Bone morphogenetic protein-7 (BMP7) is expressed in adult kidney and reduces renal fibrogenesis when given exogenously to rodents with experimental chronic nephropathies. In mesangial cells that regulate glomerular fibrosis in vivo, BMP7 inhibits transforming growth factor-β (TGF-β)-driven fibrogenesis, primarily by preventing the TGF-β-dependent down-regulation of matrix degradation and up-regulation of PAI-1. The signals and mechanisms of the BMP7 opposition to actions of TGF-β are unknown. Here we show in mesangial cells that BMP7 reduces nuclear accumulation of Smad3 and blocks the transcriptional up-regulation of the TGF-β/Smad3 target, CAGA-lux. Smad5 knock-down impairs the ability of BMP7 to interfere with the activation of CAGA-lux and the accumulation of PAI-1 by TGF-β indicating that Smad5 is required. Smad5 knock-down also reduces the rise in Smad6 upon BMP7. Forced expression of smad5 (found to be the preferred BMP7-induced receptor-activated Smad signal in mesangial cells) or of smad6 mimics BMP7 in opposing the increase in transcriptional activation of PAI-1 and its secretion upon TGF-β. This suggests a model for the BMP7-induced opposition to TGF-β-dependent mesangial fibrogenesis requiring Smad5; the model involves the inhibitory Smad6 downstream of Smad5 as well as reduced availability of Smad3 in the nucleus. BMP7 does not require signaling through Erk1/2, p38, or JNK and does not utilize the TGF-β transcriptional co-repressors Ski or SnoN in mesangial cells. These studies provide first insights into mechanisms through which BMP7 opposes TGF-β-induced glomerular fibrogenesis.

Bone morphogenetic protein-7 (BMP7), a member of the TGF-β superfamily of cysteine knot cytokines, plays pivotal roles during embryonic renal and eye development (1, 2). In adults, BMP7 expression is retained in only a few tissues, most prominently in kidney (3–5). Its physiologic functions in the adult kidney are unknown presently. Observations from this and other laboratories recently have shown that renal BMP7 expression declines progressively in early experimental diabetic or obstructive nephropathy, predating the histological onset and progression of glomerular and tubulointerstitial fibrosis (5, 6). Although renal glomerular and tubulointerstitial fibrogenesis share some of their regulatory mechanisms, they are two different processes, do not necessarily occur simultaneously, and involve different cell types.

Renal fibrosis is the terminal, mutual fate of many chronic renal diseases and causes kidney failure requiring dialysis or kidney transplantation. In experimental rodent models of obstructive or diabetic nephropathy, exogenously administered rhBMP7 reduces glomerular and tubulointerstitial fibrosis and preserves renal function (7, 8). Remarkably, therapy with rhBMP7 even can resolve early, established glomerular and interstitial fibrosis in rats with diabetic nephropathy (8). Because fibrogenesis in both of these experimental diseases is heavily TGF-β-dependent, we hypothesized that BMP7 may antagonize TGF-β-induced fibrogenic events. Indeed, in cultured mesangial cells that were incubated with TGF-β, co-incubation with BMP7 reduced accumulation of collagen and fibronectin. Some of these effects of BMP7 apparently result from inhibition of the TGF-β effects on fibrosis mediators and matrix protein degrading enzymes (MMP2) as well as their regulators (such as PAI-1) (9). Thus, BMP7 may induce signals that interfere with the actions of TGF-β.

In some cell types TGF-β and BMPs may utilize non-Smad signal pathways involving ERK, p38MAPK, or JNK-c-Jun (5, 10) in a condition-dependent manner. However, Smad2 and especially Smad3 are thought to be the primary TGF-β-induced fibrogenesis signals in most cells, and renal fibrogenesis may require Smad3 (11–13). Activation of TGF-β-receptors by their ligand causes the phosphorylation and activation of Smad substrates, Smad2 and -3, their heterodimerization with Smad4, translocation into the nucleus, and interaction with other regulatory proteins and specific gene targets (14). As a group, BMPs can utilize another series of receptor-activated (R-)Smads, namely Smad1 and Smad5 (15). Smad6 and -7 are inhibitory Smads that interfere with the phosphorylation of R-Smads and with their association with Smad4 (16). Smad6 is induced by BMPs and inhibits TGF-β and BMP signaling. Smad7 is induced by TGF-β and inhibits TGF-β signals in a negative feedback manner (17–19).

In TGF-β as well as BMP7 signaling, Smad4 acts as a transcriptional co-activator together with Smad2 and -3 or Smad1 and -5, respectively. TGF-β-dependent transcriptional activation of gene targets by Smad2 and -3 is modified further by other proteins, which include the co-repressors Ski and SnoN. SnoN appears to be a selective inhibitor of Smad3-dependent transcription. Both Ski and SnoN bind to Smad3 and may not interfere with nuclear translocation but rather prevent Smad3-induced transcriptional activation at target Smad binding ele-
EXPERIMENTAL PROCEDURES

Cell Culture Models—Experiments were performed in murine mesangial cells, which were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (3:1) containing 14 mM HEPES and 5% fetal bovine serum (23). Prior to each experiment cells were incubated in serum-free medium containing 0.1% bovine serum albumin for 24 h.

Some experiments were performed in mink lung epithelial cells (MLECs) stably expressing a PAI-1 promoter/lux construct (24). These cells were provided by Dan Rifkin, New York University, New York, NY. Cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% fetal bovine serum and G418 supplement.

Phosphorylation of Erk1/2, p38, and JNK was examined in mesangial cells in 6-well plates that were incubated without (control) or with rhBMP7, CGDH-5 kind gift from John McCartney, Curis, Cambridge, MA, rhTGF-β1, (BIOSOURCE, Camarillo, CA), or both combined each at 1 nM for 0.5–4 h. Cells were lysed in radioimmune precipitation assay buffer containing protease and phosphatase inhibitors. Aliquots of lysates were electrophoresed in SDS-polyacrylamide gels and then electrotransferred to nitrocellulose, and membranes underwent Western analysis with anti-phospho-Erk1/2 or anti-total Erk1/2 (Santa Cruz Biotechnology, Santa Cruz, CA). In other aliquots, Western blot analysis was performed with monoclonal anti-phospho-p38 and anti-total p38, or anti-phospho-JNK, or -total JNK (Santa Cruz Biotechnology). Bands were visualized with enhanced chemiluminescence and captured on x-ray film.

Nuclear Translocation of Smad3 and Smad5 upon Incubation with rhBMP7 and rhTGF-β—Mesangial cells were incubated with rhBMP7 (200 pM), rhTGF-β1 (200 pM) or both for 2 h, and cytoplasmic and nuclear extracts were prepared as described above. Aliquots underwent electrophoresis in 10% SDS-polyacrylamide gels and separated proteins were transferred to nitrocellulose. Smad5 and Smad6 were visualized by undergoing Western blot analysis with anti-FLAG antibody (Affinity Bioreagents, Golden, CO) and monoclonal anti-myc antibody (Santa Cruz Biotechnology), respectively. Subsequently, membranes were washed with deionized water, stripped with 200 mM NaOH, washed again, and blotted with monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Flanders, Pierce) and anti-histone H4 (Cell Signaling Technology, Beverly, MA) for loading control. Quantitative assessments were made after densitometry using internal controls.

Nuclear and Cytoplasmic Levels of Smad3 and Smad7 upon Incubation with rhBMP7 and rhTGF-β—Mesangial cells were incubated with rhBMP7 (200 pM), rhTGF-β1 (200 pM) or both for 2 h, and cytoplasmic and nuclear extracts were prepared as described above. Aliquots underwent electrophoresis in 10% SDS-polyacrylamide gels and separated proteins were transferred to nitrocellulose. Smad5 and Smad7 were visualized by undergoing Western blot analysis with anti-Smad1 antibody (Affinity Bioreagents), respectively. Subsequently, membranes were washed with deionized water, stripped with 200 mM NaOH, washed again, and blotted with monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase and anti-histone H4 for loading control.

Expression of Inhibiting Smad6 and Smad7 upon BMP7 and TGF-β in Mesangial Cells—Cells were incubated with serum-free medium containing rhBMP7 or rhTGF-β1 (200 pM) for 24 h. Total RNA was extracted with the RNA-Stat60 reagent and the manufacturer’s recommended method (Tel-Test, Friendswood, TX). Smad6 and -7 mRNA levels were measured by reverse transcriptase PCR using specific primers for Smad6 (sense: 5’-CTG AGA GTG ATC GCC AGA CC-3’, antisense: 5’-CTT GAG CAG CAG GTA GTC GA-3’) and Smad7 (sense: 5’-GAA GTC AAG AGG CTG TGT TGC-3’, antisense: 5’-CAG CTT GAA CAA GAA GGT GG-3’) with co-amplification of 18 S rRNA as the PCR product standard in each experiment. Results were expressed as ratios of densitometric units of mRNA/18 S rRNA.

Effect of Overexpressing Smad5 or Smad7 on TGF-β-induced PAI-1 Promoter Activation—Induction of PAI-1 is one of the mechanisms by which TGF-β contributes to fibrosis, i.e., the accumulation of extracellular matrix proteins. TGF-β raises PAI-1 levels by transcriptional activation, and this was used as an experimental model to test whether BMP7-dependent Smad5 or Smad6 contributes to the opposition to TGF-β. MLECs stably expressing the PAI-1/lux reporter construct were co-transfected with empty pcDNA3 or pcDNA3-smad5 expression vector or pcDNA3-smad6 (provided by Kohei Miyazono, Tokyo, Japan). Transfections were performed in 60-mm dishes containing 1.5 μg of plasmid DNA at a ratio of 12.5:1. Transfection efficiency was 25–30% as established by the immunofluorescence staining of cells. Transfected cells were seeded in 96-well plates. Upon attachment, cells were incubated with serum-free medium for 24 h and then with control medium or medium containing TGF-β at 50, 100, or 200 pM. Cells were incubated for 12 h, washed with ice-cold PBS, lysed in 1× lysis buffer, and frozen at –80 °C for at least 2 h. Luciferase activity was measured in cleared lysates with luciferase reagent (Promega) in a luminometer (Zyix Corporation Berthold Systems, Pfzerheim, Germany).

PAI-1 protein levels also were examined in mesangial cell media during incubation with TGF-β and forced expression of smad5 or smad7. Mesangial cells in 6-well plates were transiently transfected with empty pcDNA3 vector or the above smad5 or smad6 expression plasmids. Cells then were incubated in serum-free medium containing 0.1% bovine serum albumin in the presence or absence of TGF-β (200 pM for 48 h). Media were cleared by centrifugation. Heparin-binding proteins were precipitated from cleared media with 25 μl/ml of a 1:1 mixture of Heparin-Sepharose and Heparin Blocking Buffer at 4 °C. Precipitates were washed three times with PBS, extracted in reducing Laemmli buffer, and heated at 85 °C for 10 min. Proteins were electrophoretically separated in 10% SDS-polyacrylamide gels and electrotransferred to nitrocellulose. Blocked membranes were immunoblotted with anti-PAI-1 antibody (Santa Cruz Biotechnology).
TGF-β to examine whether BMP7 interferes with Smad3-mediated transcriptional activation. Furthermore, we tested whether Smad5 is required by BMP7 to reduce this TGF-β effect using a Smad5 knockdown approach.

We designed and tested four different Smad5 small interfering RNAs (siRNAs). Transfection of each of these Smad5 siRNAs into mesangial cells reduces Smad5 levels, but siRNA 1 is most effective (Fig. 1) and was used in the main experiments. Smad5 siRNA duplexes were constructed using the Silencer siRNA kit (Ambion, Austin, TX). Briefly, two 29-mer DNA oligonucleotide templates were synthesized containing a 21-nucleotide smad5-specific sequence and the 8-nucleotide leader sequence 5′-CCT GTG CTG TC-3′, which is complementary to the T7 promoter primer. The antisense strand of the Smad5 siRNA template 1 is 5′-AAG GCA GTG GAT GCT TTA GTG CCT GTG TC-3′, and the sense sequence template is 5′-AAG ACT AAA GCA TCC ACT GCC CCT GTG TC-3′. Templates for a non-silencing siRNA were used to generate a negative control with the same nucleotide content but without sequence homology in the GenBank™ database (antisense template, 5′-GAG GGG TTC TCT GGT GAC CCT GTC TC-3′; sense template, 5′-AAG TCA CGG AAA TCA ACG CCT GTG TC-3′). siRNA templates were hybridized to a T7 promoter primer, and the 3′-ends were extended by the Klenow fragment of DNA polymerase to create double-stranded transcription templates. Strands were transcribed by T7 RNA polymerase, and the resulting transcripts were hybridized to each other. The leader sequences were removed with single strand-specific ribonuclease.

Mesangial cells were seeded in 6-well plates at 2 × 10^5 cells/well. After 24 h cells were transfected with pgL3-9×CAGA-lux (kindly provided by Kohei Miyazono, Tokyo, Japan), 0.4 μg of plasmid DNA/well, and Effectene reagent at a ratio of 1:12.5. After 8 h cells were washed and co-transfected with Smad5 siRNA or non-silencing control siRNA at a final concentration of 25 nt using siPORT amine reagent (Ambion). After 16 h cells were transferred into 96-well plates, allowed to attach for 8 h, incubated with serum-free medium for 24 h, and then incubated with BMP7 (0, 12.5, 50, or 200 pM) in the presence or absence of BMP7 (1000 pM) for 16 h. Cells were washed and luciferase activity was measured luminometrically in cell lysates.

The dependence of BMP7 to interfere with the TGF-β-driven up-regulation of PAI-1 was examined in separate experiments. Mesangial cells were transfected with scrambled, non-silencing Smad5 siRNA or silencing Smad5 siRNA and then were incubated with TGF-β in the absence or presence of BMP7 (1000 pM) for 48 h. Accumulation of PAI-1 in cleared, conditioned medium was examined by Western blot analysis of heparin-affinity-precipitated proteins.

To elucidate whether Smad5 knock-down also blocks the increase in Smad5 upon BMP7, mesangial cells were transfected with non-silencing, scrambled control siRNA or with Smad5 siRNA as described above. Cells then were incubated with or without rhBMP7 (1000 pM) for 24 h. Cells were lysed in radiomicrome precipitation assay buffer containing protease inhibitors, aliquots were electrophoretically separated, and transferred proteins underwent Western blot analysis to visualize Smad5 and Smad6, respectively. Stripped membranes were reblotted with anti-glyceraldehyde-3-phosphate dehydrogenase for loading control. Smad6 bands were quantified as the ratio over glyceraldehyde-3-phosphate dehydrogenase density units and expressed in % of controls that were transfected with non-silencing RNA and not exposed to BMP7.

Statistical evaluations of quantitative data were made by analysis of variance and Newman-Keuls multicomparison test. Probabilities of p < 0.05 were considered to indicate statistical significance. Data are shown as mean ± S.E.

RESULTS

BMP7 and TGF-β Do Not Activate Erk1/2, p38, or JNK in Mesangial Cells—To elucidate whether BMP7 and TGF-β, per-
BGH7 Signals Opposing TGF-β

Fig. 3. R-Smad phosphorylation by BMP7 in mesangial cells. After metabolic 32P-labeling, cells were incubated with BMP7 for 30 min. Smad1 or -5 was immunoprecipitated (IP) with specific antibodies and electrophoretically resolved. Dried gels were autoradiographed. Representative autoradiograms of three experiments are shown. Levels of Smad1 or Smad5 were examined in similar studies in unlabelled mesangial cells. Smad5 was visualized directly by Western blot (WB) analysis, but for the assessment of Smad1, immunoprecipitation prior to Western blotting was required.

Fig. 4. Cytoplasmic (Cy) and nuclear (Nu) localization of Smad3 and Smad5 upon incubation of mesangial cells with BMP7 and/or TGF-β. Cells overexpressing flag-smad3 and myc-smad5 were incubated with BMP7 (200 pM), TGF-β (200 pM), or BMP7 and TGF-β, which raises the nuclear levels of Smad3, also reduces the BMP7-induced nuclear accumulation of Smad5. BMP7 tends to oppose the TGF-β-induced increase in nuclear Smad3 levels as indicated by a lower nuclear/cytoplasmic ratio of densitometric units. n = 6 blots from three independent experiments; *, p < 0.05 versus control; **, p = 0.043 versus TGF-β, gapdh, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 5. Nuclear and cytoplasmic levels of the TGF-β transcriptional co-repressors Ski and SnoN in mesangial cells. Incubation with TGF-β (200 pM) raises nuclear levels and lowers cytoplasmic levels of Ski but does not appear to cause major changes in the levels of SnoN. BMP7 does not affect the levels or the cytoplasmic/nuclear distribution of Ski or SnoN and does not interfere significantly with the effects of TGF-β. gapdh, glyceraldehyde-3-phosphate dehydrogenase.

In contrast to Ski, TGF-β does not change substantially the total levels or nuclear accumulation of SnoN in mesangial cells. BMP7 itself also does not affect levels or distribution of SnoN. These results suggest that BMP7 does not (up-)regulate levels or nuclear distribution of the TGF-β transcriptional co-repressors Ski or SnoN, i.e., BMP7 does not use this avenue to inhibit TGF-β effects.

Fig. 6. mRNA levels (quantitative reverse transcriptase PCR) of the two I-Smads, Smad6 and Smad7, in mesangial cells upon incubation with TGF-β or BMP7 (200 pM); n = 6 each; *, p < 0.05.

TGF-β and BMP7 Differentially Regulate Smad6 Expression—Similar to its effects in other cell types, BMP7 (200 pM) substantially increases expression of Smad6 in mesangial cells. In contrast, isomolar TGF-β reduces Smad6 mRNA levels significantly by about 50% below base line (Fig. 6). As expected, TGF-β increases Smad7 mRNA levels, but BMP7 has no effect on the expression of this latter inhibiting Smad (Fig. 6). Thus, Smad6 expression is differentially regulated by BMP7 and TGF-β and may be a BMP7 signal that antagonizes the fibrogenic effects of TGF-β.

Smad5 and Smad6 Raise the Resistance to TGF-β-dependent Activation of the PAI-1 Promoter—MLECs stably expressing the PAI-1-lux construct were used as a model to examine whether Smad5 or Smad6 reduces TGF-β effects on the transcriptional activation of PAI-1. As shown in Fig. 7, forced expression of smad5 substantially increases the resistance to TGF-β to increase PAI-1 promoter-driven luciferase activity. Similarly, overexpression of smad6 also reduces the ability of TGF-β to activate the PAI-1 promoter efficiently (Fig. 7).

This phenomenon also can be demonstrated in mesangial cells. In control cells that are transfected with empty pcDNA3 vector, TGF-β (200 pM) increases secreted PAI-1 protein levels (Fig. 8). Overexpression of smad5 or smad6 each reduces the amount of PAI-1 that accumulates in the media upon the addition of TGF-β.

BMP7 Requires Smad5 for Efficient Inhibition of TGF-β-dependent, Smad3-mediated Transcriptional Activation—Transcriptional activation of the 9×CAGA-lux construct by TGF-β is Smad3-dependent (25). In mesangial cells that express 9×CAGA-lux, luciferase activity is increased by TGF-β in a concentration-dependent manner (Fig. 9a). However, co-incubation with BMP7 inhibits this effect of TGF-β (Fig. 9a, top panel). Because Smad5 is the preferred BMP7 R-Smad in mesangial cells, we tested whether Smad5 knock-down would prevent this effect of BMP7. Indeed, smad5 silencing dramatically reduces the ability of BMP7 to interfere with TGF-β Smad3-driven activation of the CAGA-lux construct (Fig. 9a, bottom panel).

TGF-β also increases the secretion and accumulation of PAI-1 in conditioned mesangial cell media. Co-incubation with BMP7 partially blocks this effect of TGF-β. This ability of BMP7 to inhibit the TGF-β-driven increase of PAI-1 in mesangial cell media is altered substantially during smad5 knock-down (Fig. 9b). Thus, the dependence of BMP7 on intact Smad5 levels also is demonstrated in the regulation of PAI-1 levels, which are directly relevant to glomerular fibrogenesis.

Smad5 Knock-down Also Blocks Efficient Up-regulation of Smad6 by BMP7—In control cells that were transfected with non-silencing siRNA, BMP7 substantially increased Smad6 levels (Fig. 10). Transfection of cells with Smad5 siRNA blocks...
knock-down reduces the efficiency of BMP7 to inhibit the TGF-β/H9252 transition during exposure to TGF-β/sangial cell media. In control cells that were transfected with non-silencing Western blot analysis after affinity precipitation from me-

MLECs that stably express PAI-1/lux, compared with empty vector controls (Fig. 7). Overexpression of smad5 or smad6 reduces the accumulation of PAI-1 in mesangial cells upon TGF-β/H9252 activation in mesangial cells. Taken together, these recently published findings indicate that Smad5 transmits and is required for efficient this effect of BMP7. In conjunction with data in Figs. 7 and 9 indicating that Smad5 transmits and is required for efficient opposition of BMP7 to some effects of TGF-β, this finding suggests that Smad6 participates downstream of Smad5 and may mediate most but perhaps not all of the BMP7 opposition to TGF-β.

DISCUSSION

BMP7 is required during nephrogenesis where it promotes an epithelial phenotype at cell fate decision points (1). Renal expression of BMP7 is maintained in normal adult kidney, but the physiological renal functions of this cytokine are unknown. Circumstantial evidence suggests an anti-fibrogenic role of BMP7 in kidney. Exogenous administration of rhBMP7 to rodents with obstructive or diabetic nephropathies reduces onset and progression of glomerular and interstitial fibrosis (6–8). Hruska and co-workers (8) recently demonstrated that rhBMP7 even may cause regression of early diabetic glomerular sclerosis and interstitial fibrosis. Glomerular fibrogenesis in these and other models of progressive renal diseases is TGF-β-dependent. Hence, BMP7 may act, at least in part, by reducing TGF-β effects. Indeed, in glomerular mesangial cells, BMP7 antagonizes several TGF-β-induced, profibrogenic events and mediators that contribute to the accumulation of the extracel-

ular matrix. Moreover, in a recent series of studies, Zeis-
berg et al. (27) confirm our previous finding that BMP7 antagonizes at least some of the profibrogenic effects of TGF-β. These investigators show that BMP7 opposes TGF-β-induced epithelial-mesenchymal transition in tubular and mammary epithelial cells. Taken together, these recently published findings give rise to the possibility that BMP7 interferes with TGF-β signals or alters TGF-β signaling efficiency (perhaps by a variety of mechanisms in different cell types).

The primary and arguably most efficient fibrogenic TGF-β signals are Smad2 and -3, and between these two Smad3 appears to be the dominant fibrosis mediator for TGF-β in vitro and in vivo (11–13, 28). In some cells, non-Smad signals that are induced upon TGF-β include MEK/ERK, JNK, and/or p38 activation. They appear to function primarily as Smad2 and -3 signal modifiers and may further facilitate or oppose Smad signal efficiency. Activation of the MEK kinase 1-JNK-c-Jun pathway recently has been shown to oppose TGF-β-dependent transcriptional activation of connective tissue growth factor (29, 30). In some conditions BMP7 may activate JNK (31), but this was not confirmed in the present experiments in mesangial cells. In proximal tubular epithelial cells TGF-β as well as BMP7 activate ERK (5). In the present studies in mesangial cells, relatively low levels of TGF-β and/or BMP7 do not change the Erk1/2 phosphorylation stage; similarly, p38 phosphoryla-

tion also is not increased in mesangial cells under either condition, suggesting that the antagonistic mechanisms of BMP7 to some of the effects of TGF-β do not require Erk1/2, p38, or JNK. Hayashida et al. (32, 33) found that TGF-β can activate Erk1/2 in mesangial cells; that activation then facilitates Smad2 and -3 signals through phosphorylation in their linker region. Other investigators found that TGF-β-dependent ERK activation may oppose in a cell context-dependent manner Smad2 and -3 signals by attenuating nuclear translocation.
Effects of TGF-β on gene targets also are regulated by transcriptional repressors. Ski and SnoN have been shown to affect TGF-β transcriptional activity (35). For this reason we examined whether BMP7 increases the levels of Ski or SnoN or raises their nuclear translocation in mesangial cells. However, neither of these effects was observed in the present studies. Hence, it is unlikely that BMP7 reduces TGF-β effects through Ski or SnoN.

Smad5 appears to be the major signal for the antagonistic actions of BMP7 to TGF-β fibrogenesis as suggested by several observations from the present studies. These include increased resistance to TGF-β effects on PAI-1 in cells overexpressing Smad5 (Figs. 7 and 8). In fact, BMP7 requires Smad5 to oppose transcriptional effects of TGF-β on CAGA-Smad binding elements and secreted PAI-1 in mesangial cells as shown in Smad5 knock-down experiments (Fig. 9). The importance of Smad5 in mediating the antifibrogenic effects of BMP7 is further supported by the experiments of Zeisberg et al. (27), which showed that forced expression of Smad5 prevents TGF-β-induced epithelial to mesenchymal transition. The present experiments extend these observations to a mesenchyme-derived cell type, namely mesangial cells. They further show that here Smad5 actually is required for BMP7 opposition to TGF-β and acts largely but perhaps not exclusively through Smad6.

Smad6 is induced by BMP7 downstream of Smad5 through a Smad1- and Smad5-responsive promoter element (36). Several findings from the present experiments give rise to the involvement of Smad6 in the antagonism of TGF-β by BMP7 in mesangial cells. First, Smad6 interferes with TGF-β R-Smads and reduces Smad2/3 signal transduction to nuclear targets (17, 18, 37). However, Smad6 also inhibits BMP-Smad signaling. Second, consistent with previous observations, Smad6 provides resistance to TGF-β-induced PAI-1 promoter activation in the present studies (Figs. 7 and 8). Third, TGF-β and BMP7 regulate smad6 expression in mesangial cells differentially (Fig. 6), and smad5 knock-down hinders the rise in Smad5 upon BMP7 (Fig. 10). Thus, Smad6 participates in the effects of BMP7 to oppose TGF-β actions in mesangial cells and acts downstream of Smad5. Our findings do not exclude additional non-Smad6 pathways downstream of Smad5 but suggest that these pathways are not required and are perhaps quantitatively minor.

The transcriptional activation of PAI-1 by TGF-β, which importantly contributes to glomerular fibrogenesis, requires the interaction of Smad3 with CAGA-Smad binding elements in the PAI-1 promoter (25). Because Smad5 blocks this effect of TGF-β/Smad3, some direct or indirect interactions between these two Smads may mediate the effects of BMP7 on TGF-β actions. The nature of this Smad5/Smad3 interference is not revealed from the present experiments. Findings in Fig. 4 give rise to possible inhibition of nuclear accumulation of Smad3 by Smad5 and vice versa. Physical competition between Smad3 and Smad5 for nuclear import, i.e. interaction with importin β1 and/or the nucleopor complex perhaps, is unlikely given the large number of different proteins that use this avenue for nuclear import and the very high rate of nuclear translocation (38). Perhaps the reduction of nuclear Smad3 levels upon co-incubation with BMP7 is a secondary effect of TGF-β signal inhibition by Smad5 and/or Smad6. In this respect, a recent model of signal transport between cell surface receptors and nuclear targets may apply (39–41). In this model, activated R-Smads are transported into the nucleus, interact with target genes, are subsequently dephosphorylated, and then return to the cytoplasm for potential reactivation as long as there is ligand occupancy of the receptor and unopposed signal transduction. Applying this model, a reduced nuclear/cytoplasmic Smad3 ratio would be indicative of interruption of TGF-β/Smad signaling somewhere in the pathway, and Smad6 is an excellent candidate to cause such interruption.

In summary, the present in vitro studies indicate that BMP7-induced inhibition of some TGF-β-dependent profibrogenic activities in mesangial cells such as up-regulation of PAI-1 requires Smad5 and is mediated downstream, at least in large part, by Smad6. In its opposition to TGF-β, BMP7 does not require activation of Erk1/2, p38, or JNK1/2 and does not utilize the TGF-β transcriptional co-repressors Ski or SnoN.

Acknowledgments—We thank John McCartney (Curis, Cambridge, MA) for the provision of rhBMP7 and Dan Rifkin (New York, NY) for providing the MLsCs that stably express PAI-1/lux. We also appreciate the kind gifts of the smad3 and smad5 constructs from Serhui Souchelnytskyi and Aris Moustakas (Upsala, Sweden) and the smad6- and xCAGA-lux plasmids from Kohei Miyazono (Tokyo, Japan).

REFERENCES

1. Dudley, A. T., Lyons, K. M., and Robertson, E. J. (1995) Genes Dev. 9, 2795–2807
2. Vukicevic, S., Kopp, J. B., Layton, F. P., and Sampath, T. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9021–9026
3. Bosukonda, D., Shih, M. S., Sampath, T. K., and Vukicevic, S. (2000) Kidney Int. 58, 1902–1911
4. Simon, M., Mariesch, J. G., Harris, S. E., Hernandez, J. D., Arar, M., Olson, L., and Abboud, H. E. (1998) Am. J. Pathol. 152, F389–F392
5. Wang, S., Wu, J., and Hirschberg, R. (2000) Am. J. Physiol. 279, F130–F143
6. Morrissey, J., Hruska, K., Guo, G., Wang, S., Chen, Q., and Klahr, S. (2002) J. Am. Soc. Nephrol. 13, 531–541
7. Wang, S., Chen, Q., Simon, T. C., Strebeck, F., Chaudhary, L., Morrissey, J., Liapis, H., Klahr, S., and Hruska, K. A. (2003) Kidney Int. 63, 2037–2049
8. Wang, S., and Hirschberg, R. (2000) Am. J. Physiol. 278, F1006–F1013
9. Lai, C. F., and Cheng, S. L. (2002) J. Biol. Chem. 277, 15514–15522
10. Piek, E., Ju, W. J., Heyer, J., Escalante-Alcalde, D., Stewart, C. L., Weinstein, M., Deng, C., Kacherlapati, R., Bottiger, E. P., and Roberts, A. B. (2001) J. Biol. Chem. 276, 19945–19953
11. Roberts, A. B., Piek, E., Bottiger, E. P., Ashcroft, G., Mitchell, J. B., and Flanders, K. C. (2001) Chest 120, Suppl. 1, 43S–47S
12. Fujimoto, M., Macewaza, Y., Yokote, K., Itoh, K., Kobayashi, K., Kawamura, H., Nishimura, M., Roberts, A. B., Saito, Y., and Mori, S. (2003) Biochem. Biophys. Res. Commun. 305, 1002–1007
13. Aragona, L., and Wroblewski, G. E. (1988) Kidney Int. 33, 626–27
14. Miyazono, K., Kusanagi, K., and Inoue, H. (2001) J. Cell. Physiol. 190, 265–276
15. Kawabata, M., Imamura, T., and Miyazono, K. (1998) Cytokine Growth Factor Rev. 9, 49–61
16. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) Nature 389, 622–626
17. Kieffer, J., Maruyama, H., Fries, H., Buchler, M. W., Falb, D., and Korc, M. (1999) Biochem. Biophys. Res. Commun. 255, 263–272
18. Zhao, J., Crowe, D. L., Castillo, C., Wuewensell, C., Chai, Y., and Warburton, D. (2000) Mech. Dev. 93, 71–81
19. Watton, D., and Massague, J. (2001) Curr. Top. Microbiol. Immunol. 254, 145–164
20. Xu, W., Angelis, G., Danielpour, D., Haddad, M. M., Bischof, O., Campisi, J., Stevanovic, E., and Medrano, E. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5924–5929
21. Mizude, M., Haru, T., Furuya, T., Takeda, M., Kusunagi, K., Inada, Y., Mori, M., Imamura, T., Miyawasaki, K., and Miyazono, K. (2003) J. Biol. Chem. 278, 531–536
22. MacKay, K., Striker, L. J., Elliot, S., Blain, S., and Lo, R. (2000) Biochem. Biophys. Res. Commun. 276, 266–272
23. Dennis, S., Bhat, S., Bivin, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1994) EMBO J. 13, 3511–3520
24. Massague, J., Blain, S., and Lo, R. (2000) Cell 103, 295–309
25. Zeisberg, M., Hanai, J., Sugimoto, H., Mammoto, T., Charytan, D., Strutz, F., and Kalluri, R. (2003) J. Biol. Chem. 278, 9641–9648
26. Sato, M., Murakagi, Y., Sakai, S., Roberts, A. B., and Oshima, A. (2003) J. Clin. Oncol. 112, 1486–1494
27. Lee, M. J., Yang, C. W., Jin, D. C., Chang, Y. S., Bang, B. K., and Kim, Y. S. (2003) J. Immunol. 170, 2557–2563
28. Hayashida, T., DeCaestecker, M., and Schnaper, H. W. (2003) FASEB J. 17, 23205
33. Hayashida, T., Poncelet, A., Hubchak, S., and Schnaper, H. (1999) *Kidney Int.* **56**, 1710–1720
34. Kretzschmar, M., Doody, J., Timokhina, I., and Massagué, J. (1999) *Genes Dev.* **13**, 804–816
35. He, J., Tegen, S. B., Krawitz, A. R., Martin, G. S., and Luo, K. (2003) *J. Biol. Chem.* **278**, 30540–30547
36. Ishida, W., Hamamoto, T., Kusanagi, K., Yagi, K., Kawabata, M., Takehara, K., Sampath, T. K., Kato, M., and Miyazono, K. (2000) *J. Biol. Chem.* **275**, 6075–6079
37. Choy, L., Skillington, J., and Derynck, R. (2000) *J. Cell Biol.* **149**, 667–682
38. Kuersten, S., Ohno, M., and Mattaj, I. W. (2001) *Trends Cell Biol.* **11**, 497–503
39. Xiao, Z., Watson, N., Rodriguez, C., and Lodish, H. F. (2001) *J. Biol. Chem.* **276**, 39404–39410
40. Xiao, Z., Brownawell, A. M., Macara, I. G., and Lodish, H. F. (2003) *J. Biol. Chem.* **278**, 34245–34252
41. Inman, G. J., Nicolas, F. J., and Hill, C. S. (2002) *Mol. Cell* **10**, 283–294
Bone Morphogenetic Protein-7 Signals Opposing Transforming Growth Factor β in Mesangial Cells
Shinong Wang and Raimund Hirschberg

J. Biol. Chem. 2004, 279:23200-23206.
doi: 10.1074/jbc.M311998200 originally published online March 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311998200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 41 references, 19 of which can be accessed free at http://www.jbc.org/content/279/22/23200.full.html#ref-list-1