Bone morphogenetic protein (BMP)-7 exerts dose-dependent stimulatory and inhibitory effects during renal branching morphogenesis. Previously, we identified an inhibitory role for activin-like kinase receptors and Smad1 in BMP-dependent inhibition (Piscione, T. D., Phan, T., and Rosenblum, N. D. (2001) Am. J. Physiol. 280, F19–F33). Here we demonstrate a novel role for p38 mitogen-activated kinase (p38MAPK) in BMP7-dependent stimulatory signaling. Stimulatory doses (0.25 nM) of BMP7 increased p38MAPK activity and stimulated phosphorylation of endogenous activating transcription factor 2 (ATF2) in a p38MAPK-dependent manner in murine inner medullary collecting duct (mIMCD-3) cells. In contrast, high doses (10 nM) of BMP7 inhibited p38MAPK activity and phosphorylation of endogenous ATF2. Treatment with BMP7 exerted no significant effect on the levels of the phosphorylated forms of endogenous SAPK/JNK or p44 and p42 (ERK1 and ERK2) protein kinases. To investigate the functional importance of p38MAPK signaling, we showed that SB203580, a p38MAPK inhibitor, blocked the stimulatory effect of BMP7 on mIMCD-3 cell morphogenesis but had no effect on BMP7-dependent inhibition in a three-dimensional culture model. To identify mechanisms by which BMP7-dependent inhibitory signaling suppresses p38MAPK activity, we measured p38MAPK activity in ligand independent mIMCD-3 models of enhanced and suppressed Smad signaling. Basal activity of p38MAPK was decreased in mIMCD-3 cells and in embryonic kidney tissue expressing a constitutively active activin-like kinase receptor, but was increased in mIMCD-3 cells stably expressing a dominant negative form of Smad1. We conclude that BMP7 stimulates renal epithelial cell morphogenesis via p38MAPK and that p38MAPK activity is negatively regulated by Smad1.

Renal branching morphogenesis, defined as growth and branching of epithelial tubules during embryogenesis, is dependent on reciprocal inductive tissue interactions between the mesenchymal metanephric blastema and the epithelial ureteric bud and its daughter collecting ducts. These interactions are mediated, in part, by secreted growth factors and their cognate signaling effectors. Bone morphogenetic protein (BMP)-1-7, a member of the transforming growth factor (TGF)-β superfamily, modulates renal branching morphogenesis, consistent with its spatial expression pattern during branching morphogenesis (1) and the arrested branching phenotype observed in Bmp7 null mice (2, 3). The response of ureteric bud and collecting duct cells to BMP7 is complex and distinct from that of other members of the TGF-β superfamily. BMP7 exerts dose-dependent and opposite effects on ureteric bud morphogenesis in embryonic kidney explants treated with BMP7-agarose beads and in the murine inner medullary collecting duct (mIMCD-3) cell culture model (4, 5). In contrast, BMP2, a related TGF-β superfamily member expressed during renal embryogenesis, only inhibits ureteric bud and collecting duct morphogenesis in a monophasic dose-dependent manner (4). These findings suggest that BMP7 acts via distinct intracellular signaling pathways to exert stimulatory and inhibitory effects.

BMPs initiate intracellular signaling after binding to cell surface type I (activin-like kinase (ALK)) and type II serine/threonine kinases. Upon ligand binding, the type II receptor, BMPRII, transphosphorylates and activates the ALK receptor. ALK receptors signal via Smad proteins and mitogen-activated protein kinases (MAPK). Activation of the ALK receptor leads to association and phosphorylation of a receptor-activated Smad protein. Phosphorylation induces the receptor-activated Smad to dissociate from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated receptor-activated Smad and the co-Smad, Smad 4, and induces nuclear accumulation of this complex (6). We have demonstrated that BMPs inhibit renal epithelial cell morphogenesis after binding ALK receptors and activating receptor-dependent Smads (7, 8). In the case of BMP7, high doses activate Smad1 and induce formation of Smad1-Smad4 molecular complexes. Suppression of Smad1 signaling by overexpression of a dominant negative form of Smad1 (Smad14) abrogates the inhibitory actions of BMP7 on mIMCD-3 cell morphogenesis (5). In contrast, low doses of BMP7 fail to activate Smad1 and stimulate collecting duct morphogenesis in a manner independent of Smad1 activity (5), suggesting that BMP7-dependent stimulatory signaling occurs via a Smad1-independent pathway.

BMPs have also been shown to signal via the p38 class of MAPK in nonrenal cells (9, 10). In addition, p38MAPK activity can be regulated by BMP signaling, providing a feedback mechanism to regulate the cellular response to BMPs. For example,
in MH60 cells, the ALK receptor inhibitor, Smad6, blocks BMP2-induced activation of p38MAPK (11). Because Smad6 functions by inhibiting activation of receptor-activated Smads by ALK receptors, this observation suggests that ALK receptors can activate p38MAPK. The spatial expression of p38MAPK in both the ureteric bud and metanephric blastema (12) suggests a functional role for this MAPK during kidney development. This is supported by the observation that pharmacologic inhibition of p38MAPK in embryonic explants inhibits kidney growth and induces marked mesenchymal cell apoptosis (13). Although the effects of BMPs on p38MAPK in the embryonic kidney are undefined, the presence of massive mesenchymal cell apoptosis in the BMP7 null mouse (2) suggests the possibility that loss of BMP7-dependent regulation of p38MAPK could contribute to this phenotype.

In this paper, we investigated molecular mechanisms that control BMP7-dependent stimulation of collecting duct cell morphogenesis. We demonstrate that BMP7 regulates p38MAPK activity in a dose-dependent manner, stimulating at high doses and inhibiting at high doses. In the mIMCD-3 cell culture model, we show that BMP7-dependent stimulation of epithelial cell morphogenesis is abrogated by pharmacologic inhibition of p38MAPK. By using a ligand-independent model of BMP7 signaling in which a constitutive active form of ALK2 is stably expressed in mIMCD-3 cells, we demonstrate that ALK/Smad1 signaling negatively regulates p38MAPK activity. Our observation that phosphorylation of activating transcription factor (ATF)-2, a p38MAPK target, is decreased in embryonic kidneys expressing a constitutive active ALK receptor suggests that p38MAPK, Smad interactions occur in vivo. Our results provide a molecular explanation for BMP7 dose-dependent signaling in epithelial cells. In addition, our findings suggest a mechanism controlling the biological response to increasing doses of BMP7 during renal branching morphogenesis.

EXPERIMENTAL PROCEDURES

Immunoblot and Immunoprecipitation Assays—Proteins in cell lysates were separated by SDS-PAGE, and immunoblotted. p38MAPK ATP2, SAPK/JNK, and ERK1/2 were identified with specific antibodies directed against phosphorylated or total (phosphorylated and unphosphorylated) forms (p38MAPK, 1:250 dilution, Upstate Biotechnology, Inc.; ATP2, 1:500 dilution, SAPK/JNK and ERK1/2, 1:250 dilution, Cell Signaling). For assays of p38MAPK activity, cell lysates were incubated with protein G-agarose (Amersham Biosciences) and anti-p38MAPK antibody (Upstate Biotechnology, Inc.). After washing the immunocomplex, it was resolved on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with anti-phospho-ATF2 antibody followed by anti-rabbit horseradish peroxidase (1:10,000) and chemiluminescence (7).

Proteins were washed, separated by SDS-PAGE, and immunoblotted with a rabbit anti-Smad4 antibody (Upstate Biotechnology, Inc.) (1:1000 dilution) followed by anti-rabbit horseradish peroxidase (1:10,000) and chemiluminescence (7). In contrast, an inhibitory dose of BMP7 exerted no such effect. In fact, 10 nM BMP7 inhibited p38MAPK-dependent phosphorylation of ATF2 1.5-fold compared with control (Fig. 1, A and B). In contrast, an inhibitory dose of BMP7 exerted no such effect. In fact, 10 nM BMP7 inhibited p38MAPK-dependent phosphorylation of ATF2 1.6-fold compared with control and 2.4-fold compared with 0.25 nM BMP7 (p38MAPK activity (densitometric units), control versus 0.25 nM BMP7, 52 ± 20 versus 80 ± 16, p = 0.008; control versus 10 nM BMP7, 52 ± 20 versus 33 ± 12, p = 0.048). Analysis of the total quantity of immunoprecipitated p38MAPK and ATF2 added to each assay demonstrated that changes in phospho-ATF2 were not attributable to variation in the quantity of these proteins. Consistent with the dose-dependent stimulatory and inhibitory effects of BMP7 on p38MAPK activity, a stimulatory dose of BMP7 increased phosphorylation of p38MAPK 1.6-fold (Fig. 1, A and B).
an inhibitory dose (10 nM) of BMP7 inhibited phosphorylation of p38MAPK 1.7-fold compared with control and 2.9-fold compared with 0.25 nM BMP7 (P-p38MAPK (densitometric units), control versus 0.25 nM BMP7, 48 ± 7 versus 78 ± 7, p 0.0001; control versus 10 nM BMP7, 48 ± 7 versus 27 ± 5, p 0.0001). Taken together, these results indicate that BMP7 modulates p38MAPK activity in a biphasic dose-dependent manner.

Next, we determined whether BMP7 modulates the activity of endogenous ATF2. By using antibodies specific for the phosphorylated (activated) form of ATF2 and both phosphorylated and unphosphorylated (total) forms of ATF2, we detected endogenous phosphorylated and total ATF2 in immunoblots of cellular proteins isolated after treatment with recombinant BMP7. A stimulatory dose (0.25 nM) of BMP7 increased endogenous P-ATF2. In contrast, high dose BMP7 inhibited phosphorylation of p38MAPK. In contrast, high dose BMP7 inhibited phosphorylation of p38MAPK. In contrast, high dose BMP7 inhibited phosphorylation of p38MAPK.

**Fig. 1.** BMP7 increases phosphorylation of ATF2 in a p38MAPK-dependent manner. A, p38MAPK-dependent phosphorylation of ATF2 and phosphorylation of p38MAPK in BMP7-treated mIMCD-3 cells. p38MAPK was isolated from cell lysates by immunoprecipitation and added to a kinase assay utilizing ATF2 as a substrate. P-ATF2, T-ATF2, and p38MAPK were identified in immunoblots using specific antibodies. Phosphorylated (P-p38MAPK) and total (T-p38MAPK) forms of p38MAPK were identified in cell lysates using specific antibodies. B, left panel, quantitation of P-ATF2 identified in A. The amount of P-ATF2 was controlled for the quantity of p38MAPK in each sample. Low dose BMP7 stimulated p38MAPK-dependent phosphorylation of ATF2. In contrast, high dose BMP7 was inhibitory. *, p ≤ 0.05. n = 6 independent experiments. Right panel, quantitation of endogenous P-p38MAPK identified in A. The amount of P-p38MAPK was controlled for the quantity of T-p38MAPK in each sample. Low dose BMP7 stimulated phosphorylation of p38MAPK. In contrast, high dose BMP7 inhibited phosphorylation of p38MAPK. *, p ≤ 0.05. n = 3 independent experiments. C, levels of endogenous phosphorylated ATF2 (P-ATF2) and endogenous T-ATF2 in BMP7-treated mIMCD-3 cells. Proteins isolated from the cell lysates were analyzed with antibodies specific for phosphorylated ATF2 (P-ATF2), and total ATF2 (T-ATF2), consisting of both unphosphorylated and phosphorylated forms, by immunoblotting. D, quantitation of endogenous P-ATF2 detected in C. The amount of P-ATF2 was controlled for the quantity of T-ATF2 in each sample. Low dose BMP7 increased endogenous P-ATF2. *, p ≤ 0.0001. n = 4 independent experiments. E, effect of SB203580 (SB) on BMP7-dependent phosphorylation of ATF2. mIMCD-3 cells were treated with or without BMP7 in the absence (vehicle (V) only) or presence of 1 μM SB203580. Endogenous P-ATF2 and T-ATF2 were detected in immunoblots using specific antisera. p38MAPK activity was measured using a kinase assay as in A. F, quantitation of endogenous P-ATF2 detected in E. The amount of P-ATF2 was controlled for the quantity of T-ATF2 in each sample. Treatment with SB203580 decreased the basal levels of endogenous P-ATF2 and blocked the increase in P-ATF2 observed after treatment with 0.25 nM BMP7. *, p ≤ 0.05. n = 4 independent experiments.
BMP7 Stimulates Epithelial Cell Morphogenesis via p38MAPK

To determine whether BMP7 activates ATF2 via p38MAPK, we measured the cellular levels of phospho-ATF2 in the presence or absence of SB203580, a pharmacologic p38MAPK inhibitor (18). Treatment with SB203580 decreased the basal levels of endogenous P-ATF2 2.3-fold and abrogated the increase in P-ATF2 observed after treatment with low dose (0.25 nM) BMP7 (Fig. 1, E and F, P-ATF2/T-ATF2 (densitometric units), vehicle vs SB203580, 37 ± 12 versus 16 ± 8, p = 0.01; 0.25 nM BMP7 vs 0.25 nM BMP7 and SB203580, 60 ± 10 versus 18 ± 3, p < 0.01). Taken together, these results demonstrate that low doses of BMP7 stimulate phosphorylation of ATF2 in a p38MAPK-dependent manner.

BMP7 Does Not Control SAPK/JNK and ERK Activity in Collecting Duct Cells—To determine whether the effects of BMP7 are specific to the p38 branch of the MAPK family, we measured SAPK/JNK and ERK1/2 activation in the presence of low and high doses of BMP7. A possible role for these kinases in BMP7 signaling is suggested by the previous findings that JNK and ERK are activated by BMPs in nonrenal cells (19) and that ERK acts downstream of non-BMP growth factors including hepatocyte growth factor and EGF to control epithelial morphogenesis (20). By using antibodies specific for the phosphorylated (active) forms of SAPK/JNK, phospho-p54 and phospho-p46, and ERK, phospho-p44 and phospho-p42, and both phosphorylated and unphosphorylated forms (total) of these proteins, we detected endogenous proteins in immunoblots of cellular proteins after treatment with low or high doses of BMP7 (Fig. 2, A and B). Our results indicate no significant effect of BMP7 on SAPK/JNK or ERK activation, thus demonstrating a specific effect of BMP7 on p38MAPK.

Inhibition of p38MAPK Blocks BMP7-mediated Stimulation of Collecting Duct Cell Morphogenesis—By having determined that BMP7 stimulates and inhibits p38MAPK in a dose-dependent manner reminiscent of its actions during epithelial morphogenesis, we analyzed p38MAPK function by inhibiting p38 pharmacologically in the mIMCD-3 culture model of collecting duct morphogenesis. We have used mIMCD-3 cells, which are derived from the terminal inner medullary collecting duct of the SV40 transgenic mouse, to determine the effects of BMP7 on cellular morphogenesis and proliferation (4, 5, 7). mIMCD-3 cells were seeded in type I collagen, cultured for 48 h in the presence of a wide range of BMP7 doses with or without SB203580, and then imaged by DIC microscopy (Fig. 3A). Because we observed an effect on the number of tubular progenitors, observed in this assay as linear structures, we quantitated the number of these structures in different treatment groups (Fig. 3, B and C). Consistent with our previously published results, BMP7 increased the number of linear structures in a dose-dependent manner demonstrating maximal stimulation of 1.6-fold at a dose of 0.25 nM. This BMP7-dependent stimulatory effect was ~3-fold less potent that that observed after treatment with EGF. The greater potency of EGF may due to its property of stimulating both ERK and p38MAPK (21, 22). In contrast, BMP7, at doses greater than 1 nM, inhibited with a maximal inhibitory effect of 1.5-fold at a dose of 20 nM (Fig. 3, A and B). Remarkably, SB203580 totally abrogated the stimulatory effects of BMP7 on mIMCD-3 cell morphogenesis but did not interfere with the inhibitory effects of BMP7 (Fig. 3C). The lack of any BMP7-dependent stimulatory response in the presence of SB203580 demonstrates that p38MAPK is required for the stimulatory effects of BMP7.

In addition to the inhibitory effect of SB203580 on BMP7-dependent stimulation of mIMCD-3 cell morphogenesis, we observed a 25% decrease in the number of linear structures formed in the presence of SB203580 alone (Fig. 3, B versus C, 100 versus 78 tubule progenitors) and a diminished morphogenetic response to EGF in the presence of SB203580 (Fig. 3, B versus C, 480 versus 340 tubule progenitors). Although the observation that p38MAPK is activated by EGF in nonrenal cells (22) may explain the decreased morphogenetic activity of mIMCD-3 cells cultured in the presence of SB203580, our data do not eliminate the possibility that SB203580 exerts a more general toxic effect on cellular function impacting negatively on mIMCD-3 cell morphogenesis.

ALK2 Inhibits Collecting Duct Cell Morphogenesis in a Ligand-independent Manner—Doses of BMP7 >1 nM exert a dominant inhibitory effect on p38MAPK activation and collect-
ing duct cell morphogenesis (see above and Ref. 5). We hypothesized that this dominance may be explained by negative regulation of p38MAPK activity by Smad1 because high doses, but not low doses of BMP7, act via Smad1 (5). To study interactions between Smad and p38MAPK signaling, we generated a ligand-independent model of ALK signaling designed to avoid potentially confounding effects of BMP7 signaling via both pathways. The ALK receptor, ALK2, binds BMP7 and activates Smad1 signaling (23). A mutant ALK2 allele, Alk2Q207D, is characterized by a point mutation in its cytoplasmic GS domain and constitutively activates Smad1 in a ligand-independent manner (24). We generated stably transfected mIMCD-3 cell lines expressing tagged versions of wild-type ALK2 and ALK2Q207D, both of which were detected as a 68-kDa band by Western analysis using anti-FLAG monoclonal antibody (Fig. 4A). Several different clones were isolated. Clones expressing high levels of ALK2WT (clone 42) or ALK2Q207D (clone 26) were analyzed further first by examining the subcellular distribution of ALK3-HA-FLAG using immunofluorescence and confocal microscopy (Fig. 4B). In contrast to untransfected mIMCD-3 cells, we observed a punctate pattern of expression along the border of 81% of cells stably expressing ALK2WT and 86% of cells stably expressing ALK2Q207D. This pattern was consistent with localization of the transfected ALK2 to the peripheral cell membranes.

Next, we determined the functional effects of ALK2Q207D expression on Smad signaling by using an assay of Smad1-Smad4 molecular complex formation (Fig. 4C). Smad1-Smad4 molecular complexes were detected at very low levels under basal conditions in ALK2WT-expressing cells. In contrast, these complexes were undetectable in unstimulated mIMCD-3 cells. This difference is consistent with the prior observation that overexpression of wild-type type I BMP receptors can lead to autoactivation of receptors on the cell surface (14). Treatment of ALK2WT-expressing cells with 20 nM BMP7 resulted in the formation of Smad1-Smad4 molecular complexes as observed in mIMCD-3 cells. In ALK2Q207D-expressing cells, we detected high levels of Smad1-Smad4 molecular complexes under basal conditions (no ligand). This result was consistent with constitutive signaling by the mutant ALK2 receptor. The amount of Smad4 detected in association with Smad1 was increased after treatment with high dose BMP7 suggesting enhanced signaling via endogenous wild-type ALK2 or another ALK receptor. Our demonstration that equivalent amounts of Smad4 and Smad1 were expressed in ALK2WT- and ALK2Q207D-expressing cells indicates that these differences in Smad4 associated with Smad1 are not due to differences in the absolute amounts of Smad1 and Smad4 expressed in these cells.

We further investigated ALK2Q207D function by investigating its effects on BMP7-dependent collecting cell morphogenesis in the mIMCD-3 cell culture model (Fig. 5). First, we ana-
lyzed whether overexpression of ALK2 disrupted the dose-response to BMP7 by treating ALK2 WT-expressing cells with increasing doses of BMP7 and counting the number of linear structures formed (Fig 5, A and B). Although we observed a decrease in the absolute number of tubule progenitors consistent with our previous results in mIMCD-3 cells transfected with wild-type ALK3 (8) and wild-type Smad1 (5), the dose-dependent stimulatory and inhibitory responses to BMP7 were intact. In contrast, ALK2Q207D-expressing cells demonstrated a persistent “inhibitory” tubulogenic profile compared with ALK2WT-expressing cells (Fig. 5, A and C). The number of tubule progenitors formed by ALK2Q207D-expressing cells under basal conditions was less than one-half that observed in cultures of ALK2WT-expressing cells (Fig. 5, C versus B). In addition, ALK2Q207D-expressing cells were unresponsive to stimulatory doses of BMP7 (Fig. 5, A and C). Yet formation of tubule progenitors could be stimulated with EGF, demonstrating the viability of these cells. However, the decreased number of tubule progenitors formed by ALK2Q207D cells as compared with ALK2WT cells in response to EGF indicated a dominant effect of Smad signaling over hepatocyte growth factor signaling observed by us previously (8). Taken together, these results demonstrate that ALK2Q207D signals via Smad1 and inhibits collecting duct cell morphogenesis in a ligand-independent manner.

Activation of Receptor-activated Smads Negatively Regulates p38MAPK—We investigated interactions between the Smad signaling pathway and p38MAPK in mIMCD-3 cells in which Smad signaling is enhanced or suppressed. In ALK2Q207D-expressing cells, Smad1 signaling is up-regulated in a ligand-independent manner (see above and Fig. 4C). We measured p38 MAPK-dependent phosphorylation of ATF2 in mIMCD-3 ALK2Q207D cells in the absence of exogenous BMP7 (Fig. 6, A and B). p38MAPK-dependent phosphorylation of ATF2 was decreased 1.8-fold, suggestive of a negative regulatory effect of Smad1 on p38MAPK activity (p38MAPK activity, control cells versus ALK2Q207D cells, 118 ± 20 versus 68 ± 17, p = 0.0003). Negative regulation of p38MAPK by Smad1 predicted that p38 MAPK activity would be increased in cells with decreased Smad signaling. Previously, we demonstrated that stable expression of a dominant negative Smad1 allele, Smad1Δ458, in mIMCD-3 cells decreased ligand-dependent activation of Smad1 (5). We measured p38 MAPK activity in mIMCD-3 cells expressing Smad1Δ458 cells and demonstrated a 1.8-fold increase in p38MAPK-dependent ATF2 phosphorylation (p38MAPK activity, control cells versus Smad1Δ458 cells, 118 ± 20 versus 210 ± 17, p < 0.0001). Taken together, these results
BMP7 Stimulates Epithelial Cell Morphogenesis via p38MAPK

A.

Control (no BMP7)  
0.25 nM BMP7  
10 nM BMP7  
10 ng/ml EGF

B.

Number of Tubular Progenitors (percent expressed as untreated)

\[ 0 \quad 0.1 \quad 0.25 \quad 0.5 \quad 1.0 \quad 10 \quad 10 \]

\[ [BMP7] \quad [EGF] \]

ALK2(WT)

\[ (78) \quad (108) \quad (130) \quad (105) \quad (51) \quad (54) \quad (240) \]

\( [BMP7] \quad [EGF] \quad \text{ng/ml} \)

C.

Number of Tubular Progenitors (percent expressed as untreated)

\[ 0 \quad 0.1 \quad 0.25 \quad 0.5 \quad 1.0 \quad 10 \quad 10 \]

\[ [BMP7] \quad [EGF] \quad \text{ng/ml} \]

ALK2(Q207D)

\[ (29) \quad (24) \quad (24) \quad (24) \quad (23) \quad (82) \]

\( [BMP7] \quad [EGF] \quad \text{ng/ml} \)

Fig. 5. mIMCD-3 cells stably expressing ALK2\textsuperscript{Q207D} exhibit an inhibitory phenotype when induced to form tubule progenitors. mIMCD-3 cells expressing ALK2\textsuperscript{Q207D} were cultured for 48 h in type I collagen with culture medium supplemented with either no ligand, BMP7, or EGF. A, structures formed were imaged by DIC microscopy (×100 magnification). B and C, quantitation of effects of BMP7 on formation of tubule progenitors by mIMCD-3 cells expressing ALK2\textsuperscript{WT} or ALK2\textsuperscript{Q207D}. The number of linear structures present in four randomly selected microscopic fields is shown in parentheses below each bar. The bar graph shows the number of structures formed in each treatment group as a % of control (no BMP7). B, stable expression of ALK2\textsuperscript{WT} decreased the absolute number of tubule progenitors but did not interfere with the dose-dependent stimulatory and inhibitory effects of BMP7. C, stable expression of ALK2\textsuperscript{Q207D} inhibited in a manner identical to treatment with high doses of BMP7. *\( p < 0.05\), \( n = 3 \) independent experiments.

The arrest of branching morphogenesis observed in the dysplastic kidneys of Bmp7 null mice provides compelling evidence that BMP7 is required for normal renal development (2, 3). Our previous demonstration that recombinant BMP7 exerts dose-dependent stimulatory and inhibitory effects on renal branching morphogenesis suggested the existence of distinct signaling pathways that transduce these effects (4). This dual signaling model was strengthened by our results indicating differential signaling by high and low doses of BMP7 via Smad1. High doses (≥0.5 nM) of BMP7 require Smad1 to inhibit tubule growth and branching. In contrast, low doses (<0.5 nM) of BMP7 stimulate these processes in a Smad1-independent manner (5). In this work, we further elucidate the mechanisms controlling dose-dependent signaling BMP7. We demonstrate a distinct and novel role for p38MAPK in BMP7-dependent stimulation. Doses of BMP7 that stimulate collecting duct cell morphogenesis stimulated p38MAPK activity. In contrast, doses of BMP7 that inhibit epithelial cell morphogenesis inhibit inhibited p38MAPK. Furthermore, we show that BMP7 regulates the phosphorylation of endogenous ATF2 in a p38MAPK-dependent manner. The functional significance of these results was investigated in the mIMCD-3 culture model in which we demonstrated that pharmacologic inhibition of p38MAPK activity blocks BMP7-dependent stimulation of mIMCD-3 cell morphogenesis but does not affect BMP7-dependent inhibition.

Our results demonstrate opposing functions for p38MAPK and BMP-activated Smads in collecting duct cells. p38MAPK-Smad interactions have been investigated previously in non-renal cells. The majority of studies have focused on the effects of TGF-β. Evidence supporting cooperative interactions includes the finding that TGF-β induces phosphorylation of ATF2 in a p38MAPK-dependent manner and formation of ATF2-Smad4 molecular complexes (17). In prostatic epithelial cells, p38MAPK is required for TGF-β- and Smad3-dependent cell adhesion and nuclear translocation of Smad3 (26). In human epithelial cells, ATF3 and Smad3 act cooperatively to control Id1 promoter activity (27). In gingival fibroblasts, p38MAPK and Smad3 act cooperatively to control collagenase-3 gene expression, and nuclear translocation of Smad3 is dependent on p38MAPK activity (28). In cultured pancreatic cells, TGF-β or an activated TGF-β-responsive ALK receptor can activate p38MAPK and Smad3 is required for p38MAPK activation (29).

A limited number of studies have investigated interactions between BMP-dependent Smad signaling and p38MAPK. BMP-activated Smads and ATF2 act cooperatively to control β-myosin heavy chain gene expression and cardiomyocyte differenti-
A. mIMCD-3 Cells

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B. P-ATF2 / p38<sup>MAPK</sup>

![Graph showing P-ATF2 / p38<sup>MAPK</sup> levels in cells expressing different constructs.]

C. Embryonic Kidney

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D. P-ATF2 / T-ATF2

![Graph showing P-ATF2 / T-ATF2 levels in embryonic kidneys from control and Alk3 transgenic mice.]

**Fig. 6.** The activity of p38<sup>MAPK</sup> and ATF2 is regulated by ALK/Smad signaling. A, p38<sup>MAPK</sup>-dependent phosphorylation of ATF2 was measured in wild-type mIMCD-3 cells and in mIMCD-3 cells stably expressing Alk2<sup>Q207D</sup> or a dominant negative acting Smad1 allele (Smad1<sup>Δ458</sup>). P-ATF2, T-ATF2, and p38<sup>MAPK</sup> were detected in protein lysates using specific antibodies. B, quantitation of P-ATF2 detected in A. The amount of P-ATF2 was controlled for the quantity of immunoprecipitated p38<sup>MAPK</sup> in each sample. p38<sup>MAPK</sup>-dependent phosphorylation of ATF2 was suppressed by stable expression of ALK2<sup>Q207D</sup> and was enhanced by expression of Smad1<sup>Δ458</sup>. *, p < 0.005. n = 4 independent experiments. C, top, immunofluorescence images of whole mount preparations of embryonic kidneys isolated from control and Alk3 transgenic mice at E13.5. Ureteric bud branches are stained with D. biflorus agglutinin. Bottom, immunoblots of tissue protein lysates generated from kidneys of E13.5 mice. D, quantitation of P-ATF2 detected in C. The amount of P-ATF2 was controlled for the quantity of T-ATF2 in each sample. The level of endogenous P-ATF2 was suppressed in the embryonic kidneys of Alk3 transgenic mice, isolated at the stage of branching morphogenesis. *, p < 0.01. n = 4 independent experiments.

A study of peroxisome proliferator-activated receptor-γ regulation during adipogenesis shows that both BMP2 and p38<sup>MAPK</sup> act cooperatively during the differentiation of undifferentiated mesenchymal cells into adipocytes. However, the role of Smad1 in BMP2-dependent p38<sup>MAPK</sup> activation was not addressed directly (31).

In contrast to these studies, others that show TGF-β-dependent induction of Smad and p38<sup>MAPK</sup> signaling do not demonstrate a dependence of p38<sup>MAPK</sup> effects on Smads and vice versa. Expression of a dominant negative form of Smad3 or the ALK inhibitor, Smad7, did not interrupt TGF-β-dependent p38<sup>MAPK</sup> activation in NMuMG mammary epithelial cells (32). Similarly, in the MDA-231 cell model of breast cancer, both TGF-β and p38<sup>MAPK</sup> control expression of parathyroid hormone-related protein, but p38<sup>MAPK</sup> activation is not Smad-dependent (33). Taken together, these studies consistently demonstrate that TGF-β and BMP2 activate both Smad and p38<sup>MAPK</sup>-dependent signaling pathways. However, the interdependency
of these pathways appears to be due, in part, to the cellular context in which signaling is initiated. Although our study provides additional insight into the interplay between p38MAPK and Smad signaling, it does not address the molecular mechanism that transduces BMP7-dependent activation of p38MAPK distinct from Smad activation. It is possible that the dose-dependent effects of BMP7 might be explained by binding to distinct ALK receptors in a manner dependent on the dose of BMP7. Such a model would further suggest that distinct ALK receptors have differential activity in activating Smads and p38MAPK.

Previous work (34) has shown that BMP7 binds to ALK2, ALK3, and ALK6 with different affinities. Each of these ALK receptors has been demonstrated to signal via the Smad pathway. Furthermore, a study of TGF-β-dependent activation of p38MAPK in which a mutant TGF-β-binding ALK receptor that cannot bind Smads but retains kinase activity demonstrated that ligand-dependent activation of p38MAPK was not interrupted (32). Thus, it is possible that ALK receptors transduce signals to both p38MAPK and Smads but that the kinetics of p38MAPK and Smad activation are dependent on the dose of ligand presented to the receptor.

Recent studies have suggested a possible mechanism by which BMP7 could trigger p38MAPK at doses lower than those required for Smad activation. In C2C12 and MC3T3 cells, BMP heteromeric complexes exist on the cell surface in the absence of ligand (35). Ligand binding to these preformed complexes triggers Smad activation. In contrast, receptor complexes that form only upon interaction with ligand trigger p38MAPK-dependent signaling (36). These observations raise the possibility that different doses of BMP7 differentially engage preformed versus ligand-stimulated receptor complexes. Alternatively, activation of p38MAPK by low doses of BMP7 may be independent of ALK receptor activation. Studies of BMP7 signaling in osteoblastic cells demonstrate that up-regulation of alkaline phosphatase, a p38MAPK target, is blocked by antibodies directed against α1 and α2 integrins (37). These results suggest that BMP7 bound to integrins could trigger p38MAPK activation. Further studies will be required to test elements of these signaling models.

We have proposed previously (5) that BMP7 acts in a dose-dependent activity gradient to control rates of cell proliferation, tubule growth, and branching during kidney development. This hypothesis is based on our findings that BMP7 controls ureteric bud and collecting duct branching and cell proliferation in a dose-dependent manner and that cell proliferation in these tubules is spatially regulated. The studies reported here suggest a model in which differential cellular responses within a BMP7 concentration gradient are controlled by distinct signaling pathways. The p38MAPK pathway would control responses to low doses of BMP7 leading to increased cell proliferation and branching whereas the Smad pathway would control responses to high doses of BMP7 suppressing cell proliferation and branching. Suppression of p38MAPK activity by high doses of BMP7 would serve to integrate the dose-dependent cellular response to BMP7.

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REFERENCES

p38MAPK Acts in the BMP7-dependent Stimulatory Pathway during Epithelial Cell Morphogenesis and Is Regulated by Smad1
Ming Chang Hu, David Wasserman, Sunny Hartwig and Norman D. Rosenblum

J. Biol. Chem. 2004, 279:12051-12059. doi: 10.1074/jbc.M310526200 originally published online January 12, 2004

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