gronroos et al., 2002; Simonsson et al., 2005). Since Tle4 was able to interact histone deacetylase 1 (HDAC1) (Choi et al., 1999), it may help erase the acetylation marker from Smad7 to facilitate its ubiquitination and further degradation.

If Tle4 mediates BMP7 signaling by repressing Smad7, the activation of BMP7 reporter by Tle4 is actually the derepression of the basal BMP7 activity. Indeed, in 293 cells, we detected a basal level of phosphorylated Smad1, 5 and 8. Furthermore, Smad4 knockdown reduced the effect of Tle4 on BMP7 reporter, although the extent was limited. Here, using the BMP receptor inhibitor will be a better choice than Smad4 knockdown. Smad1, 5 and 8 themselves have DNA binding domain, thus may directly mediate gene transactivation (Korchynskiy and ten Dijke, 2002). In contrast, the BMP receptor inhibitor, such as DMH1, could specifically and efficiently block BMP signaling by inhibiting the phosphorylation of Smad1, 5 and 8, thus resulting in a clearer background (Hao et al., 2010). Since Smad7 can also block TGF- β signaling (Hayashi et al., 1997), it will be interesting to test whether Tle4 overexpression can activate TGF- β reporter, 3TP-luc, as well.

It is noticeable that Tle4 could activate BMP reporter even stronger than BMP7 administration, suggesting that it may use other mechanism, besides repressing Smad7, to mediate BMP signaling. Tle4 may directly mediate activation of BMP reporter and endogenous Id1 gene, since recent study showed that Tle3 was presented at the promoters

of activated genes (Villanueva et al., 2011). If this is the case, it is important to determine which transcriptional factor binds to the BMP binding element (SBE) and recruits Tle4. The first candidate will be Smad proteins. However, Smad proteins lacked the common interaction motif for Gro/Tle proteins, such as WRPW tetrapeptides and Engrailed homology 1 (Eh1) sequences (Buscarlet and Stifani, 2007). Besides the SBEs, the BMP7 reporter also contains a GC rich region, which may be the binding site for other transcriptional factors. A chromatin immunoprecipitation (ChIP) assay for Tle4 will confirm the presence of Tle4 at the promoter region of Id1 gene.

Although Tle4 could modulate the BMP signaling, the BMP signaling pathway does not affect the amount of endogenous Tle4 proteins. In fact, BMP7 did not influence the expression of any Tle proteins, from Tle1 to Tle3 (data not shown). Since the basal Tle4 protein level is low in 293 cells, it seemed that BMP signaling pathway did not require Tle proteins to activate target genes. Thus, to further address the biological importance of Tle4 in BMP signaling, the metanephric mesenchymal cells and its derived epithelia may be a better tool, because they have relative high endogenous Tle4 expression (Cai et al., 2003). It will be valuable to test whether Tle4 knockdown in those cells affects their response to BMP7 for proliferation and cell survival (Dudley et al., 1999).

Finally, although Gro/Tle proteins are important corepressors and studied in different types of cancers (Buscarlet and Stifani, 2007), their involvement in renal fibrosis is totally unknown. We even do not know whether those proteins are expressed in adult kidneys. However, considering the amplification effect of Gro/Tle proteins on BMP signaling, it is interesting to check their expression during renal fibrosis. Since the target

of Tle4, the Smad7 proteins, is involved in the regulation of both TGF- β and BMP signaling, the precise function of Tle4 in renal fibrosis may be complicated, but it may serve as another regulation point to balance the TGF- β and BMP signaling *in vivo*.

In summary, this dissertation analyzed both the mechanisms and regulations of TGF- β superfamily mediated target gene expression in renal epithelia. The work provided further insight into the TGF- β signaling and may provide new clues for the medical treatment of TGF- β associated diseases, such as renal fibrosis and cancer.

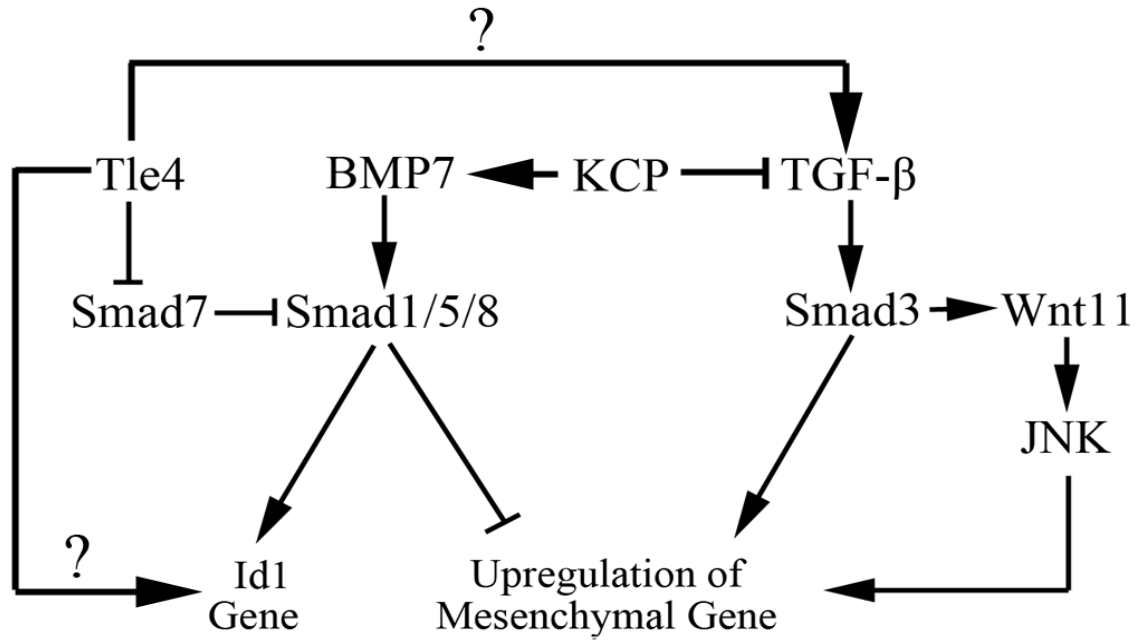


Figure 5-1. **Schematic diagram summarizing the mechanisms and regulation of TGF- β superfamily mediated gene expression in this thesis.** Arrows indicates the promotion, “ \perp ” means the inhibition and “?” means uncertain.

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