Smad expression during kidney development

P. Vrljicak, D. Myburgh, A. K. Ryan, M. A. van Rooijen, C. L. Mummery, and I. R. Gupta. Smad expression during kidney development. Am J Physiol Renal Physiol 286: F625–F633, 2004. First published December 2, 2003; 10.1152/ajprenal.00152.2003.—Signal- ing by the transforming growth factor (TGF)-β superfamily is important during kidney development. Here, we describe the spatial and temporal expression patterns of the Smads, the transcription factors that translate TGF-β signals into gene expression. RT-PCR data and in situ hybridization analysis showed that the receptor-regulated (R) Smads (Smad1, -2, -3, -5, and -8), the common partner Smad (Smad4), and the inhibitory (I) Smads (Smad6 and -7) were all expressed during mouse kidney development from embryonic day 12 until the end of nephrogenesis at postnatal day 15. Each Smad had a distinct spatial distribution. All were expressed by mesenchymal cells in the nephrogenic zone and were downregulated once these cells began to epithelialize. The common partner Smad, Smad4, was present in uninduced mesenchymal cells and at ureteric bud tips. The bone morphogenetic-responsive R-Smads, Smad1, -5, and -8, were mainly expressed in the nephrogenic zone, whereas the TGF-β-responsive R-Smads were predominantly noted in the medullary interstitium. Expression of the I-Smad Smad7 was also seen in mesenchymal cells in the interstitium. Based on the observed patterns of expression, we speculate that individual or combinations of Smads may play specific roles in cell-fate determination during kidney development.

transforming growth factor-β signaling; mRNA

TRANSFORMING GROWTH FACTOR-β (TGF-β) superfamily ligands affect critical processes during organ development, including cell proliferation, differentiation, cell-fate determination, apoptosis, and morphogenesis by activating discrete signaling pathways (2, 18). The signaling pathway for all TGF-β members begins with ligand binding to a cell-surface receptor complex of type I and type II serine-threonine kinases. The type I receptor then phosphorylates a group of transcription factors known as Smads, which initiate target gene transcription (2, 26). The eight Smads that have been identified so far in mammals can be divided into three subclasses: the receptor-regulated Smads (R-Smads), the common partner Smad (Smad4), and the inhibitory Smads (I-Smads). R-Smads Smad1, -5, and -8 are activated during bone morphogenetic protein (BMP) signaling, whereas Smad2 and -3 are part of the TGF-β and activin signaling pathways. Smad4, the common partner, couples with the phosphorylated form of an R-Smad, and then the complex translocates to the nucleus to regulate transcription. The I-Smads Smad6 and -7 negatively regulate TGF-β and BMP signaling by forming stable interactions with the activated type I receptor or by preventing Smad4 from coupling with activated R-Smads (14, 16, 19, 27).

The murine ureteric bud develops as an offshoot of the mesonephric duct at embryonic (E) day 10.5 (E10.5) and induces the adjacent mesenchyme to become the metanephric mesenchyme (38). Reciprocal inductive interactions between the ureteric bud and the metanephric mesenchyme are required to establish the structural organization of the kidney. The ureteric bud undergoes successive branching events to form the collecting duct network, and at the tips of the branching ureteric bud the adjacent metanephric mesenchyme is induced to form the majority of each nephron (1). Members of the TGF-β superfamily have been shown to be important for these processes. Targeted inactivation of either bone morphogenetic protein (Bmp)-7 or Bmp-4 leads to severe murine kidney malformations (9, 11, 22, 25). In addition, culture of embryonic kidney explants in the presence of TGF-β growth factors such as TGF-β1, activin, and BMP-2, -4, or -7 affects whole organ growth and the ability of the ureteric bud to undergo branching morphogenesis (30, 34, 35, 37, 44). Despite the importance of these signaling pathways, we know little about the Smads, the signal mediators downstream of the ligand/receptor complex, and their function during kidney development. Mouse knockouts have been of limited success in elucidating the function of many of the Smads because the embryos die before the formation of the final metanephric kidney (4, 41, 42, 45, 48, 49).

Here, we describe the spatial and temporal expression patterns, using RT-PCR and in situ hybridization, of the R-Smads (Smad1, -2, -3, -5, and -8), the common partner, Smad4, and the I-Smads (Smad6 and -7) during kidney development. In mice, the metanephric kidney develops at E11. At E12, nephrogenesis begins and continues until the first 2 wk of the postnatal period (38). We demonstrate that the R-Smads, Smad4, and the I-Smads are expressed in the mouse kidney from E12 until the end of nephrogenesis. Within the nephrogenic zone, Smads are expressed by cells at the tips of the ureteric bud and by mesenchymal cells. However, once these mesenchymal cells begin to undergo epithelialization, Smad expression is downregulated. From our expression data, we speculate that individual or combinations of Smads may play specific roles in determining cell fate during kidney development.

MATERIALS AND METHODS

Animals and tissue collection. Pregnant CD1 mice were obtained from Charles River Laboratories. Embryos and their metanephric
kidneys were microdissected at E12, obtained from whole kidneys at embryonic (Fig. 1. Expression of Smads during kidney development. Total RNA was in accordance with the rules and regulations of the Canadian Council (and H11003) with 10 μl g/ml yeast tRNA, 1% SDS, and 50 g/ml) were added for overnight digestion at 65°C. After incubation, slides were exposed to 10 μg/ml biotinylated D. biflorus agglutinin (Vector Labs) overnight at 4°C. The following day, sections were treated with the streptavidin complex provided by the Vectastain ABC kit (Vector Labs) followed by application of 3,3′-diaminobenzidine according to the manufacturer’s recommendations. RT-PCR. The presence of Smad1, -2, -3, -4, -5, -6, -7, and -8 mRNAs was analyzed by RT-PCR. RNA was extracted with the RNeasy kit (Qiagen). RT-PCR was performed using the OneStep RT-PCR kit (Qiagen). Primer sequences were as follows: Smad1 (sense: ATGAGTGGACAGCCGCTGTGT; antisense: CTGTTGGAGAACAATGTTGA); Smad2 (sense: CCCACTCCATTCCAGAAAC, antisense: GAGCTTGTCGTCACATTGTTG); Smad3 (sense: GTTGGGACAGCTGGAAGAG, antisense: GTTAGTGGAGGAGGAGGAC); Smad4 (sense: AAGGTTGGGAGAAGTGAAC, antisense: ATGCTTGGACATTCTGTTG); Smad5 (sense: GGAACCTGAGCCACAATGGA, antisense: CTGTTGGGAGAGTGGGATA); Smad6 (sense: CACACTGGATCCTGCGAGTTGA; antisense: AAAGTGGACACCTTGAGGAG); Smad7 (sense: TCTTGCTGTGCAAATTGTTG; antisense: AGTAAAGGAGGAGGGGAG); Smad8 (sense: CACCGACCACTTCCATTGTTCA; antisense: CTCCTGAGGAGTGGTGA); Smad9 (sense: CACACGACCACTTCCATTGTTCA; antisense: CTCCTGAGGAGTGGTGA); and β-actin (sense: ACACTGGATCCTGCGAGTTGA; antisense: AAAGTGGACACCTTGAGGAG). RT-PCR was performed directly from the RNA. RT was performed using 200 ng of total RNA with 100 ng of reverse primer at 50°C for 30 min. For the

Fig. 1. Expression of Smads during kidney development. Total RNA was obtained from whole kidneys at embryonic (E12, E15, E18), postnatal (P1, P15), and adult (Ad.; >2 mo) stages and used for RT-PCR. A: transcripts for all receptor-regulated Smads (R-Smads) and Smad4 are detected at each stage examined. B: transcripts for the inhibitory Smads (I-Smads; Smad6 and Smad7) are detected at each stage examined. β-Actin is amplified from each sample as an internal control.

kidneys were microdissected at E12, E15, and E18, at postnatal days (P1 and P15), and at adulthood (2 mo or older). Animals were treated in accordance with the rules and regulations of the Canadian Council of Animal Care guidelines. In situ hybridization. Whole mouse embryonic kidneys were fixed overnight in 4% paraformaldehyde in PBS, washed with PBS-0.1% Tween 20 (Sigma), and dehydrated in methanol. In situ hybridization was performed as described previously with some modifications (47). Briefly, the tissue was bleached with 6% hydrogen peroxide, treated with 10 μg/ml protease K (Invitrogen), and then washed in 0.2% glycine before re fixation in 4% paraformaldehyde/0.2% glutaraldehyde. For 60 min, the tissue was prehybridized in a solution of 50% formamide, 5× SSC (pH 4.5), 50 μg/ml yeast tRNA, 1% SDS, and 50 μg/ml heparin. cRNA probes (1 μg/ml) were added for overnight hybridization at 65°C. The tissue was then washed with a solution of 50% formamide, 5× SSC (pH 4.5), and 1% SDS, followed by a solution of 50% formamide and 2× SSC at 65°C. Blocking in 10% sheep serum in Tris-buffered saline (TBS)-0.1% Tween 20 was followed by incubation in a 1:2,000 dilution of alkaline phosphatase-conjugated anti-digoxigenin (DIG) antibody (Roche). Samples were developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) in 0.1 M NaCl, 0.1 M Tris (pH 9.5), 50 mM MgCl₂, and 0.1% Tween 20. Samples were visualized as whole mounts using a dissecting microscope and then cryosectioned at 20-μm thickness.

Probe synthesis. Complete or partial coding sequences for the following mammalian cDNAs were linearized and in vitro transcribed in the antisense direction using the following combination of enzymes. Gdfn and c-Ret (a gift from Frank Costantini, Columbia University) were digested with HindIII and BamHI and transcribed using SP6 and T3 (Promega), respectively. Mouse Bmp-4 was linearized with EcoRI and transcribed with SP6. Rat Smad1, Smad2, and Smad3 (gifts from Jean-Jacques Lebrun, McGill University) were digested using HindIII and transcribed using SP6. Smad4 (a gift from Christian Sirard, McGill University) was linearized with HindIII and transcribed with T3. Smad5 (a gift from An Zwijsen, Flanders Inter- university Institute for Biotechnology) was digested with BamHI and transcribed with T7. Smad8 (a gift from Christian Sirard) was subcloned into pCRII vector (Invitrogen), linearized with HindIII, and transcribed using T7. Tgf-β1 (a gift from Fuad Ziyadeh, University of Pennsylvania) was cut with XhoI and transcribed with SP6. Smad6 was digested with EcoRI and transcribed with T7. Smad7 (gift from An Zwijsen) was digested with XhoI and transcribed with T7. Probes were labeled with DIG-labeled UTP (Roche) according to the manufacturer’s specifications.

Dolichos biflorus agglutinin for labeling ureteric bud elements. Visualization of ureteric bud derivatives was performed using Dolichos biflorus agglutinin (DBA) staining. Cryosections were washed in 1× TBS and treated with 0.25% trypsin in 0.272 M CaCl₂ for 30 min at 37°C. After incubation, slides were exposed to 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidases. After a brief TBS wash, tissue was exposed to 10 μg/ml biotinylated D. biflorus agglutinin (Vector Labs) overnight at 4°C. The following day, sections were treated with the streptavidin complex provided by the Vectastain ABC kit (Vector Labs) followed by application of 3,3′-diaminobenzidine according to the manufacturer’s recommendations.

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RESULTS

Temporal expression. To determine the temporal sequence of Smad expression, RNA was extracted from whole kidneys at various times during embryonic and postnatal development using RT-PCR. The R-Smads (Smad1, -2, -3, -5, and -8), Smad4, and the I-Smads (Smad6 and -7) were detected at all embryonic and postnatal stages examined, including the adult kidney (Fig. 1).

Distribution of the common partner, Smad4, in relation to the TGF-β ligands, TGF-β1, and Bmp-4. The spatial expression of Smads in the developing kidney was characterized to determine whether they were expressed in the same cell types as TGF-β ligands. Whole mount in situ hybridization was performed on kidneys at E12 (data not shown) and E15 with probes for all R-Smads, Smad4, I-Smads, Bmp-4, and Tgf-β1 and compared with the expression patterns for c-Ret and Gdnf. As previously reported, c-Ret was expressed at the growing tips of the ureteric bud, and Gdnf was detected in uninduced mesenchymal cells adjacent to ureteric bud tips (17, 29).

Kidneys were cryosectioned and then colabeled with biotinylated D. biflorus to identify ureteric bud cells. As shown in Fig. 2, Tgf-β1, Bmp-4, and Smad4 were all expressed at E15. Differences in their expression patterns were readily apparent when these kidneys were sectioned. In whole-mount preparations, Tgf-β1 was ubiquitously expressed. This was confirmed in the cryosections where Tgf-β1 was detected in mesenchymal cells within the interstitium, in uninduced mesenchymal cells in the nephrogenic zone, and at the tips of the ureteric bud. Bmp-4 was detected mostly in the cortex in whole-mount preparations (Fig. 2), but in the cryosections, intense expression was noted in the nephrogenic zone, and a fainter signal was observed within the medullary interstitial compartment (Fig. 2). Bmp-4 was expressed in the mesenchyme adjacent to the ureteric bud tips and in the tips themselves but not in mesenchymal cells undergoing epithelialization. Smad4 was strongly expressed in the nephrogenic zone and markedly reduced in the medulla in whole-mount preparations (Fig. 2). In the cryosections, Smad4 expression was similar to Bmp-4: a strong signal was detected in the uninduced mesenchyme and the ureteric bud tips within the nephrogenic zone. In contrast to Bmp-4, Smad4 was more weakly expressed by mesenchymal cells in the interstitium. Smad4 was noted to be weakly expressed in the mesenchymal cells surrounding the ureter (Fig. 2), as previously described for Bmp-4 (10, 25). Similar results were obtained at E12 for Tgf-β1, Bmp-4, and Smad4 (data not shown).

Distribution of R-Smads. The BMP-responsive R-Smads, Smad1, -5, and -8, were all expressed by mesenchymal cells in the nephrogenic zone and at the ureteric bud tips at E15 (Fig. 3). A weaker signal was detected by all three probes in mesenchymal cells in the medulla and around the ureter. The TGF-β-responsive Smads, Smad2 and -3, had different expression patterns. Smad2 was strongly expressed in mesenchymal cells in the medulla at E15 and was difficult to detect in other regions of the kidney (Fig. 3). In contrast, Smad3 labeled strongly throughout all compartments of the kidney except for condensing mesenchymal structures where it was downregulated (Fig. 3). Smad3 was expressed in mesenchymal cells in both the nephrogenic zone and the medullary interstitium and in cells from ureteric bud tips. Similar results for Smad1, -2, -3, -5, and -8 were obtained at E12 (data not shown).

DISCUSSION

A number of lines of evidence confirm that members of the TGF-β superfamily are important during murine kidney development. Targeted inactivation of Bmp-7 or Bmp-4, for example, leads to small, abnormal kidneys (9, 11, 22, 25). Furthermore, results from experiments using cultured embryonic kidney explants demonstrate that TGF-β1, BMP-2, BMP-4, BMP-7, and activin affect organ growth and renal branching morphogenesis (30, 34, 36, 37, 44). Although Smads have been shown to be the intracellular mediators for all TGF-β signaling pathways, little is known about their role during kidney development. The results reported here demonstrate that the R-Smads, Smad4, and I-Smads were expressed in the mouse kidney throughout development. Each Smad showed a distinct pattern of expression, and many of them were shown to co-occur in the same cell type (Fig. 5). Smads were expressed in the nephrogenic zone by uninduced mesenchymal cells adjacent to the ureteric bud tips and by mesenchymal cells in the peripheral cortex that were destined to become stromal cells. In mesenchymal cells adjacent to the ureteric bud tips, Smad expression was downregulated once they began to differentiate. Medium to high levels of expression for all of the R-Smads and Smad7 were detected within mesenchymal cells in the medullary interstitium, whereas Smad4 and -6 were weakly expressed within this cell type. All of the Smads were detected within the ureteric bud tips.

Characterizing the mRNA expression patterns for the Smads is an important step toward understanding how these genes are expressed and regulated. Our mRNA results complement protein expression data reported by Oxburgh and Robertson (28), although they differ in some significant ways. Our images
clearly demonstrate that each Smad has a particular pattern of expression (Fig. 5). Perhaps due to the greater sensitivity of in situ hybridization, we found that Smad5 and -8 mRNA transcripts are present in mesenchymal cells and ureteric bud tips, a result that has not been clearly demonstrated by protein immunohistochemistry. In addition, we noted that all Smads were expressed in the tips of the growing ureteric bud at E12 and at E15. This observation differs somewhat from that of Oxburgh and Robertson, who were unable to detect Smads at E11.5 in the ureteric bud, using RT-PCR or immunohistochemistry, although Smads were observed in ureteric bud derivatives or collecting ducts at E15.5. Thus either the Smads become turned on in ureteric bud tips only after the first branching event has passed at ~E12 or the methods differ in sensitivity. Oxburgh and Robertson observed that all of the Smads were downregulated in condensing mesenchyme but...
then reappeared in more mature structures such as glomeruli and tubules. Judging from their images, protein expression in glomeruli and tubules was only observed for Smad2, -3, and -4. We were unable to detect any significant Smad mRNA expression in either the condensing mesenchymal structures or their mature counterparts, suggesting that transcription was reduced in these locations.

It is unclear from the reported RNA and protein expression data whether TGF-β ligands signal via paracrine or autocrine pathways or both during kidney development. Clearly, Bmp-4 is expressed in the same mesenchymal cell types that express the BMP-responsive Smads and Smad4 (Figs. 2 and 3). These results run counter to other reports in the literature that show Bmp-4 is expressed by condensing mesenchymal structures and not by cells at the ureteric bud tip (10, 25, 34). In these other reports, cell types were identified by histology rather than colabeling with known cell-specific markers. It is possible that the signal reported in cells of condensing mesenchymal structures actually originated from ureteric bud tips. Other differences in our expression data may be due to differences in the probes themselves or the detection system used for in situ hybridization (e.g., radioisotope vs. DIG labeling and detection). We are unaware of any study that documents the expression of BMP-4 protein in the developing kidney. Two reports have examined the expression of the BMP type I receptors, known as activin-like kinase (ALK)-3 and -6. Dewulf et al. (8) found expression of ALK-6 mRNA in ureteric bud cells and ALK-3 in both derivatives of the ureteric bud and mesenchymal cells at E12.5. Martinez et al. (23) reported similar findings using in situ hybridization, although they detected ALK-6 expression in mesenchymal cells as well as ureteric bud cells. They also detected expression of the BMP type II receptor in mesenchymal and ureteric bud cells. Collectively, the published results and those reported here suggest that all of the BMP signaling components are present in mesenchymal cells during kidney development. Thus BMP signaling within mesenchymal cells is likely autocrine.

TGF-β1 mRNA was predominantly expressed in the interstitium and at ureteric bud tips. Smad2 was mostly expressed in mesenchymal cells of the interstitium, whereas Smad3 was ubiquitously expressed (Figs. 2 and 3). Although both TGF-β-responsive Smads were expressed by mesenchymal cells in the interstitium, the common partner, Smad4, was only weakly expressed in these cells. This could reflect a discrepancy between transcription and translation such that there may be significant levels of protein present despite a reduction in the number of mRNA transcripts. However, the immunohistochemical data from Oxburgh and Robertson (28) also suggest that there is little Smad4 protein within the medullary interstitium. This, therefore, raises the possibility that there may be other common partner Smads that mediate TGF-β signaling in these mesenchymal cells. Our expression data are consistent with those reported by Clark et al. (7), who demonstrated expression of TGF-β1 mRNA in rat kidney at similar time points in mesenchymal cells of the nephrogenic zone and in epithelial cells at the tips of the ureteric bud. However, they did not see expression of the ligand in the interstitium itself, but this may be due to differences in our respective probes and/or differences between the two species. The mRNA for the TGF-β1 type I receptor, ALK-5, is expressed in the interstitium of the developing kidney (7). In contrast, the type II receptor is detected in mesenchymal cells within the nephrogenic zone and the interstitium and in ureteric bud cells (6). All of this evidence suggests that TGF-β1 signaling within mesenchymal cells in the interstitium is also autocrine.

When murine embryonic kidney explants are grown in the presence of TGF-β1, BMP-2, or activin, renal branching morphogenesis is inhibited (12, 30, 35, 36). The effect of BMP-7 on renal branching morphogenesis is more complicated: some authors have reported no significant effect (44), whereas others have found it to be stimulatory or inhibitory depending on the dose (30). We observed some expression of all Smads within the tips of the ureteric bud. Future work will need to establish whether TGF-β ligands are being secreted by mesenchymal or ureteric bud cells to determine whether signaling in ureteric bud cells is paracrine or autocrine. If the source of ligand were indeed from mesenchymal cells, then this would suggest a mechanism by which mesenchymal cells could regulate the extent of ureteric bud branching. Smads are highly expressed in uninduced mesenchyme but downregulated once these cells begin to differentiate (28). We speculate that mesenchymal cells secrete TGF-β ligands that then relay signals to the ureteric bud tips to inhibit branching and prepare the ureteric bud tip to become the distal segment of the future nephron.

The I-Smads, Smad6 and -7, are induced by TGF-β and BMP signaling and negatively regulate these pathways (40, 46). Although the I-Smads are known to be important for the function of glomerular cells in the adult kidney (5, 39, 43), we now demonstrate that they are also present during development in mesenchymal cells and at ureteric bud tips. The expression pattern of Smad6 parallels that of Smad4 in that the strongest signal is seen in mesenchymal cells within the nephrogenic zone. In contrast, Smad7 is detected in all cell types, including mesenchymal cells within the interstitium. Taken together, it is plausible that TGF-β and BMP signaling within all cell types of the developing kidney can be regulated by at least one of the I-Smads.

By combining our results with the rest of the literature, a model for TGF-β and BMP signaling can be proposed. All components of the BMP pathway including its R-Smads, Smad1, -5, and -8, are most highly expressed within mesenchymal cells within the nephrogenic zone and at the ureteric bud tips. In contrast, the components of the TGF-β signaling pathway including Smad2 and -3 are mostly detected within mesenchymal cells of the medullary interstitium and at the ureteric bud tips. This suggests that these pathways may be spatially segregated so that BMP signaling predominates in the nephrogenic zone, whereas TGF-β signaling predominates in the interstitium. The patterns of expression of the I-Smads also support this model. Smad6 is mostly reported to be a BMP inhibitor (13, 14, 20), and thus its expression in the nephrogenic zone would be consistent with this function. In contrast, Smad7 is reported to be both a TGF-β and BMP inhibitor (13), which fits with its more ubiquitous expression within the developing kidney.

According to the results, each Smad has a particular expression pattern that may influence cellular differentiation. Mesenchymal cells are known to become epithelialized in the presence of a signal from the ureteric bud. In contrast, in the absence of a ureteric bud signal, the same cells may undergo apoptosis. Mesenchymal cells can also differentiate to become stromal or interstitial cells that express molecular markers such
as BF-2, Pod-1, and retinoic acid receptor-α and -β (15, 24, 32, 33). Some of the factors that are known to induce mesenchymal-to-epithelial transformation include Wnt4 (21) and leukemia inhibitory factor (3, 31). Plisov et al. (31) have shown that leukemia inhibitory factor with TGF-β2 and FGF2 enhances Wnt signaling by promoting Tcf1/Lef1 DNA-binding activity. Furthermore, they demonstrated in electrophoretic mobility shift assays that Smad4 was able to interact with Tcf1 and Lef1. These results suggest that TGF-β signaling plays a role in mesenchymal cell differentiation. We speculate that the types of Smads expressed and their relative levels may influence whether a mesenchymal cell will become epithelialized, undergo apoptosis, or become an interstitial stromal cell. By colabeling ureteric bud derivatives with *D. biijorus*, our data also demonstrate that all Smads are expressed in the tips of the growing ureteric bud and can therefore affect renal branching morphogenesis. In the future, conditional kidney-specific knockouts for individual Smads will be important tools with which to clarify the roles of these transcription factors in effecting mesenchymal and ureteric bud cell fates.

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