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BMP-2/ALK3 and HGF signal in parallel to regulate renal collecting duct morphogenesis

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SUMMARY

Bone morphogenetic protein (BMP)-2 and hepatocyte growth factor (HGF) exert antagonistic effects on renal collecting duct formation during embryogenesis. A current model proposes HGF inhibits BMP-2 signaling at the level of Smad1 in a common target cell. Here, we show that BMP-2 and HGF control collecting duct formation via parallel pathways. We examined the interactions between BMP-2 and HGF in the mIMCD-3 model of collecting duct morphogenesis. During tubule formation, HGF rescued the inhibitory effects of BMP-2 and of a constitutive active form of the BMP-2 receptor, ALK3, stably expressed in mIMCD-3 cells. To determine whether the effect of HGF occurs through known mediators which act downstream of the BMP-2/ALK3 complex, we examined the effect of HGF on BMP-2-induced Smad1 phosphorylation, Smad1/Smad4 complex formation, and Smad1 nuclear translocation. Neither HGF nor other receptor tyrosine kinase ligands (EGF, FGF-4) induced phosphorylation of endogenous Smad1 in mIMCD-3 cells or in Mv1Lu, MC3T3-E1 or P19 cells. Furthermore, none of these ligands blocked induction of the BMP-responsive promoter, Tlx2. Thus, HGF overcomes the inhibitory effects of BMP-2 on collecting duct morphogenesis without interrupting any of the known signaling events in the BMP-2 dependent Smad1 signaling pathway. We conclude that BMP-2/ALK3 and HGF function to control parallel pathways downstream of their respective cell surface receptors. Integration of these signals likely occurs at the level of transcriptional or post-transcriptional events.

Key words: Bone morphogenetic protein-2, Activin-like-kinase 3, Hepatocyte growth factor, Renal branching morphogenesis

INTRODUCTION

The formation of complex tissues during embryonic development is dependent on the simultaneous actions of growth factors which exert synergistic or antagonistic effects on their cellular targets (Neubüser et al., 1997; Buckland et al., 1998). The manner in which these activities are integrated in a particular target cell is fundamental to understanding these interactions. In the developing kidney, the branched network of collecting ducts is formed by a process termed branching morphogenesis, defined as growth and branching of epithelial tubules during embryonic development (Saxen, 1987). Renal branching morphogenesis arises via reciprocal mesenchymal-epithelial tissue interactions between the mesenchymal metanephric blastema and the epithelial ureteric bud (and its derivative collecting ducts). These interactions are mediated by growth factors belonging to several different protein superfamilies, including bone morphogenetic proteins (BMP) and ligands that activate receptor tyrosine kinases (Lechner and Dressler, 1997). In the kidney, BMP-2 is expressed by induced metanephric mesenchymal cells in apposition to developing collecting ducts (Dudley and Robertson, 1997) and inhibits collecting duct morphogenesis (Piscione et al., 1997). Hepatocyte growth factor (HGF), a ligand for the receptor tyrosine kinase, C-MET, is expressed in an overlapping temporal and spatial pattern compared to BMP-2 (Woolf et al., 1995) but stimulates collecting duct growth and branching (Santos et al., 1994).

The molecules which act downstream of BMPs have recently been defined in both transfected cell lines and in BMP responsive nonrenal cells (Massagué, 1996; Macías-Silva et al., 1998). Binding of ligand to the type I activin-like kinase receptors, ALK2, ALK3 or ALK6, induces phosphorylation of Smad1 which is then followed by formation of complexes between Smad1 and Smad4, and nuclear translocation of Smad1 (Hoodless et al., 1996). In the embryonic mouse kidney, ALK3 and Smad1 RNA transcripts are expressed in the collecting ducts during branching morphogenesis (Dewulf et al., 1995; Dick et al., 1998). This observation, together with the demonstration that BMP-2 binds to the collecting duct cell surface via a molecular complex containing ALK3 (Piscione et al., 1997) suggests a role for these signaling molecules in renal

branching morphogenesis. Previous work using genetically constituted nonrenal cells suggests that HGF inhibits the actions of BMP-2 by inhibiting nuclear translocation of Smad1 (Kretzschmar et al., 1997). However, the mechanisms by which these antagonistic signals are integrated by cells which express signaling components downstream of BMP-2 and HGF under physiologic conditions is undefined.

In this paper, we defined interactions between HGF and BMP-2 in a physiologically relevant cell model of renal tubulogenesis (mIMCD-3 cells). In this model, tubule formation is stimulated by HGF and inhibited by BMP-2 in an identical manner to that observed in vivo (Cantley et al., 1994; Sakurai et al., 1997; Piscione et al., 1997). Our results demonstrate that HGF rescues tubule formation by mIMCD-3 cells treated simultaneously with BMP-2 and in mIMCD-3 cells which stably express a constitutively active form of the BMP-2 receptor, ALK3. To determine the mechanisms underlying these effects, we defined signaling events downstream of the BMP-2/ALK 3 complex and the effect of HGF on these events. We show HGF has no effect on endogenous Smad1 phosphorylation, Smad1/Smad4 complex formation, Smad1 nuclear translocation or BMP-2 induced transcriptional activation of a BMP response element. Our results indicate that HGF overcomes the inhibitory effects of BMP-2 during collecting duct morphogenesis by signaling via a parallel pathway and not by disrupting the known targets of the BMP-2 signaling pathway, as previously reported. Integration of signals downstream of BMP-2 and HGF likely occurs at the level of transcriptional or post-transcriptional events.

MATERIALS AND METHODS

Cell lines and transfection

mIMCD-3 cells were co-transfected with plasmids encoding hemagglutinin (HA)-tagged wild-type (WT), constitutive inactive (K261R), or constitutive active (Q233D) ALK3 receptors (generous gift from L. Attisano) and a puromycin expression vector using a calcium phosphate transfection method. Puromycin resistant clones which stably expressed wild-type or mutant forms of ALK3 were identified by screening protein lysates by western blotting. HA-tagged ALK3 was detected using a mouse monoclonal antibody to HA (Amersham, 1:1000 dilution), and chemiluminescence as recommended by the manufacturer (ECL kit; Amersham). P19 cells were cultured in α-MEM containing 7.5% calf serum and 2.5% fetal bovine serum (FBS). MC3T3-E1 cells were maintained in α-MEM and 10% FBS and Mv1Lu cells were maintained in α-MEM with non essential amino acids and 10% FBS.

Mouse inner medullary collecting duct (mIMCD-3) model of tubulogenesis

Tubulogenesis assays were performed in type I collagen in 96-well culture plates as previously described (Piscione et al., 1997). Cell culture medium was supplemented with either 5 nM BMP-2, 20 μg/l HGF or both. After 48 hours, gels were fixed in 4% formaldehyde, washed in PBS, and then imaged by differential interference contrast (DIC) microscopy. The number of tubular structures in an area of standard dimensions was determined in four randomly selected positions of the gel for each treatment condition by an observer blinded to the treatment condition. Data were then analyzed using the Statview statistical analysis program (version 4.01; Abacus Concepts, Berkeley, CA). The mean difference from control for various treatment groups were analyzed by Student's *t*-test (two-tailed).

The spatial orientation of mIMCD-3 structures in type I collagen gels was determined using confocal microscopy. After 48 hours of culture, gels were fixed for 10 minutes in 4% formaldehyde, and then treated sequentially with 0.1% Triton X-100 and oregon green-conjugated phalloidin (1:50 dilution, Molecular Probes, Inc.) to identify actin filaments. Images of structures were obtained using a Leica confocal laser microscope. Cross-sectional and longitudinal images of mIMCD-3 structures derived from gels cultured under identical conditions were obtained from 8 μm thick sections counterstained with hematoxylin and eosin.

Immunofluorescence

To detect HA-tagged ALK3 expression in stably transfected mIMCD-3 cells, HA-tagged clones (confirmed by western analysis) were grown in monolayer on glass coverslips in Dulbecco's modified Eagle medium (DMEM)-F12 supplemented with 5% FBS, penicillin (100 U/ml), streptomycin (100 U/ml), and puromycin (1.0 g/l) in 5% CO_2 at 37°C for 12 hours. Next, cells were fixed in 4% formaldehyde in PBS, washed in PBS, permeabilized with 0.1% Triton X-100 for 20 minutes at room temperature, and then blocked with 10% goat serum in PBS for 60 minutes at room temperature. Cells were incubated with anti-HA monoclonal antibody (1:100 dilution) for 60 minutes at room temperature, washed with PBS, and then incubated with FITC-conjugated goat anti-mouse antibody (Jackson Immunologicals; 1:200 dilution) for 60 minutes at room temperature. Mutant clones were photographed using a Zeiss Axioskop confocal laser microscope.

To study nuclear translocation of Smad1, untransfected mIMCD-3 cells were grown in monolayer on glass coverslips in DMEM-F12 supplemented with 0.2% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) in 5% CO_2 at 37°C for 12 hours. Cells were treated for 1 hour with either 5 nM BMP-2 (provided by Genetics Institute), 20 μg/l HGF (Collaborative Biomedicals) or both and then fixed in 4% formaldehyde. After 3 washes in PBS, the cells were permeabilized with methanol for 15 minutes at room temperature, and then blocked with 10% goat serum and 3% BSA in PBS for 1 hour at room temperature. Cells were incubated with anti-Smad1 antibody (Macías-Silva et al., 1998) (1:50 dilution) overnight at 4°C, washed with PBS, and then incubated with FITC-conjugated goat anti-rabbit antibody (Jackson Immunologicals; 1:200 dilution) for 60 minutes at room temperature. Coverslips were mounted and imaged using a Zeiss Axioskop confocal laser microscope.

Phosphate labeling and immunoprecipitation of mIMCD-3 cellular proteins

For [^{32}P]phosphate labeling, cells were washed and preincubated with phosphate-free medium containing 0.2% dialyzed FBS. The cells were then incubated with media containing 1 mCi/ml [^{32}P]PO_4 for 2 hours at 37°C. Cell lysates were subjected to immunoprecipitation with either anti-Smad1 antibody or anti-Smad4 antibody followed by adsorption to Protein A-Sepharose (Pharmacia). Immunoprecipitated proteins were washed, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. In some experiments, analysis of immunoprecipitated proteins was performed by immunoblotting using a rabbit anti-Smad4 antibody (Macías-Silva et al., 1998) (1:1000 dilution) followed by anti-rabbit HRP (1:10,000) and chemiluminescence.

Transcriptional response assay

P19 cells were transiently transfected with a Tlx2-lux reporter construct using a calcium phosphate DNA precipitation method (Hoodless et al., 1996). Cells were seeded at 20% confluency in 24-well plates and transfected overnight with 0.1 μg reporter DNA per well. To induce the luciferase reporter, cells were treated overnight with the appropriate ligands, lysed, and luciferase activity was measured using the luciferase assay system (Promega) in a Berthold Lumat LB 9501 luminometer. All transfections were normalized to β-galactosidase activity by cotransfection of 0.01 μg of a cytomegalovirus β-galactosidase (pCMV β-gal) plasmid per well.

Reverse transcription-PCR

P19 cell total RNA was isolated using commercially available reagents (Qiagen) and reverse transcribed using an oligo dT primer (Gibco-BRL) and reverse transcriptase (Gibco-BRL). First strand cDNA was used as substrate in PCR using deoxynucleotide primers and thermal conditions specific for C-MET (Woolf et al., 1995), fibroblast growth factor receptor-2 (FGFR2) (Abbass et al., 1997), and epidermal growth factor receptor (EGFR) (Itoh et al., 1998). PCR products were detected by agarose gel electrophoresis.

RESULTS

Collecting duct cells form tubular structures in three-dimensional collagen matrix

The collecting tubule is a physiologically relevant model for investigating the mechanisms by which antagonistic growth factor mediated signals are integrated by a common target cell (Piscione et al., 1997; Santos et al., 1994). SV40 transformed mouse inner medullary collecting duct cells (mIMCD-3) form branched tubules in a three-dimensional type I collagen matrix (Cantley et al., 1994; Piscione et al., 1997) and serve as an in vitro model in which to investigate the actions of growth factors on collecting tubule formation. Indeed, the responses of mIMCD-3 cells to morphogens is identical to the effects observed in the intact kidney (Santos et al., 1993; Piscione et al., 1997).

We defined the three-dimensional organization of mIMCD-3 cells in collagen matrix by confocal and conventional microscopy (Fig. 1). Confocal imaging of mIMCD-3 structures in collagen gels at 8 μm intervals of depth demonstrated that the vast majority of mIMCD-3 structures form within the matrix rather than at the interface of matrix with the underlying plastic or overlying culture medium. Brightfield imaging of 8 μm histologic sections of the gel matrix demonstrated that the mIMCD-3 structures are multicellular and elongated with cells organized in a single layer around a central lumen. Taken together, these observations demonstrate that mIMCD-3 structures generated within type I collagen matrix are tubules.

HGF is dominant over BMP-2 during collecting tubule formation

We investigated competition between BMP-2 and HGF in the mIMCD-3 culture model of collecting duct morphogenesis (Fig. 2A). Imaging by DIC microscopy of tubules formed after 48 hours of culture showed that HGF stimulated the formation of thin and highly branched tubules, as previously described (Piscione et al., 1997). In contrast, BMP-2 treated tubules were short and unbranched. Treatment with both HGF and BMP-2 increased the number of tubules formed but these tubules were short and unbranched like BMP-2-treated tubules. Quantitation (Fig. 2B) was performed by counting the number of tubules present in four randomly selected regions of the outer perimeter of the collagen gel. This analysis revealed that HGF increased tubule formation by 31% compared to cells untreated with ligand ($P<0.05$), whereas 5 nM BMP-2 inhibited tubule formation by 82% ($P<0.05$). In the presence of HGF, BMP-2 was a less potent inhibitor and only inhibited tubulogenesis by 42% compared to 82% inhibition with BMP-2 alone ($P<0.05$). Thus, HGF is able to rescue collecting tubule formation from almost total inhibition by BMP-2.

ALK3 is the functional BMP-2 receptor in collecting duct cells

Fetal bovine serum contains follistatin (Phillips et al., 1997) and fetuin (Saunders et al., 1994), both of which inhibit the actions of BMPs by competing with cell surface BMP receptors for ligand binding (Ferguson et al., 1998; Demetriou et al., 1996). Since these extracellular protein interactions may have biased BMP-2/HGF ligand competition in favor of HGF, we studied interactions between the BMP-2 and HGF signaling pathways by

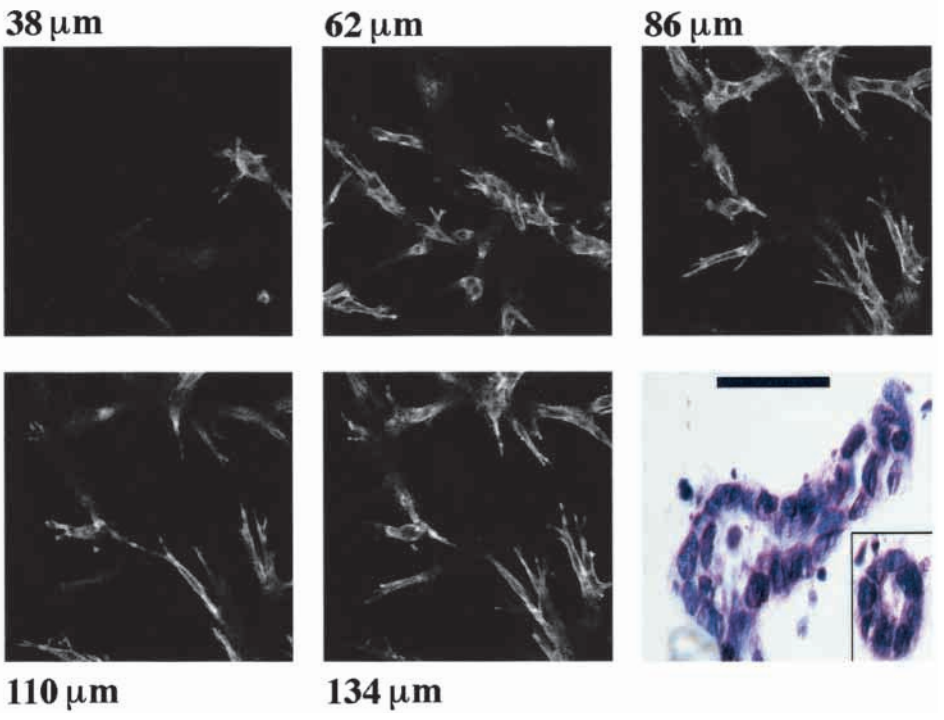
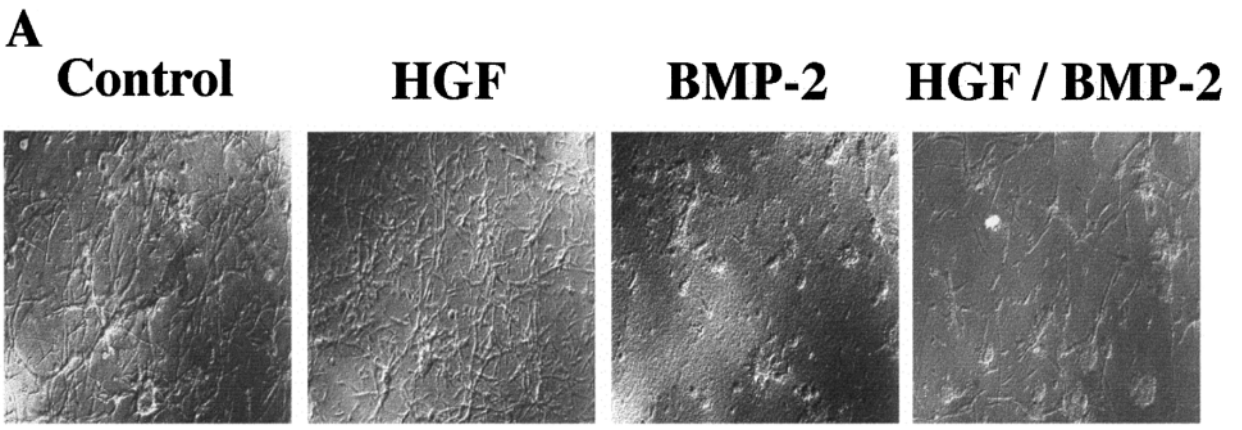


Fig. 1. mIMCD-3 cells form tubular structures in three-dimensional culture. mIMCD-3 cells were cultured for 48 hours in type I collagen and culture medium. Fixed gels stained with oregon green phalloidin were imaged at 8 μm intervals through the depth of the gel by confocal microscopy. Representative images at 24 μm intervals, with depth position in gel indicated above each panel, demonstrate that mIMCD-3 structures are formed within the gel matrix (magnification ×400). Lower right-hand panel: Longitudinal and cross-sectional images of 8 μm gel sections counterstained with hematoxylin and eosin demonstrate that the mIMCD-3 structures are multicellular and elongated with cells organized in a single layer around a central lumen (magnification ×1000). Bar, 20 μm.



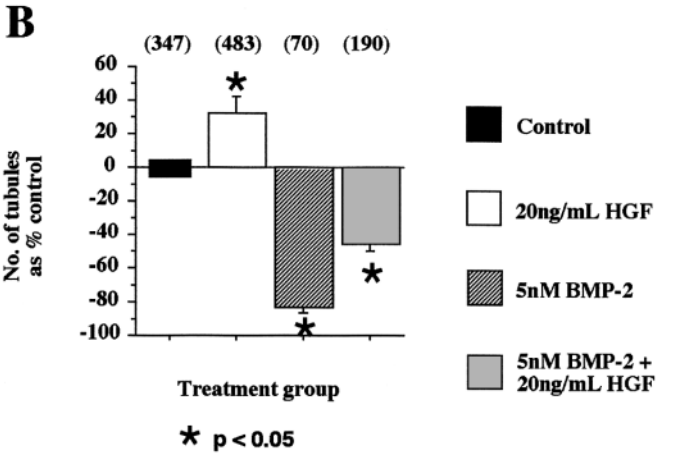


Fig. 2. Effect of HGF on renal branching morphogenesis in the presence of BMP. (A) Untransfected mIMCD-3 cells were cultured for 48 hours in type I collagen and culture medium supplemented with either no ligand, 5 nM BMP-2, 20 μg/l HGF, or both BMP-2 and HGF. Structures formed in type I collagen were imaged by DIC microscopy (×100 magnification). (B) The number of tubules (linear structures) formed by untransfected mIMCD-3 cells cultured in type I collagen in the presence of BMP-2 or HGF was quantitated. The mean number of tubular structures present in 4 randomly selected microscopic fields is shown in parentheses above each bar.

generating mIMCD-3 cells in which the BMP-2 signaling pathway is activated in a ligand independent manner. Previously, we demonstrated that BMP-2 is bound to the surface of collecting duct cells by a molecular complex which includes the type I activin-like kinase receptor, ALK3 (Piscione et al., 1997). All type I receptors, including ALK3, consist of a highly conserved 30 amino acid region, the GS domain, immediately upstream of the kinase domain. Within the GS domain, a mutation at position 233, changing a glutamine to an aspartic acid, confers a constitutive active phenotype on the ALK3 receptor. In contrast, changing a lysine to an arginine residue at position 261 generates a kinase deficient ALK3 receptor. This mutant is able to bind ligand but cannot signal (Weiser et al., 1995).

To establish the functional significance of BMP-2 binding to ALK3 we stably transfected mIMCD-3 cells with either wild-type (WT), constitutive active (Q233D) or constitutive inactive (K261R) ALK3 receptor modified at the carboxy terminus with the hemagglutinin epitope (HA). mIMCD-3 cell clones expressing HA-ALK3 were identified by the presence of a 58 kDa band detected by immunoblotting using an anti-HA monoclonal antibody (Fig. 3A). The size of this band is consistent with the size of HA-tagged ALK3 (58 kDa) predicted by its primary structure (ten Dijke et al., 1993; Hoodless et al., 1996). The subcellular distribution of HA-ALK3 was examined further using immunofluorescence and confocal microscopy. A punctate pattern with maximal intensity along the borders of cell membranes was observed in 82% of stably transfected cells (Fig. 3B), consistent with localization of HA-ALK3 to cell membranes. Next, we induced mIMCD-3 cells expressing HA tagged WT, K261R, and Q233D forms of ALK3 to form tubules in type I collagen (Fig. 3C). In the absence of BMP-2, both the WT and the K261R expressing cells formed branched tubular structures. In contrast, cells expressing ALK3 (Q233D) aggregated, but rarely formed tubules. Quantitation of tubulogenesis was performed by counting the number of linear tubular structures per defined surface area of the collagen gel (Fig. 3D). Since transfecting mIMCD-3 cells decreases their tubulogenic activity and overexpression of wild-type type I BMP receptors can lead to autoactivation of receptors on the cell surface (Attisano et al., 1993), we compared the activity of ALK3 (Q233D) and ALK3 (K261R) expressing cells to those expressing WT ALK3. mIMCD-3 cells expressing HA tagged ALK3 (Q233D) formed only a few tubules, representing a 94% reduction compared to the control (WT ALK3 clones, $P<0.05$). These results indicate that the activated ALK3 receptor inhibits tubulogenesis, a finding which is consistent with our previous demonstration that BMP-2 inhibits tubule formation and is bound to mIMCD-3 cells by ALK3 (Piscione et al., 1997). In contrast, mIMCD-3 cells expressing the inactive K261R mutant form of ALK3 formed 41% more tubules than mIMCD-3 cells expressing HA tagged WT receptors ($P<0.05$). This result may reflect dominant negative activity of ALK3 (K261R) in the presence of autocrine BMP activity. Taken together, these results establish an inhibitory function for ALK3 in the collecting duct downstream of BMP-2.

HGF is dominant over the activated BMP-2 dependent signaling pathway during collecting tubule formation

The isolation of mIMCD-3 cells which signal constitutively via ALK3 in the absence of BMP-2 provided an opportunity to test the competition between the HGF and BMP-2 signaling pathways in the absence of soluble extracellular BMP inhibitors. As shown in Fig. 4A, mIMCD-3 cells expressing

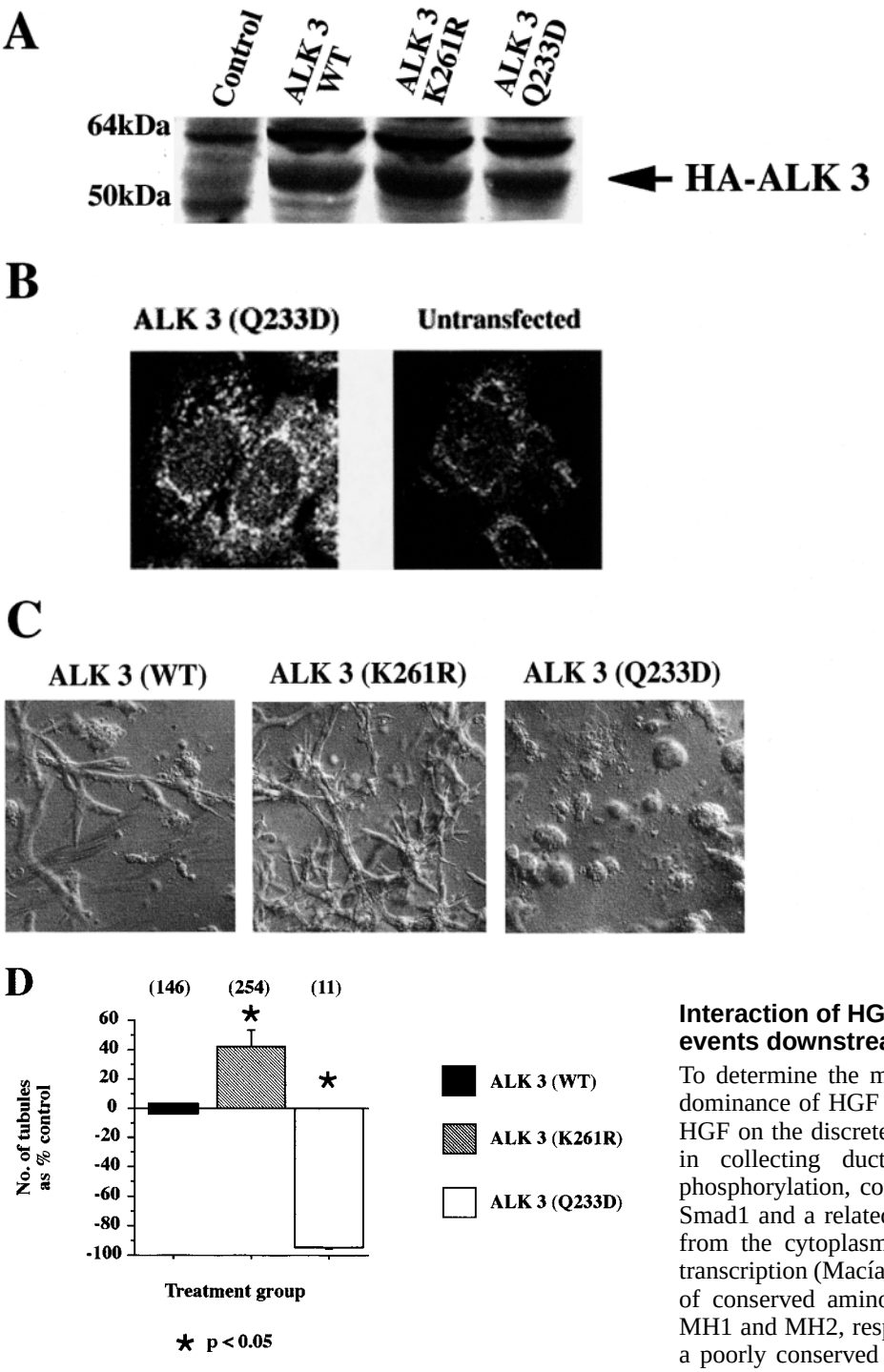


Fig. 3. ALK3 functions to inhibit collecting duct cell morphogenesis. (A) mIMCD-3 cells were stably transfected with plasmids encoding HA-tagged WT, K261R, or Q233D ALK3. Proteins from cell lysates were analyzed by immunoblotting using an anti-HA monoclonal antibody. (B) Representative HA-tagged ALK3 mIMCD-3 clones were grown in monolayer. HA was detected by confocal laser microscopy using a mouse monoclonal anti-HA antibody and a fluorescein-conjugated secondary antibody (×480 magnification). (C) Stably transfected mIMCD-3 clones expressing either WT, K261R (constitutive inactive), or Q233D (constitutive active) ALK3 receptors were cultured in type I collagen with DMEM-F12 and 5% fetal calf serum and then imaged by DIC microscopy (×100 magnification). (D) The number of tubules (linear structures) formed by WT, K261R, and Q233D clones was quantitated. The mean number of tubules present in 4 randomly selected microscopic fields is shown in parentheses above each bar.

the constitutive active form (Q233D) of ALK3 formed very few tubules under basal conditions. This result is consistent with the observed effect of BMP-2 on tubule formation by untransfected mIMCD-3 cells (Figs 1, 2). Addition of 5 nM BMP-2 to cultures of the ALK3 (Q233D) expressing cells led to a further reduction in tubule formation (Fig. 4B, hatched bar) (P<0.05). This response is consistent with the prediction that BMP-2 can activate endogenous ALK3 receptors which are co-expressed with the mutant receptor on the cell surface. In contrast, addition of 20 μg/l HGF resulted in the formation of branched tubules by ALK3 (Q233D) expressing cells (202% increase compared to untreated ALK3 (Q233D) expressing cells) (P<0.05) (Fig. 4B). This stimulatory effect of HGF was reduced only to a minor degree by treatment of ALK3 (Q233D) expressing cells with both HGF and BMP-2. Taken together, these results establish the dominance of HGF over the BMP-2 signaling pathway during collecting duct morphogenesis.

Interaction of HGF with intracellular signaling events downstream of BMP-2/ALK3

To determine the mechanism which underlies the biological dominance of HGF over BMP-2, we determined the effect of HGF on the discrete molecular events downstream of BMP-2 in collecting duct cells. These events include Smad1 phosphorylation, complex formation between phosphorylated Smad1 and a related protein, Smad4, translocation of Smad1 from the cytoplasm to the nucleus, and activation of gene transcription (Macías-Silva et al., 1998). Smad proteins consist of conserved amino- and carboxy-terminal domains termed MH1 and MH2, respectively. These domains are separated by a poorly conserved intervening segment known as the linker region (Hoodless et al., 1996). The MH1 domain interacts physically with the MH2 domain and thereby inhibits the transcriptional activity of the MH2 domain (Baker and Harland, 1996; Liu et al., 1996; Hata et al., 1997). Phosphorylation of serine residues in the MH2 domain by an activated type I BMP receptor (e.g. ALK3) presumably relieves these autoinhibitory interactions to activate the receptor-regulated Smad. This, in turn, induces receptor regulated Smads including Smad1 to partner with Smad4 and then to translocate to the nucleus (Kretzschmar et al., 1997).

We first investigated the effects of BMP-2 on endogenous Smad1 phosphorylation in mIMCD-3 cells. mIMCD-3 cells

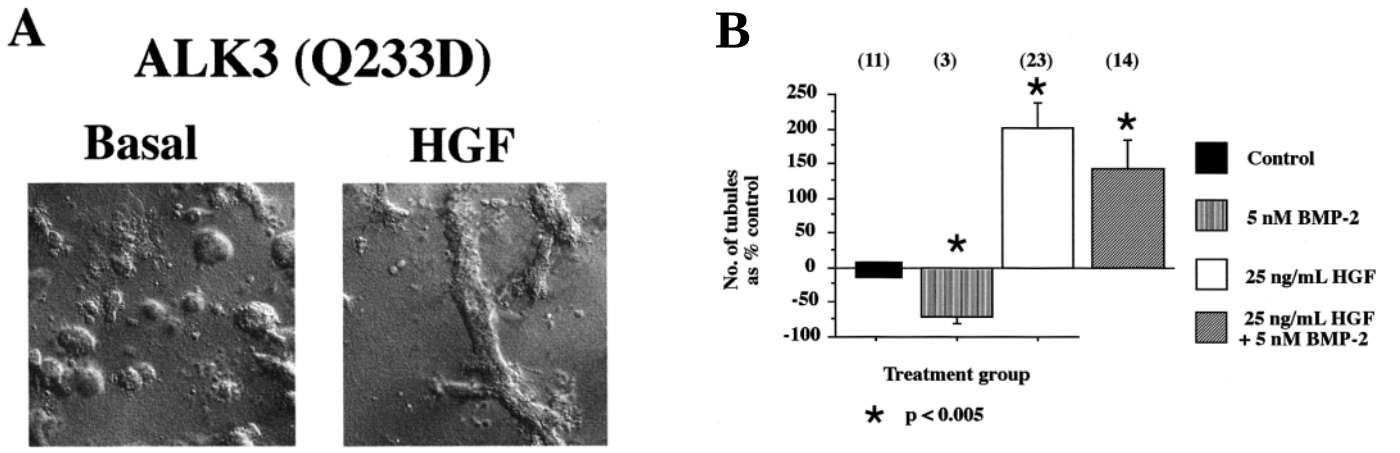


Fig. 4. Effect of HGF on tubule formation by collecting duct cells expressing constitutive active (Q233D) ALK3. (A) mIMCD-3 cells stably transfected with the ALK3 (Q233D) receptor were cultured in type I collagen in culture medium supplemented with 5 nM BMP-2 or 20 μg/l HGF for 7 days and imaged by DIC microscopy (×100 magnification). (B) Quantitative analysis of tubule formation by ALK3 (Q233D) expressing mIMCD-3 cells exposed to no ligand, 5 nM BMP-2, 20 μg/l HGF or 5 nM BMP-2/20 μg/l HGF. The mean number of tubules present in 4 randomly selected microscopic fields is shown in parentheses above each bar.

were labeled with [^{32}P]phosphate and then treated with 10 nM BMP-2 for 30 minutes. Cellular proteins were immunoprecipitated with anti-Smad1 antibody and the immunoprecipitated proteins were then analyzed by SDS-PAGE. As shown in Fig. 5A (upper panel), BMP-2 induced a marked increase in Smad1 phosphorylation as indicated by both an increase in radioactive signal and an upward mobility shift in Smad1 migration. The mobility shift is a direct result of Smad1 phosphorylation causing it to migrate more slowly than unphosphorylated Smad1 (Hoodless et al., 1996). In contrast, we observed little change in the phosphorylation of Smad1 after treatment with HGF. The slight effects occasionally observed (Fig. 5A, bottom panel) may reflect fluctuations in the yield of Smads isolated in the immunoprecipitation procedure. Consistent with this, we observed no shift in the mobility of Smad1 in HGF treated samples in contrast to the clear shift that is induced when overexpressed Smads are phosphorylated by MAPK in COS cells (data not shown). Simultaneous treatment with HGF and BMP-2 resulted in a marked increase in Smad1 phosphorylation and a shift in Smad1 mobility to a similar degree to that observed with BMP-2 alone. Furthermore, incubation of cells with HGF for 30 minutes prior to treatment with BMP-2 achieved the same result (Fig. 5A, upper panel, right hand lane). These data suggest that HGF signaling does not target endogenous Smad1 for phosphorylation in mIMCD-3 cells and further indicate that HGF does not interfere with BMP-mediated phosphorylation of Smad1.

To determine whether other BMPs which signal via Smad1 (Macías-Silva et al., 1998) induce the same effects, Smad1 phosphorylation was analyzed after treatment of mIMCD-3 cells with BMP-7. We have previously shown that, like BMP-2, high doses of BMP-7 (>0.5 nM) inhibit collecting duct morphogenesis (Piscione et al., 1997). As shown in Fig. 5A (lower panel), 10 nM BMP-7 induced both phosphorylation and a mobility shift of Smad1. Similar to our observations with BMP-2, phosphorylation of Smad1 in mIMCD-3 cells treated with BMP-7 was not affected by treatment with HGF (Fig. 5A, lower panel). Since epidermal growth factor (EGF) has also been previously reported to interrupt nuclear translocation and the transcriptional activity of Smad1 by inducing phosphorylation in the Smad1 linker region, we analyzed endogenous Smad1 phosphorylation in mIMCD-3 cells after treatment with EGF. Similar to HGF, 100 μg/l EGF did not increase Smad1 phosphorylation. In contrast, in the same experiments, 10 nM BMP-7 induced both an increase in Smad1 phosphorylation and a change of Smad1 mobility (Fig. 5B). To investigate the generality of these findings, we measured ligand-dependent phosphorylation of Smad1, Smad2 and Smad4 in several other cells lines derived from different lineages. These include Mv1Lu (derived from mink lung epithelial cells), MC3T3-E1 (a mesenchymal cell) and P19 cells (a mouse embryonic carcinoma cell line). In each of these cell lines, Smad phosphorylation was assessed after treatment of the cells with HGF, EGF, fibroblast growth factor (FGF), FBS or BMP-7. As shown in Fig. 5C, in each of these cell lines, HGF, EGF or FGF-4 had little effect on endogenous Smad1 phosphorylation. This sharply contrasted with the strong induction of Smad1 phosphorylation observed in response to BMP-7. Similarly, none of these treatments induced phosphorylation of Smad2 or Smad4. Interestingly, we observed the same effect after treatment with fetal calf serum, suggesting that serum contains a BMP-like activity which results in Smad1 activation. Phosphopeptide mapping of endogenous Smad1 from BMP-7 and FBS treated P19 cells revealed that phosphorylation occurred on residues in the carboxy terminal in an identical fashion as previously described for transfected Smad1 (data not shown). Taken together, these data establish that BMP-2 signaling in mIMCD-3 cells directs phosphorylation of Smad1, and that combined treatment with HGF and BMP-2 does not disrupt this event.

Next, we determined the effect of BMP-2 on Smad1/Smad4 complex formation, a prerequisite for cytoplasmic to nuclear translocation of Smad1. Proteins in cell lysates were immunoprecipitated with an anti-Smad1 antibody (Macías-Silva et al., 1998) and then immunoblotted with an anti-Smad4 antibody (Macías-Silva et al., 1998) (Fig. 6A). Under basal conditions, Smad4 was not detected in association with Smad1,

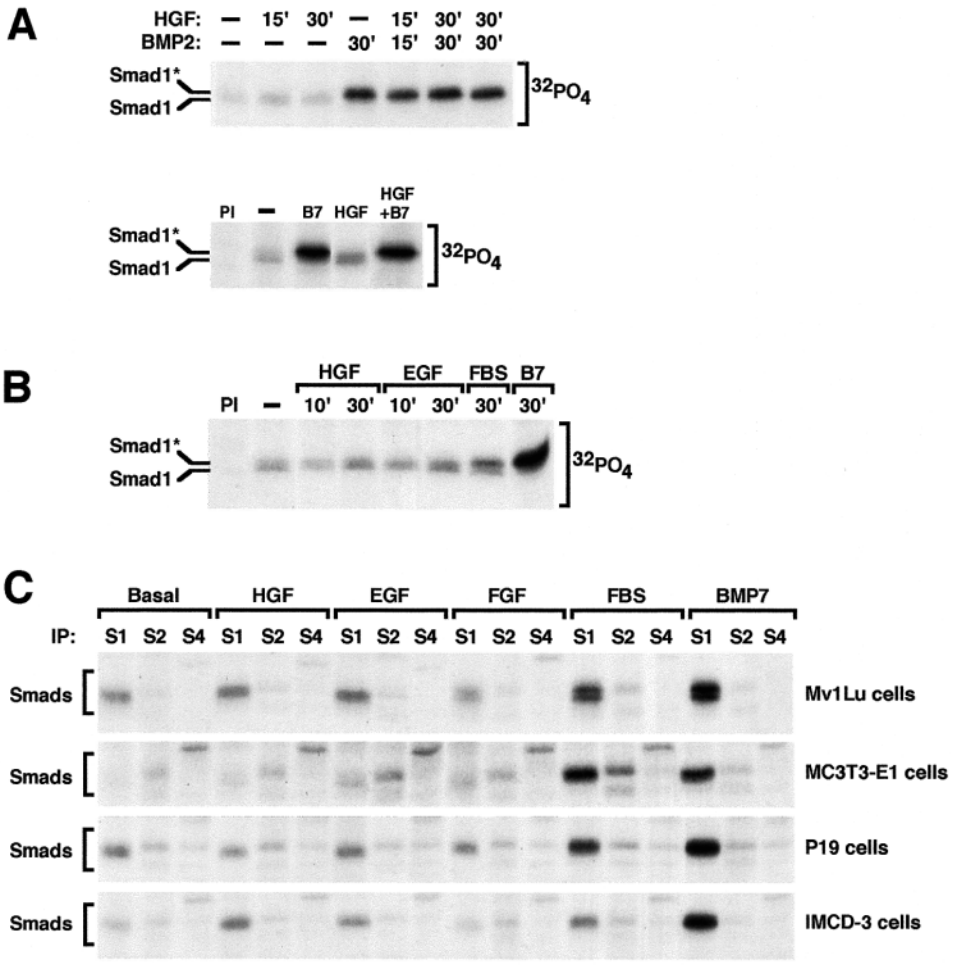


Fig. 5. BMP-2 dependent Smad1 phosphorylation is not disrupted by HGF, FGF or EGF. (A) Untransfected mIMCD-3 cells were labeled with [^{32}P]phosphate and incubated in 0.2% FBS + DMEM-F12 in the presence of either 10 nM BMP-2, 100 μg/l HGF, both BMP-2 and HGF or no ligand for 15-30 minutes (upper panel) or with 10 nM BMP-7, 100 μg/l HGF or both BMP-7 and HGF or no ligand (lower panel). After immunoprecipitation with an antibody to Smad1, proteins were analyzed by SDS-PAGE and autoradiography. (B) Untransfected mIMCD-3 cells were labeled with [^{32}P]phosphate and incubated in 0.2% FBS-DMEM-F12 in the presence of either 10 nM BMP-7, 100 μg/l HGF, 100 μg/l EGF, 10% fetal bovine serum or no ligand for 10 or 30 minutes. After immunoprecipitation with an antibody to Smad1, proteins were analyzed by SDS-PAGE and autoradiography. (C) Untransfected mIMCD-3 cells, Mv1Lu cells, MC3T3-E1 cells, and P19 cells were labeled with [^{32}P]phosphate and incubated in 0.2% FBS + DMEM-F12 in the presence of either 10 nM BMP-7, 100 μg/l HGF, 100 μg/l EGF, 100 μg/l FGF, 10% fetal bovine serum or no ligand for 30 minutes. After immunoprecipitation with an antibody to Smad1, Smad2 or Smad4, proteins were analyzed by SDS-PAGE and autoradiography.

indicating that Smad1/Smad4 complexes do not exist in untreated mIMCD-3 cells. In contrast, treatment with 5 nM BMP-2 resulted in the formation of heteromeric complexes of Smad1 and Smad4 while treatment with 20 μg/l HGF did not induce complex formation. Furthermore, in cells treated with both BMP-2 and HGF, the level of heteromeric complex formation was similar to cells treated with BMP-2 alone. To confirm that equivalent levels of Smad1 were immunoprecipitated, anti-Smad1 precipitates were immunoblotted using the anti-Smad1 antibody. In all treatment groups unphosphorylated Smad1 was clearly detected, while in BMP-2 treated cells a slower migrating form of Smad1 (*Smad1) was detected, thus confirming that activation of Smad1 occurred in cells in which Smad1/Smad4 complexes were detected. Taken together, these data indicate that BMP-2 induces endogenous Smad1/Smad4 complex formation in collecting duct cells and that HGF does not physically disrupt Smad1/Smad4 protein interactions.

Smad1/Smad4 complexes translocate to the nucleus where they function to activate BMP responsive genes. Thus, we determined whether HGF altered BMP-2-induced cytoplasmic to nuclear translocation of endogenous Smad1 in collecting duct cells using immunofluorescence and confocal laser microscopy. Under basal conditions, Smad1 protein was detected in a punctate distribution throughout the cytoplasm of mIMCD-3 cells similar to previous reports for Smad5 (Nishimura et al., 1998) and Smad2/3 (Nakao et al., 1997; Tsukazaki et al., 1998). However, after a 60 minute treatment with 5 nM BMP-2, translocation of Smad1 protein was identified as a shift in fluorescent signal from cytoplasm to nucleus in 77% of 60 cells analyzed (Fig. 6B). No signal was detected using preimmune antisera and fluorescein-conjugated secondary antibody (data not shown). In contrast, when the cells were stimulated with 20 μg/l HGF, there was no nuclear translocation of Smad1 protein. Moreover, in cells treated with both HGF and BMP-2, nuclear translocation of Smad1 protein was clearly detected and was indistinguishable to that seen in cells treated with BMP-2 alone. Together, these data indicate that HGF does not alter biological responses to BMP-2 by interfering with Smad1 activation and translocation.

To confirm these conclusions, we tested BMP-dependent induction of a BMP target promoter (Tang et al., 1998). This provided an opportunity to determine whether HGF signaling interferes with transcription directed by this promoter. Tlx2 is a homeobox gene of the HOX11 class and is strongly induced by BMP-2 during mouse gastrulation and in P19 cells, a mouse embryonic carcinoma cell line (Tang et al., 1998). The previous observation that P19 cells express FGF receptors (Ameerun et al., 1996), and our demonstration that P19 cells express mRNA transcripts for C-MET, FGFR2 and EGFR, the receptors for HGF, FGF-4 and EGF, respectively (data not shown), provided a basis to determine whether interactions between receptor

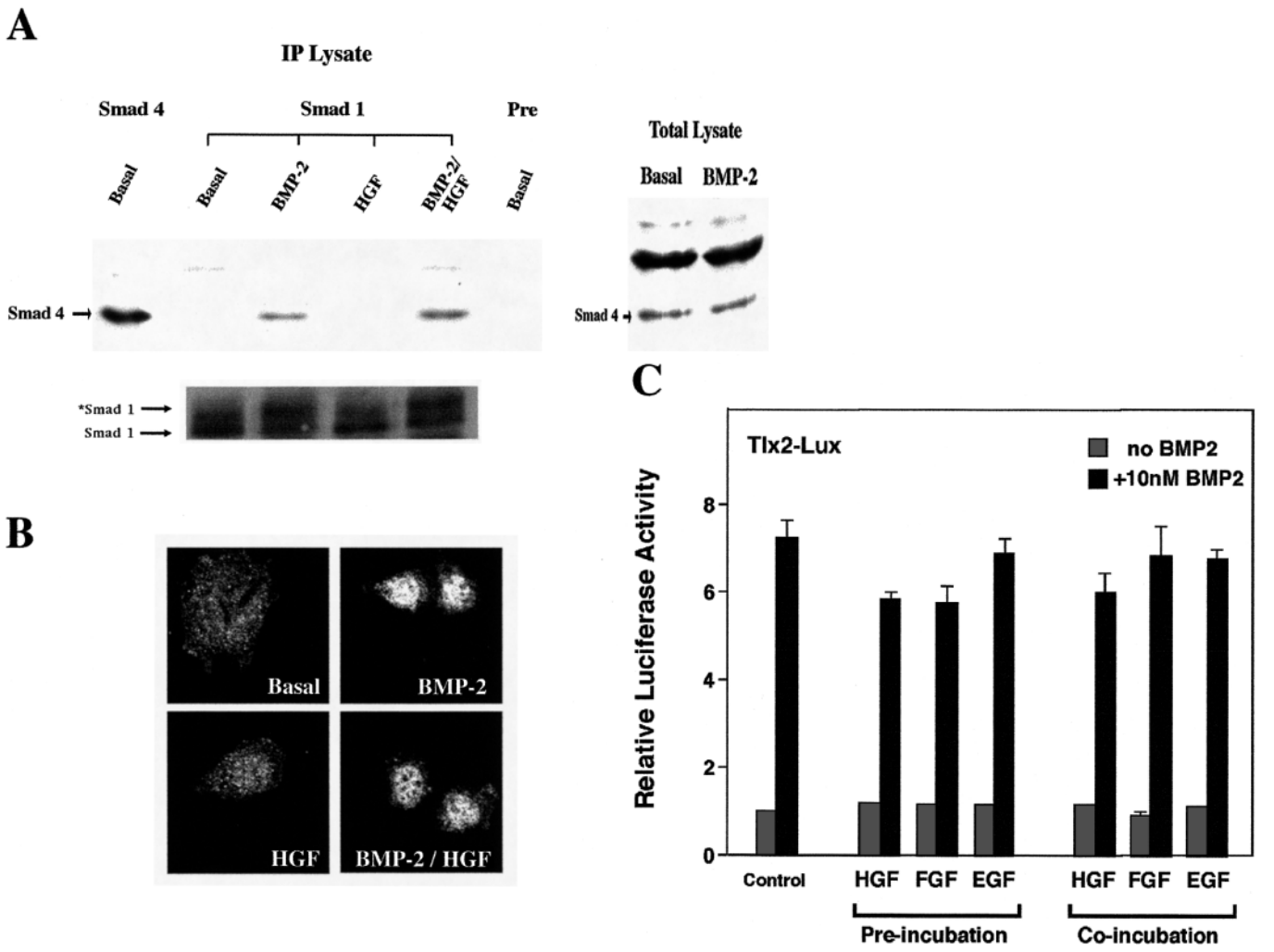


Fig. 6. BMP-2 dependent signaling events are not disrupted by HGF. (A) Untransfected mIMCD-3 cells were incubated in 5% FBS + DMEM-F12 then treated for 1 hour with either no ligand, 5 nM BMP-2, 20 μg/l HGF or both HGF and BMP-2. Proteins in cell lysates were analyzed with an anti-Smad4 antibody by immunoblotting. Alternatively, cell lysate proteins were immunoprecipitated with an antibody to Smad1, separated by SDS-PAGE, and then analyzed by western blotting using an antibody to Smad4 or Smad1. (B) Untransfected mIMCD-3 cells were grown in monolayer in 0.2% FBS + DMEM-F12 then treated for 1 hour with either no ligand, 5 nM BMP-2, 20 μg/l HGF, or both HGF and BMP-2. Immunostaining was performed using an antibody to Smad1, followed by a fluorescein-conjugated secondary antibody. Images were obtained by confocal laser microscopy (×480 magnification). (C) P19 cells were transiently transfected with a Tlx2-luciferase reporter construct and then treated with HGF, FGF, or EGF in the absence or presence of BMP-2. After 20 hours of incubation luciferase activity was measured. Luciferase activity was controlled for the activity of β-galactosidase.

tyrosine receptor signaling and BMP-2 occurred at the level of transcription. For this, P19 cells, transiently transfected with the reporter gene pTlx2-luciferase, were incubated with BMP-2 or were co-incubated with BMP-2 together with HGF, FGF or EGF. In addition, we also pre-incubated the cells for 30 minutes with HGF, FGF, or EGF prior to addition of BMP-2. As shown in Fig. 6C, BMP-2 induced the Tlx2 promoter 4.5-fold. This induction was not significantly decreased by any of the three receptor tyrosine kinase receptor ligands we tested. Taken together, our experiments indicate that while HGF is dominant over the inhibitory effect of BMP-2 on renal branching morphogenesis, HGF does not disrupt the known early biochemical signaling events that are activated in response to BMP-2. Our results suggest that integration of signals downstream of BMP-2 and HGF likely occurs at the level of transcriptional or post-transcriptional events.

DISCUSSION

During renal development, collecting duct cells exist within a complex environment of growth factors and must integrate these diverse signals to form a tubular network. Several types of evidence strongly suggest that both BMP-2 and HGF regulate renal branching morphogenesis but with opposite effects, as observed in nonrenal embryonic tissues (Niswander and Martin, 1993; Neubüser et al., 1997). BMP-2 is expressed in induced metanephric mesenchyme in apposition to developing collecting ducts (Dudley and Robertson, 1997) that express the BMP receptor, ALK3 (Dewulf et al., 1995), and inhibits growth and branching of collecting tubules (Piscione et al., 1997). HGF is expressed in the metanephric mesenchyme adjacent to developing collecting ducts and stimulates growth and branching of collecting ducts which

express its receptor, C-MET (Woolf et al., 1995; Santos and Nigam, 1993; Piscione et al., 1997).

We have determined the interactions between BMP-2 and HGF in the mIMCD-3 model of collecting duct morphogenesis. As discussed above, inner medullary collecting duct cells form branched tubules when suspended in extracellular matrix and respond to growth factors in an identical manner to that observed in embryonic kidney explants (Santos and Nigam, 1993; Piscione et al., 1997). Moreover, the relative simplicity of the IMCD-3 cell model provides a means to test the direct actions of growth factors in cells derived from the ureteric bud. Such an endeavor is precluded by the cellular complexity and presence of reciprocal tissue interactions between the metanephric blastema and the ureteric bud in the intact developing kidney. Yet, the properties of growth factor signaling and tubular morphogenesis characteristic of organogenesis in vivo are maintained in the mIMCD-3 model.

In this work, we demonstrate that HGF is dominant over the BMP-2 dependent signaling pathway during collecting tubule formation. To identify the molecules involved in the interaction between the HGF and BMP-2/Smad signaling pathways, we define the signaling pathway downstream of ALK3 in collecting duct cells. We demonstrate, surprisingly, that HGF does not interrupt any of the discrete signaling events initiated by activation of the ALK3 receptor including cytoplasmic to nuclear translocation of Smad1. Further, we find no evidence that HGF, or other ligands that signal via tyrosine kinase receptors, induce phosphorylation of endogenously expressed Smads. These results are inconsistent with a currently proposed model of HGF-Smad interaction arising from studies in mink lung epithelial cells (R-1B/l17) in which SMAD proteins were genetically overexpressed (Kretzschmar et al., 1997). HGF and EGF were shown to induce phosphorylation of Smad1 in its linker region in a MEK1 dependent manner and to inhibit nuclear translocation of Smad1 (Kretzschmar et al., 1997), thereby blocking the BMP-2 signaling pathway. Our observations of signaling via endogenously expressed Smad proteins in a physiological model of collecting duct morphogenesis do not support this model. Our results which indicate that HGF does not alter BMP-2 dependent Smad1 signaling suggest that interactions between the Smad1 and HGF signaling pathways occur downstream of nuclear translocation of the Smad1/Smad4 complex.

HGF may act to disrupt the discrete events of the BMP-2 signaling pathway by signaling to the nucleus in a parallel but dominant pathway. The molecular events, which underlie the morphogenetic effects of HGF, have been described in MDCK cells. Binding of HGF to C-MET induces phosphorylation of phosphoinositide triphosphate (PIP-3) kinase (Santos et al., 1993; Cantley et al., 1994) and phosphorylation of Stat3, a transcription factor originally described in the JAK-STAT pathway. Phosphorylation of Stat3 induces homodimerization and translocation to the nucleus (Boccaccio et al., 1998). In fact, Stat3 is a critical molecule that determines the protubulogenic properties of HGF. Thus, signaling via Stat3 may evoke a more potent parallel transcriptional response than signalling via Smad1, accounting for the dominant effects of HGF over BMP-2 in mIMCD-3 cells. Alternatively, the activated BMP-2 and HGF pathways may converge on common nuclear target genes and Stat3 may abrogate inhibition of 'tubulogenesis' genes by Smad1 and its partner proteins. Recent evidence indicates that the transcriptional coactivator, p300, physically interacts with Stat3 and Smad1 and this interaction is required for the synergistic effects of leukemia inhibitory factor (LIF) and BMP-2 on primary neural progenitor cells (Nakashima et al., 1999). p300, also termed CREB binding protein (CBP), is a downstream target of protein kinase A (PKA) acting via CREB. Our recent observation that PKA is required for BMP-2 activity during collecting duct morphogenesis and that BMP-2 induces CREB phosphorylation (Gupta et al., 1999) raises the possibility that HGF exerts its dominant effects by disrupting interactions between p300 and Smad1. Future experiments are required to investigate the effects of HGF on p300 activity in the context of BMP-2 signaling during renal branching morphogenesis.

Our work demonstrates, for the first time, that ALK3 is the functional BMP-2 receptor in the renal collecting tubule and inhibits tubule formation. BMPs induce cellular responses by forming heteromeric complexes with type I and type II cell surface transmembrane serine/threonine kinase receptors (Ruberte et al., 1995). The cellular response to a BMP is defined by the particular member of the type I receptor family to which it binds, since the type I receptor transduces a signal to downstream intracellular target molecules (Wrana et al., 1994). Among the candidate BMP-2 type I receptors identified to date, both ALK3 and ALK6 have been identified in the developing renal collecting system (Dewulf et al., 1995). Our demonstration that BMP-2 is bound to the mIMCD-3 cell surface by ALK3 (Piscione et al., 1997) provided a basis for testing the function of ALK3. Expression of a constitutive active (Q233D) form of ALK3 strongly inhibited tubule formation in an identical manner to that seen with treatment of mIMCD-3 cells which only express endogenous ALK3 with BMP-2. Our results using cells which express a constitutive inactive (K261R) form of ALK3 support this conclusion since tubule formation by these cells was enhanced above control levels. This enhancement may be due to stimulatory signaling by BMP-7 in the presence of a downregulated BMP-2/ALK3/Smad1 signaling pathway. Low doses of BMP-7, derived either from collecting duct cells themselves (Dudley and Robertson, 1997) or fetal bovine serum (Macías-Silva et al., 1998) stimulate renal branching morphogenesis (Piscione et al., 1997). While the cognate ligand and function of ALK6 in the kidney are as yet unknown, studies in nonrenal tissues suggest that the function of ALK6 may be distinct from ALK3. In the developing limb ALK3 and ALK6 play distinct roles. ALK6 is required for early steps during mesenchymal condensation and cartilage formation while ALK3 controls later cartilage differentiation (Zou et al., 1997).

The spatial and temporal patterning of the renal collecting system is precisely controlled (Saxen, 1987). Our previous work as well as that of others suggests that this development is controlled simultaneously by stimulatory and inhibitory peptide growth factors. Our current work provides insight into how these signals are propagated and integrated within the collecting duct cell and provides a basis for further investigating the genetic control of tubulogenesis.

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