BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis

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1Division of Gastroenterology and Nutrition and 2Program in Developmental Biology, 3Division of Nephrology, Hospital for Sick Children, 4Division of Nephrology, Toronto Hospital, and 5Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario, Canada M5G 1X8

Piscione, Tino D., Thomas D. Yager, Indra R. Gupta, Branko Grinfeld, York Pei, Liliana Attisano, Jeffrey L. Wrana, and Norman D. Rosenblum. BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis. Am. J. Physiol. 273 (Renal Physiol. 42): F961–F975, 1997.—The bone morphogenetic proteins, BMP-2 and OP-1, are candidates for growth factors that control renal branching morphogenesis. We examined their effects in embryonic kidney explants and in the mIMCD-3 cell model of collecting duct morphogenesis (mIMCD-3 cells are derived from the terminal inner medullary collecting duct of the SV40 mouse). Osteogenic protein-1 (OP-1), at a dose of 0.25 nM, increased explant growth by 30% (P = 0.001). In contrast, 100-fold greater concentrations of OP-1 (28 nM) decreased explant growth by 10% (P < 0.001). BMP-2 was entirely inhibitory (maximum inhibition of 7% at 5 nM, P < 0.0004). In an in vitro model for branching morphogenesis utilizing the kidney epithelial cell line, mIMCD-3, low doses of OP-1 (<0.5 nM) increased the number of tubular structures formed by 28 ± 5% (P = 0.01), whereas concentrations >0.5 nM decreased that number by 22 ± 8% (P = 0.02). All concentrations of BMP-2 (0.05–10 nM) were inhibitory (maximum inhibition at 10 nM of 88 ± 3%, P < 0.0001). Stimulatory doses of OP-1 increased tubular length (P = 0.003) and the number of branch points/structure (3.2-fold increase, P = 0.0005) compared with BMP-2. To determine the molecular basis for these effects, we demonstrated that BMP-2 is bound to mIMCD-3 cells by the type I serine/threonine kinase receptor, ALK-3, and that OP-1 bound to an ~80-kDa protein using ligand-receptor affinity assays. To demonstrate that OP-1 can exert both stimulatory and inhibitory effects within a developing kidney, embryonic explants were treated with agarose beads saturated with 2 µM OP-1. OP-1 decreased the number of ureteric bud/collecting duct branches adjacent to the beads by 58 ± 1% (P < 0.0001). In contrast, the number of branches in tissue distal to the OP-1 beads was enhanced, suggesting a stimulatory effect at lower doses of OP-1. We conclude that OP-1 and BMP-2 directly control branching morphogenesis and that the effects of OP-1 are dependent on its local concentration within developing kidney tissue.

bone morphogenetic protein-2; osteogenic protein-1; bone morphogenetic protein-7; inner medullary collecting duct; tubulogenesis

RENAL BRANCHING morphogenesis is defined as the growth and branching of epithelial tubules during embryonic development (30). In the kidney, branching morphogenesis results from reciprocal mesenchymal-epithelial tissue interactions between the mesenchymal metanephric blastema and the epithelial ureteric bud (and its derivative collecting ducts). These interactions are mediated, in part, by peptide growth factors. Recent evidence suggests that bone morphogenetic proteins (BMPs), a family of growth factors within the transforming growth factor-β (TGF-β) superfamily, play a role in controlling these tissue interactions (7, 8, 19, 34).

BMPs constitute the largest subgroup within the TGF-β superfamily. They are related to other TGF-β-related families including the activins and TGF-βs by the homology among their carboxy-terminal domains (reviewed in Ref. 14). The BMPs are subdivided into three subgroups: 1) BMP-2/4, 2) BMP-5, BMP-6, and BMP-7 (also named osteogenic protein-1, OP-1), and 3) BMP-3 and BMP-8 (16). BMPs induce cellular responses by forming heteromeric complexes with type I and type II cell surface transmembrane serine/threonine kinase receptors (28). 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 (37). Binding assays in model cell systems suggest that ALK-3 and ALK-6 are candidate type I receptors for BMP-2 (18) and that ALK-2 is a candidate receptor for OP-1 (33).

A large body of evidence in multiple species indicates that BMPs are involved in controlling morphogenetic steps at multiple stages of development (14). Several types of evidence suggest a role for BMPs during mammalian kidney development. BMP-2/4 and OP-1 and ALK-3 and ALK-6 are expressed in a temporal and spatial pattern that is consistent with a role in inductive mesenchymal-epithelial tissue interactions (2, 6, 7, 24, 35). OP-1 induces the differentiation of epithelial elements when added to explanted uninduced metanephric blastema (34). Mutational inactivation of the murine Op-1 gene results in underdevelopment and disorganization of both the mesenchymal- and epithelial-derived elements in the embryonic kidney. This phenotype suggests that OP-1 may function to control aspects of renal development other than inductive tissue interactions (7, 19). However, the cellular complexity of the developing kidney and the ongoing nature of reciprocal mesenchymal-epithelial interactions severely limit the ability to distinguish the effects of OP-1 at the primary versus secondary level. Therefore, the Op-1−/− renal phenotype does not provide direct evidence regarding the role of OP-1 in branching morphogenesis. The function of BMP-2 and its candidate ALK receptors is also undefined. Mutational inactivation of BMP-2 and its type I serine/threonine kinase...
that the effects of OP-1 are dependent on its local concentration. We characterized the direct effects of these BMPs on developing collecting ducts in the mIMCD-3 model (4). Consistent with our results in explants, OP-1 and BMP-2 are bound to mIMCD-3 cells by the type II serine/threonine kinase receptor, ActRII/IIB. BMP-2 is also bound by the type I serine/threonine kinase receptor, ALK-3, and OP-1 is bound by an ~80-kDa protein. Since peptide growth factors exist in a concentration gradient in some developing tissues (11), we tested the possibility that OP-1 exerts a stimulatory or inhibitory effect on branching morphogenesis in embryonic kidney tissue depending on its local concentration. Using agarose beads saturated with micromolar amounts of OP-1, we demonstrated an inhibition of ureteric bud/collection duct branching adjacent to the beads but stimulation in tissue distal to the beads. Taken together, our results suggest that OP-1 and BMP-2 directly control renal branching morphogenesis and that the effects of OP-1 are dependent on its local concentrations within developing kidney tissue.

METHODS

Embryonic kidney organ culture and treatment of organs with recombinant proteins. Mouse embryos were surgically dissected from embryonic day 12 (E12) pregnant CD1 mice. Embryonic kidneys were isolated by microdissection and were cultured on 0.45 µm polyethylene terephthalate membranes (Falcon) (40) in 12-well multiwell plates in the presence of Richter’s modification of Dulbecco’s modified Eagle’s medium-Ham’s F-12 nutrient mixture (DMEM-F12, BRL Life Technologies) containing 50 µg/ml transferrin (Sigma) (25). Culture medium was changed every 48 h. BMP-2 (provided by Genetech) and OP-1 (provided by Creative Biomolecules) were either added directly to the culture medium or were absorbed at 37°C for 30 min onto Affi-Gel blue agarose beads (100–200 mesh, 75–150 µm diameter; Bio-Rad), previously washed once with phosphate-buffered saline, pH 7.4 (PBS). Treated beads were washed once in Richter’s modified DMEM-F12 medium and manually placed on organ cultures (31). The effect on kidney growth was defined by measuring the surface area of the explants using image analysis software (NIH Image) and by imaging 5-µm paraffin-embedded tissue sections stained with hematoxylin and eosin.

Wholemount immunostaining of embryonic kidneys. Embryonic kidneys were fixed in 4% formaldehyde in PBS for 10 min, washed with PBS four times for 10 min each wash, and then stored in blocking buffer consisting of 1% goat serum and 0.0001% Tween-20 in PBS. Kidneys were then incubated for 2 h with fluorescein-conjugated Dolichos biflorus agglutinin (20 µg/ml) (Vector Labs) in blocking buffer and then washed with PBS four times for 10 min each wash. The effect of ligand on the collecting system was defined by counting the number of ureteric bud/collection duct branches formed on either side of the ureteric bud.

mMCD cell culture. The mMCD-3 cell line is derived from the terminal inner renal medullary collecting duct of the SV40 transgenic mouse. mMCD-3 cells retain several differentiated characteristics of this nephron segment, as previously described (26). Monolayer cultures of mMCD-3 cells (obtained from American Tissue Culture Collection) were maintained in DMEM-F12 supplemented with 5% fetal bovine serum (Hyclone), penicillin (100 U/ml), and streptomycin (100 U/ml) in 5% CO2 at 37°C. For assays of tubulogenesis, collagen gels were prepared on ice by mixing 4 µl of 1 M N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Sigma), 8 µl of 1 M NaHCO3, 40 µl DMEM-F12, 200 µl rat type I collagen (Collagen, Collaborative Biomedical Products), and 25,000–250,000 mMCD-3 cells. Aliquots (50 µl/well) were seeded in 96-well culture plates. After the gels solidified at 37°C, 100 µl of DMEM-F12 containing 5% fetal bovine serum were added to each well. Cultures were then maintained at 37°C in 5% CO2 and the medium was changed every 48 h.

Effect of growth factors on mMCD-3 tubulogenesis. Serial dilutions of activin A and TGF-β1 (kindly provided by Genetech), OP-1, and BMP-2 were prepared in DMEM-F12 and added to newly established cultures of mMCD-3 cells embedded in collagen gels. After 48 h in culture, gels were fixed in 4% formaldehyde in PBS for 10 min at room temperature. Fixed gels were then washed four times in PBS and stored in blocking buffer at 4°C. After fixation, gels were directly imaged by differential interference contrast (DIC) microscopy using an Axioskop microscope and plan-Neofluar objectives (Carl Zeiss). Representative microscopic fields were photographed with a MC80 magnetic shutter camera (Carl Zeiss) using fine-grain black and white film (Kodak T-Max 400 or Ilford Delta 400) at x100 magnification. The effect of each ligand on mMCD-3 tubulogenesis was determined by counting the number of continuous, elongated, linear structures per quadrat of photograph.

Morphometric analysis of mMCD-3 structures. Collagen gels were prepared containing 5,000 cells/gel. Cell culture medium containing ligand was immediately added to wells containing solidified gels to achieve the following concentrations: OP-1, 0.25 nM; and BMP-2, 5 nM. Two or seven days later, gels were fixed and stained with bis-benzamide (Hoechst No. 33258, Sigma) for 30 min at 4°C in the dark and then destained with PBS for 15 min at room temperature in the dark. In some experiments, mMCD-3 nuclei were detected using fine-grain black and white film (Kodak T-Max 400 or Ilford Delta 400) at x100 magnification. The effect of each ligand on mMCD-3 tubulogenesis was determined by counting the number of continuous, elongated, linear structures per quadrat of photograph.

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absence of any nuclei in a structure extending beyond a branch point.

Reverse transcription-polymerase chain reaction cloning of BMPs and receptor serine/threonine kinases. Poly(A)^+ mRNA was isolated from E13 mouse metanephroi by the oligo(dT) method using a commercial kit (Fast Track, Invitrogen). Oligo(dT)-primed first-strand cDNA was synthesized using 200 ng poly(A)^+ mRNA as substrate and reverse transcriptase (Superscript II, GIBCO-BRL). For reverse transcription-polymerase chain reaction (RT-PCR) cloning of cDNAs encoding BMPs, E13 metanephric cDNA was used as a substrate for PCR using degenerate oligonucleotides directed against conserved sequences present in the subfamily of TGF-β members that includes the BMPs, Vg-1 and decapentaplegic (1). A 130-bp DNA band was generated and cloned into pBluescript. Recombinant plasmids were purified and sequenced by the dyeoxy chain-termination method using a commercial kit (Pharmacia). For RT-PCR cloning of cDNAs encoding receptor serine/threonine kinases, first-strand E13 metanephric cDNA was used as substrate for PCR using degenerate oligonucleotides directed against conserved sequences present in the family of receptor serine/threonine kinases (9). A 450-bp PCR product was generated and cloned into pBluescript. Recombinant plasmids were isolated and sequenced.

Detection of mIMCD-3 serine/threonine kinase receptor RNAs. Ribonuclease (RNase) protection assays were performed using specific antisense riboprobes for TβRI (ALK-5), TβRII, ActRIB (ALK-4), ActRII, and c-met. Riboprobes were prepared from linearized plasmid templates treated with proteinase K (50 µg/ml at 37°C for 30 min). Riboprobes were then synthesized by performing in vitro transcription with T3 polymerase in the presence of [32P]UTP (Amersham). Following digestion of template DNA with RNase-free deoxyribonuclease I, full-length radiolabeled transcripts were isolated by gel purification. RNase protection assays were performed using a commercial kit (Ambion). A quantity of 10 µg of mIMCD-3 total RNA was coprecipitated with 5 × 10^6 cpm of riboprobe, resuspended in 20 µl of hybridization buffer (80% formamide, 100 mM sodium citrate, 300 mM sodium acetate, and 1 mM EDTA) and hybridized overnight at 50°C. For a positive control, 5 µg of mouse liver total RNA was hybridized with 5 × 10^6 cpm of an antisense mouse β-actin riboprobe. For a negative control, 10 µg of yeast RNA was hybridized with each riboprobe. Following hybridization, RNase digestion was carried out at 37°C for 30 min. The precipitated RNA was dissolved in 8 µl of 80% loading buffer, heat-denatured at 90°C for 3 min, and electrophoresed in a 8 M urea/6% acrylamide gel.

RT-PCR was used to detect mIMCD-3 mRNA encoding the ALK-2, ALK-3, and ALK-6 receptors. First-strand cDNA was synthesized as described above. PCR reactions were then performed using the following primers, all of which encode members that includes the BMPs, Vg-1 and decapentaplegic (1). A 130-bp DNA band was generated and cloned into pBluescript. Recombinant plasmids were purified and sequenced by the dyeoxy chain-termination method using a commercial kit (Pharmacia). For RT-PCR cloning of cDNAs encoding receptor serine/threonine kinases, first-strand E13 metanephric cDNA was used as substrate for PCR using degenerate oligonucleotides directed against conserved sequences present in the family of receptor serine/threonine kinases (9). A 450-bp PCR product was generated and cloned into pBluescript. Recombinant plasmids were isolated and sequenced.

Ligand-receptor affinity binding studies. Human recombinant BMP-2, OP-1, and TGF-β1 (R & D Systems) were iodinated as described by Frolik et al. (10). For receptor binding studies, 0.1 nM [125I]-labeled TGF-β, 10 nM [125I]-OP-1, or 10 nM [125I]-BMP-2 were incubated with mIMCD-3 cell monolayers and were affinity cross-linked using disuccinimidyl suberate as previously described (20). For immunoprecipitations, cells were lysed in lysis buffer [20 mM tris (hydroxymethyl)ammonium chloride, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100] in the presence of protease inhibitors and centrifuged to remove debris. [125I]-BMP-2- and [125I]-OP-1-labeled cell extracts were incubated with polyclonal antibodies to ALK-1, ALK-2, ALK-3, and ALK-6 (generously provided by P. ten Dijke and K. Miyazono (32, 33)), and TGF-β1-labeled cell extracts were incubated with polyclonal TβRI or TβRII antibodies (generously provided by J. Massagué). Lysates were incubated with antibodies for 1 to 2 h at 4°C and collected on protein A-Sepharose beads (Pharmacia). The immunoprecipitates were washed five times in cold lysis buffer and then resuspended in sample buffer for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Statistical analysis. Data were analyzed using the Statview statistical analysis program (version 4.01; Abacus Concepts, Berkeley, CA). For the dose-response analyses and explant treatment experiments, mean differences between the effect of the various ligands were examined by Student's two-tailed t-test. Differences between measurements obtained in the morphometric analyses of mIMCD branching structures were examined by analysis of variance. Significance was taken at a value of P < 0.05 (two-tailed).
nM OP-1 attenuated the development of the ureteric bud and appeared to reduce the mass of mesenchymal cells. Despite a reduction in kidney surface area, the architecture of BMP-2-treated explants was preserved. However, the tissue appeared to be more compact than control, and the ureteric bud was somewhat attenuated compared with that observed in 0.25 nM OP-1-treated explants. Together these data indicate a direct role for OP-1 and BMP-2 in regulating embryonic kidney development. However, the cellular complexity of the organ culture explant limits the ability to determine the direct effects of BMP-2 and OP-1 on cell types within the kidney and specifically on tubulogenesis. Therefore, we developed an in vitro model using mIMCD-3 cells to investigate the direct effects of BMPs on branching morphogenesis.

BMP-2 and OP-1 exert opposite effects on tubulogenesis in vitro. mIMCD-3 cells are derived from the terminal inner medullary collecting duct of the SV40 mouse (26) and form branching structures in three-dimensional matrices (4). The mIMCD-3 cell model provides an opportunity to test the activity of controlled quantities of ligands on collecting duct morphogenesis without interaction with other cell types. mIMCD-3 cells cultured in type I collagen form branching tubular structures when induced with 5% fetal bovine serum (Fig. 4). We defined intermediate structures formed after induction of mIMCD-3 cells in collagen gels by directly imaging structures at different times after cultures were initiated. Multicellular spheroid structures were observed within 6 h of induction (Fig. 4, A and D), elongated structures by 18 h (Fig. 4, B and E), and elongated branching forms by 72 h (Fig. 4, C and F). Subcellular structures, specifically large cytoplasmic processes, were observed at the ends of structures before and during branch formation (Fig. 4G). Cross sections of elongated branched mIMCD-3 structures revealed that they consist of interconnected cells organized around a central lumen (Fig. 4H); therefore, they can be defined as tubules.

We determined the effect of BMP-2 and OP-1 on mIMCD-3 tubulogenesis by adding these ligands to the
culture medium which bathed the collagen gels. The apoptotic response of mIMCD-3 cells cultured in collagen gels in the absence of serum precluded analysis of the effect of these ligands in the absence of serum. Therefore, in these experiments the effects of BMP-2 and OP-1 were compared in the presence of 5% serum.

To determine whether mIMCD-3 tubulogenesis was regulated in vitro in a manner consistent with effects observed in whole organ explants cultured in vitro, we first tested the effects of known positive and negative regulators of tubulogenesis. TGF-β and activin A are known inhibitors of tubulogenesis in embryonic kidney explants (4, 27), and hepatocyte growth factor (HGF) is stimulatory (29). Consistent with these effects on intact organs, serum alone (Fig. 5A) and 20 ng/ml HGF (Fig. 5B) stimulated mIMCD-3 tubulogenesis. In contrast, 0.5 nM TGF-β and 1 nM activin A inhibited mIMCD-3 tubulogenesis (Fig. 5, D and E). BMP-2 and OP-1 altered both the number and phenotype of the structures that were formed within 48 h. This is precisely quantitated below; here, we focus on the broad phenotype. Treatment with 0.25 nM OP-1 appeared to increase the number of structures formed compared with serum alone (Fig. 5C). In contrast, 2.5 nM BMP-2 produced a marked reduction in the number of mIMCD-3 structures formed (Fig. 5F). These results suggested that BMP-2 and OP-1 have opposite effects on the morphogenesis of mIMCD-3 tubular structures in collagen gels.

To precisely define the effects of BMP-2 and OP-1 on mIMCD-3 tubule formation, we performed dose response analyses of mIMCD-3 tubule formation in the presence of varying doses of these ligands. We assessed the number of mIMCD-3 tubular structures formed...
48 h after induction in collagen gels in the presence of serum supplemented medium containing OP-1, BMP-2, TGF-β, or activin A (Fig. 6). For each ligand, four experiments were performed, and we counted up to 144 ml MCD-3 linear structures in 4 different scaled photographic fields. OP-1 affected ml MCD-3 tubule formation in a biphasic concentration-dependent manner (Fig. 6A). At low concentrations, OP-1 increased the number of ml MCD-3 tubular structures formed above control values with a maximum effect observed at 0.25 nM (28 ± 5% increase, P = 0.01). However, at concentrations greater than 0.5 nM, OP-1 inhibited the number of ml MCD-3 tubular structures formed with maximal inhibition observed at 10 nM (81 ± 8% inhibition, P =
In contrast, the effect of BMP-2 was entirely inhibitory with maximal inhibition at a dose of 10 nM (88 ± 3% inhibition, P < 0.0001) (Fig. 6B). BMP-2 was also inhibitory at concentrations as low as 0.05 nM and was never observed to stimulate the formation of mMCD-3 tubules. Both TGF-β and activin A inhibited the formation of serum-induced mMCD-3 tubular structures in a concentration-dependent manner consistent with previous studies (4, 27) (Fig. 6, C and D). These results demonstrate that OP-1 exerts direct but opposite effects on mMCD-3 tubulogenesis in a dose-dependent manner. In contrast, BMP-2 is inhibitory and more potent than high-dose OP-1 and other members of the TGF-β superfamily.
BMP-2 and OP-1 modulate mIMCD-3 tubulogenesis in a dose-dependent manner. mIMCD-3 cells (50,000 cells/gel) were cultured in type I collagen in presence of serum with TGF-β1 (C), activin A (D), BMP-2 (B), or OP-1 (A) and imaged by DIC 48 h after initiation of cultures. Numbers of tubules (linear structures) formed are expressed as percentage of control (5% fetal bovine serum alone) in 4 independent experiments. A: OP-1 is stimulatory at concentrations ≤0.5 nM [28 ± 5% increase (max), P = 0.01] and inhibitory at concentrations >0.5 nM (1 nM, 13 ± 4% inhibition; P = 0.05; 2.5 nM, 19 ± 11% inhibition; P = 0.16; 10 nM, 22 ± 8% inhibition, P = 0.02). B: BMP-2 is inhibitory at all concentrations tested (1 nM, 45 ± 13% inhibition, P = 0.04; 2.5 nM, 62 ± 8% inhibition, P = 0.005; 5 nM, 80 ± 3% inhibition, P = 0.0001; 10 nM, 88 ± 3% inhibition, P < 0.0001). C: TGF-β1 is inhibitory at all concentrations tested (0.5 nM, 45 ± 12% inhibition, P = 0.03; 1 nM, 67 ± 12% inhibition, P = 0.01). D: activin A is inhibitory at all concentrations tested (2.5 nM, 45 ± 14% inhibition, P = 0.05; 5.0 nM, 39 ± 7% inhibition, P = 0.009; 10 nM, 55 ± 13% inhibition, P = 0.02).

BMP-2 and OP-1 modulate the phenotype of mIMCD-3 tubular structures. Our initial results suggested that BMP-2- and OP-1-treated structures differ in their phenotype. Therefore, we analyzed their structural characteristics. Representative structures imaged simultaneously by DIC and immunofluorescence microscopy are shown in Fig. 7. Morphometric measurements were made using photographed images of 155 structures generated by 2 days and 7 days after induction in collagen gels consisting of a low density of cells (5,000 cells/gel). At this low concentration of cells, mIMCD-3 structures can be imaged at high resolution without optical interference by other adjacent structures. Compared with the images of higher cell-density gels (Fig. 5), structures formed in low-density gels contain fewer cells and develop more slowly as a function of time.

We analyzed the morphometric characteristics of mIMCD-3 tubules during the early phase of tubulogenesis (2 days) and when mature tubules with lumens were formed (7 days). OP-1-treated structures differed from BMP-2-treated structures in the number of branch points per structure and in length (Table 1). Two days after induction of tubulogenesis, OP-1-treated structures were more highly branched than BMP-2-treated structures [OP-1 (n = 48) vs. BMP-2 (n = 44): 0.6 ± 0.1 vs. 0.07 ± 0.05 branch points/structure, respectively; P < 0.0001] (Fig. 7, A–D). Only 3/44 BMP-2-treated structures contained branch points or were branched. Further analysis of structures arising from branch points revealed that only three of all the branches generated in OP-1- and BMP-2-treated structures contained nuclei (Fig. 7, C and D). This suggests that an early step in the formation of a branch is the formation of a cell process and that BMP-2 inhibits the formation of these cell processes. In addition, OP-1-treated structures were longer than BMP-2-treated structures [OP-1 (n = 48) vs. BMP-2 (n = 44): length, 40.6 ± 4.9 vs. 29.5 ± 3.1 μm; P = 0.06]. Despite this difference in length, the number of nuclei/tube did not differ between OP-1- and BMP-2-treated structures [OP-1 (n = 48) vs. BMP-2 (n = 44), 1.8 ± 0.3 vs. 1.8 ± 0.4 nuclei/tube]. This suggests that OP-1 increases cell size.

In contrast to 2-day structures, 7-day structures contained a much larger number of cells [OP-1 (n = 43) and BMP-2 (n = 20), 76 ± 12 and 38 ± 14 nuclei/structure, respectively]. Consistent with our analysis of 2-day structures, OP-1-treated structures were more highly branched than BMP-2-treated structures [OP-1 vs. BMP-2, 13 ± 2 vs. 4 ± 1 branch points/structure; P < 0.0001] (Fig. 7, E–H). The branches arising from these branch points consisted of true branches (contain nuclei) and pseudo branches (consist of cell processes only). Both true and pseudo branches were more numer-
ous in OP-1-treated structures compared with BMP-2-treated branches (OP-1 vs. BMP-2 true branches, 7 ± 1 vs. 2 ± 1 true branches/structure (P < 0.0003); pseudo branches, 5 ± 1 vs. 1 ± 1 pseudo branches/structure (P < 0.0001)). Also consistent with our analysis of 2-day structures was our finding that 7-day OP-1-treated structures were longer than BMP-2-treated structures, probably secondary to the larger number of cells in OP-1-treated tubules (OP-1 vs. BMP-2: length, 191 ± 14 vs. 118 ± 16 µm; P = 0.003). However, although BMP-2 inhibited tubular growth and branching, the smooth borders and width of BMP-2-treated tubules were more similar to native tubules than those treated with OP-1 (Fig. 7, E and G) (30). This suggests that BMP-2 plays a role in modulating the final shape of

Table 1. Effect of OP-1 and BMP-2 on the phenotype of mIMCD-3 cell structures

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2 Days</th>
<th>7 Days</th>
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<tr>
<td></td>
<td>OP-1</td>
<td>BMP-2</td>
</tr>
<tr>
<td></td>
<td>0.25 nM (n = 48)</td>
<td>5 nM (n = 44)</td>
</tr>
<tr>
<td>Length of structure, µm</td>
<td>41 ± 5</td>
<td>30 ± 3</td>
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<tr>
<td>Number of branch points/structure</td>
<td>0.6 ± 0.1</td>
<td>0.07 ± 0.05*</td>
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Values are means ± SE; n = number of structures analyzed. OP-1, osteogenic protein-1; BMP-2, bone morphogenetic protein-2; mIMCD-3, a cell line derived from the terminal inner medullary collecting duct of the SV40 mouse. *P < 0.0001, compared with 0.25 nM OP-1 at 2 days. †P < 0.003, compared with 0.25 nM OP-1 at 7 days. ‡P < 0.0001, compared with 0.25 nM OP-1 at 7 days.
developing branched tubules. Taken together, our analysis of early and mature tubular structures suggests
1) BMP-2 and OP-1 exert different activities during the formation of branched tubules, 2) the formation of a true branch is preceded by the elaboration of a cytoplasmic process by the cell, and 3) BMP-2 inhibits the formation of these branches possibly by interfering with cell process formation.

mIMCD cells express mRNAs encoding BMP receptors. Having demonstrated that BMP-2 and OP-1 control the number and type of mIMCD-3 structures formed in collagen gels, we sought to define the molecular basis for these effects. Since the specificity of the effects of these ligands resides, in part, with the cell surface receptors which bind them, we first identified receptors that are expressed in the E13 metanephros, a stage at which collecting duct morphogenesis is occurring. Since members of the TGF-β superfamily are known to signal via a highly conserved family of cell surface transmembrane serine/threonine kinases, we used a RT-PCR cloning strategy to identify receptor mRNAs (9). By this method we identified cDNAs encoding TβRI (ALK-5), TβRII, ActR1B (ALK-2), ActRIIB, ALK-3, and ALK-6 (Table 2). Next, we determined by RNase protection and RT-PCR assays whether these mRNAs are also expressed by mIMCD-3 cells (Fig. 8; Table 2). RNase protection assays using exact match cRNA probes demonstrated protection of RNA fragments of 200, 380, 390, and 400 bp in size predicted for TβRI (ALK-5), TβRII, ActR1B (ALK-2), and ActRIIB, respectively. These are the known receptors for TGF-β and activin A, respectively. Positive controls in this assay included β-actin and c-met, the receptor for HGF (3). RT-PCR and Southern analysis using exact match primers and cDNA probes demonstrated that mIMCD-3 cells express ALK-3 and ALK-6, type I receptors known to bind BMP-2 (Fig. 8, B and C). Interestingly, we did not detect expression of ALK-2, a candidate OP-1 receptor. Together, these results demonstrate that the E13 metanephros and mIMCD-3 cells express mRNAs encoding type I and type II receptors, which bind TGF-β superfamily members including BMP-2.

BMP-2 binds to mIMCD-3 cells via ALK-3. To further define, at the protein level, the receptors that bind BMP-2 and OP-1, we performed ligand-receptor cross-

### Table 2. Expression of serine/threonine kinase receptor mRNAs in E13 metanephros and mIMCD-3 cells

<table>
<thead>
<tr>
<th>Receptor</th>
<th>E13 Metanephros</th>
<th>mIMCD-3</th>
<th>Candidate Ligand</th>
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<tbody>
<tr>
<td>ALK 1</td>
<td>−</td>
<td>ND</td>
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<td>ALK 2</td>
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<td>+</td>
<td>+</td>
<td>BMP-2, OP-1</td>
</tr>
<tr>
<td>TβRII</td>
<td>+</td>
<td>+</td>
<td>TGF-β</td>
</tr>
<tr>
<td>ActRIIB</td>
<td>+</td>
<td>+</td>
<td>Activin A, OP-1</td>
</tr>
</tbody>
</table>

Embryonic day 13 (E13) metanephros were detected by reverse transcription-polymerase chain reaction (RT-PCR). mIMCD-3 cells were detected by RNase protection or RT-PCR. ND, not done; TGF-β, transforming growth factor-β.

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Fig. 8. mIMCD-3 cells express a broad repertoire of type I and type II serine/threonine kinase receptor mRNAs. A: RNase protection assay demonstrating protection of mIMCD-3 RNAs using 32P-labeled antisense RNA probes for c-met, TβRI, TβRII, ActR1B, ActRIIB, and actin. B: agarose gel of DNA bands generated by reverse transcription-polymerase chain reaction (RT-PCR) of mIMCD-3 poly(A)+ mRNA using exact-match oligonucleotide primers for ALK-3 and ALK-6. C: Southern analysis of agarose gel, shown in B, demonstrating hybridization of 32P-labeled ALK-3 and ALK-6 cDNA probes to candidate ALK-3 and ALK-6 DNA bands generated by RT-PCR of mIMCD-3 RNA. MW, molecular weight markers.
linking with immunoprecipitation using antisera directed against type I and type II receptors (Fig. 9). Isolated receptors were resolved by SDS-PAGE. In these experiments, mIMCD-3 cell receptor affinity labeled with $^{125}$I-TGF-$\beta$ served as a positive control. As expected, we observed three labeled products corresponding to betaglycan, type II and type I receptors, typically observed in a wide variety of cell lines responsive to TGF-$\beta$ (38). The identities of T$\beta$RII and T$\beta$RI were confirmed by immunoprecipitation of total cell lysates with specific receptor antibodies (Fig. 9, middle). Since TGF-$\beta$ receptors typically form stable heteromeric complexes, immunoprecipitates using anti-T$\beta$RII antibodies contained affinity-labeled species corresponding to T$\beta$RII and additional species corresponding to betaglycan and T$\beta$RI, as observed previously (36). Similarly, immunoprecipitates of anti-T$\beta$RI antibodies contained labeled products corresponding to both T$\beta$RI and T$\beta$RII.

mIMCD-3 cells labeled with $^{125}$I-BMP-2 yielded three labeled products, a high-molecular-weight band at $\sim$180 kDa, a diffuse 80–85-kDa band, and a faster migrating 75-kDa band (Fig. 9, left). The two larger proteins likely represent the two alternatively spliced forms of BMPR-II (18). Alternatively, the 80- to 85-kDa form may also represent ActRII and ActRIIB, which under the appropriate conditions also bind BMP-2 (L. Attisano and J. L. Wrana, unpublished observations). To identify the specific BMP-2 binding type I receptors, lysates from affinity-labeled cells were subjected to immunoprecipitation with specific type I receptor antibodies. Only anti-ALK-3 immunoprecipitates contained affinity-labeled complexes. Right: total cell lysate after labeling of cells with $^{125}$I-OP-1 contains five labeled products, 200, 180, 150, 85, and 80 kDa in size. Immunoprecipitation of the cell lysate with ActRIII/IB antibodies demonstrates that OP-1 binds this type II receptor as in the cell line C5.18. Immunoprecipitation of cell lysate with specific type I receptor antibodies demonstrates that the 80-kDa protein (p80) is not ALK-1, ALK-2, ALK-3, or ALK-6.

Fig. 9. BMP-2 and OP-1 bind to mIMCD-3 cell surface proteins. $^{125}$I-labeled ligand was cross-linked to mIMCD-3 cells in monolayer. Labeled ligand-receptor complexes were immunoprecipitated using antisera directed against type I and type II receptors and analyzed by SDS-PAGE. Middle total cell lysate after labeling of cells with $^{125}$I-TGF-$\beta$ contains 3 labeled products, $>180$, 80–85, and 69 kDa in size. Immunoprecipitation of cell lysate with anti-T$\beta$RII yielded two bands, the size of T$\beta$RI and T$\beta$RII. Immunoprecipitation of cell lysate with anti-T$\beta$RII yielded a major band the size of T$\beta$RI and a minor band the size of T$\beta$RII. Left: total cell lysate after labeling of cells with $^{125}$I-BMP-2 contains 3 labeled products, 180, 80–85, and 75 kDa in size. Immunoprecipitation of the cell lysate with specific type I receptor antibodies demonstrating that only anti-ALK-3 immunoprecipitates contain affinity-labeled complexes. Right: total cell lysate after labeling of cells with $^{125}$I-OP-1 contains five labeled products, 200, 180, 150, 85, and 80 kDa in size. Immunoprecipitation of the cell lysate with ActRIII/IB antibodies demonstrates that OP-1 binds this type II receptor as in the cell line C5.18. Immunoprecipitation of cell lysate with specific type I receptor antibodies demonstrates that the 80-kDa protein (p80) is not ALK-1, ALK-2, ALK-3, or ALK-6.
to p80, we performed competition assays using unla-
beled OP-1. As previously observed (39), addition of
unlabeled OP-1 did not displace binding of labeled OP-1
but rather increased binding to the cell lysates (data
not shown). The basis for this phenomenon is currently
unclear (39). To attempt to determine the identity of
p80, we performed immunoprecipitation with antibod-
ies directed against the type I receptors, ALK-1, ALK-2,
ALK-3, and ALK-6. However, none of these antibodies
identified p80. These results suggest that the OP-1
signaling pathway in mIMCD-3 cells includes ActRII/
IIB and another as yet unidentified receptor that may
correspond to p80.

OP-1 exerts both stimulatory and inhibitory effects on
branching morphogenesis in the developing kidney. On
the basis of our results in embryonic kidney explants
and in the mIMCD-3 model of collecting duct morpho-
genesis, we hypothesized that OP-1 exerts opposite
effects on branching morphogenesis in different areas
of developing kidney tissue depending on its local
concentration. To test this directly, we delivered recom-
binant OP-1 and BMP-2 in a spatially restricted man-
ner to developing murine E12 kidneys by saturating
agarose beads with a micromolar amount of ligand and
placing ligand-saturated beads on the kidney surface
(Fig. 10) (25). Consistent with our previous results, the
growth of kidney explants treated with high doses of
OP-1 or BMP-2 was significantly less than that of
kidneys treated with beads saturated with bovine
serum albumin (Table 3). Since branched tubular struc-
tures are formed symmetrically on either side of the
ureteric bud, we tested the effect of these BMPs on
branching morphogenesis by applying them to one side
of the kidney. We then compared the number of ureteric
bud/collecting duct branches that formed in the side
adjacent to the beads to that on the untreated contralat-
eral side. On the sixth day in culture, control kidneys
contained a nearly equivalent number of
D. biflorus agglutinin-stained collecting system branches on either
side of the ureteric bud (ratio of mean number on each
side $\pm$ 0.01; Table 3). In contrast, the number of
branches on the side receiving either 2 µM BMP-2 or 2
µM OP-1 beads was 64 $\pm$ 2% and 58 $\pm$ 1% less than that
on the untreated contralateral side ($P < 0.0001$).
Furthermore, in the tissue immediately adjacent to

Table 3. Effect of BMP-2 and OP-1 on embryonic
kidney growth and development

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>2 µM BMP-2</th>
<th>2 µM OP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in diameter, %</td>
<td>3.0 ± 0.5</td>
<td>-4.0 ± 0.4*</td>
<td>-4.0 ± 0.4*</td>
</tr>
<tr>
<td>No. branches: side of bead vs.</td>
<td>92 ± 1</td>
<td>64 ± 2†</td>
<td>58 ± 1†</td>
</tr>
<tr>
<td>opposite side, %</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 separate experiments. Numbers of ureteric bud/collecting duct branches were identified by staining with Dolichos biflorus agglutinin. *$P < 0.0001$, control vs. BMP-2 or OP-1. †$P < 0.0001$, control vs. BMP-2 or BMP-7.
BMP-2 and OP-1 beads, which presumably received the highest dose of ligand, no ureteric bud/collection duct structures were observed. In contrast, we consistently observed that the collecting system in the region of the kidney distal to the OP-1 beads was more highly branched than in any region of the control or BMP-2-treated explants. In this region, the concentration of OP-1 is predicted to be lower than in areas adjacent to the beads secondary to diffusion of the ligand. We attempted to demonstrate directly that agarose beads soaked in low doses of OP-1 stimulate branching morphogenesis in explants. We did not observe any stimulation under these conditions. This negative result is likely explained by the relatively narrow dosage range in which OP-1 is stimulatory, the strong affinity of OP-1 for the extracellular matrix (35), and the slow kinetics of peptide release from agarose beads (13, 31). Polypeptides such as epidermal growth factor and fibroblast growth factor are bound to agarose beads with an efficiency of 90–95%. Approximately 50% of bound protein is released within the first 24 h, whereas 10% is released during every subsequent 24-h period. Thus these release kinetics combined with the narrow dose response range and sequestration of growth factor by the extracellular matrix could easily mitigate against a stimulatory effect in explant tissue. However, taken together with our in vitro data, our results strongly support our hypothesis that OP-1 exerts different morphogenetic effects, depending on its local concentration in developing kidney tissue.

**DISCUSSION**

Previous studies have demonstrated the spatial expression of OP-1 and BMP-2 early in mouse kidney development and have suggested a role for these BMPs in mesenchymal-epithelial interactions and their morphogenetic consequences (2, 8). During early murine kidney development, OP-1 mRNA is expressed in the ureteric bud and in the induced metanephric mesenchyme. In contrast, BMP-2 mRNA expression is restricted to the metanephric mesenchyme. Later, during maturation of mesenchymal-derived elements, OP-1 is expressed in maturing glomeruli, whereas BMP-2 expression decreases markedly. Although functional tests have indicated a role for OP-1 in the induction of the metanephric blastema (7, 19, 34), there is no direct evidence that either OP-1 or BMP-2 controls branching morphogenesis.

Our experiments in embryonic kidney explants (Figs. 1–3) and in an in vitro model of collecting duct morphogenesis (Figs. 5 and 6) demonstrate that OP-1 exerts opposite effects on branching morphogenesis in a dose-dependent manner. Picomolar amounts of OP-1 stimulate, whereas nanomolar amounts inhibit. In vitro, low doses of OP-1 (0.25 nM) increase mMCD-3 tubule length and branching, and 100× higher doses attenuate these effects. The effects of 0.25 nM OP-1 and 10 nM OP-1 on embryonic kidney explants are consistent with the effects in vitro. In contrast, the effects of BMP-2 are monophasic and inhibitory. BMP-2 inhibits the formation of tubules. However, its qualitative effects are more complex. Although BMP-2 inhibits branch formation and linear growth, it generates a more sculpted, thicker tubular trunk in vivo and in vitro (Figs. 3 and 7). Thus OP-1 and BMP-2 may act cooperatively to regulate both the number and the structural characteristics of developing branched tubules.

The mechanism that underlies the stimulatory effect of OP-1 (<0.25 nM) on tubulogenesis is undefined. Further studies are needed to determine the effect of OP-1 on cellular mechanisms which appear to be critical during tubulogenesis. These include cell proliferation, cell process formation, and cell-cell adhesion (22). The mechanisms that underlie the inhibitory effect of BMP-2 and OP-1 (>0.25 nM) are also undefined. However, the observation that activation of the ALK-3 receptor mediates apoptosis in the developing limb (41) suggests that treatment of mMCD-3 cells in collagen gels with BMP-2 may induce apoptosis via activation of ALK-3, thereby decreasing the number of cells capable of forming tubular structures. Further studies are required to test this possibility and to determine whether the inhibitory effect of high-dose OP-1 is mediated by a similar mechanism.

Our data provide insights into the mechanisms of branch formation. The structures that arise from branch points in immature OP-1-treated structures are cellular processes. More mature structures consist of true branches (consisting of one or more cells) that arise from these cellular processes. Immature BMP-2-treated structures rarely consist of a branch point. Thus our data suggest that BMP-2 controls branch formation by inhibiting the formation of cellular processes. Recent studies have defined some of the molecular elements involved in filopodia formation (23) and in the formation of tubulin-based processes (22), and this information may provide a basis to test the effects of BMP-2 and OP-1 on these pathways. Thus further studies are required to characterize whether these cellular processes are actin or microfilament based.

OP-1 also increases the length of mMCD-3 structures. In our analysis of 2-day, immature structures and 7-day, mature structures, we found no significant difference in the number of resident nuclei within OP-1- vs. BMP-2-generated tubules. This suggests that properties other than cell number (e.g., cell shape, cell size) determine the length of these structures. Further studies will be required to test whether the effects of these BMPs on elements of the cytoskeleton are related to this observed effect on cell shape.

The differential effects of BMP-2 and OP-1 on branching morphogenesis are surprising, since both of these growth factors signal via a highly conserved family of cell surface receptors. To determine the molecular basis for these effects, we identified type I and II serine/threonine cell surface receptors, which can act as binding partners for BMP-2 and OP-1 (Fig. 9). These results provide a biochemical basis for the phenotypic effects we observed. We demonstrate that the BMP-2 cell surface receptor complex consists of ALK-3 and a type II receptor, the size of which is consistent with either BMPRII or ActRIIB. These results are consistent
with the recent demonstration that ligand-dependent phosphorylation of MADR1, a downstream effector of BMP-2, requires the coexpression of type I and type II BMP-2 receptors and can be mediated by either ActRIIB or BMPRII in conjunction with either ALK-3 or ALK-6 (15). Our results strongly suggest that in mIMCD-3 cells, ALK-3 is the type I receptor that signals in the BMP-2 pathway. Our results also demonstrate that the mIMCD-3 OP-1 receptor complex consists of the type II receptor, ActRII/IIIB. We were not able to identify type I receptors (ALK-2, ALK-3, ALK-6) in this complex using specific antibodies. This may be because receptors such as ALK-2 are expressed in lower than detectable quantities. Our inability to detect receptors with these antibodies is not explained by cross-species differences, since the ALK-6 antibody is directed against a mouse peptide (32), the ALK-3 antibody identifies ALK-3 in the mIMCD-3 BMP-2 receptor complex, and the ALK-2 antibody is directed against a human peptide that is identical in mouse ALK-2. We did identify an unknown protein, p80, in the OP-1 receptor complex. However, the failure of unlabeled OP-1 to competitively displace radiolabeled OP-1 as previously described (39) limited our ability to determine whether p80 is a specific OP-1 receptor. This unusual property of OP-1 is possibly due to its high affinity for the extracellular matrix (35).

Our demonstration that BMP-2 and OP-1 have opposite effects on mIMCD-3 tubulogenesis is consistent with previous observations that members of the TGF-β superfamily can induce different biological responses (16). For example, during patterning of the Xenopus embryo, activins can function to induce dorsal mesoderm while BMP-4 induces ventral mesoderm (12). The specificity of these responses appears to be mediated by the particular type I receptor that is activated by a particular BMP-type II receptor complex. Type I receptors with highly related kinase domains can mediate similar biological responses, whereas more distantly related type I receptors may mediate distinct responses (5). Such is the case for BMP-2 signaling, which is regulated by ALK-3 and ALK-6 but not by ALK-2 and ALK-5 (15). At the level of protein homology, the kinase domains of ALK-3 and ALK-6 are highly related to each other but share less identity with those of ALK-1, ALK-2, ALK-4, and ALK-5 (33). These observations support a hypothesis that predicts that at low concentrations, OP-1 signals via a type I receptor other than ALK-3 and ALK-6. Our studies demonstrate that the mIMCD-3 OP-1 receptor, p80, is not ALK-1, ALK-2, ALK-3, or ALK-6. In addition, our demonstration that OP-1 stimulates mIMCD-3 tubulogenesis and increases the number of branches and the length of structures suggests that the OP-1 receptors signal via a stimulatory pathway distinct from the BMP-2 pathway. The elements in both stimulatory and inhibitory pathways downstream of BMP-2 are largely unknown. Future studies aimed at identifying these elements will provide a necessary biochemical basis for the effects of these BMPs at the cellular level.

The growth and branching of the ureteric bud and its daughter collecting ducts appears to be tightly regulated during renal development. The expression of growth factors that signal via stimulatory or inhibitory pathways may serve as a mechanism to control these processes. Our observations that BMP-2 and OP-1 differentially regulate both the number and the phenotype of branched tubules in a dose-dependent manner suggest that both these BMPs are important regulatory molecules for these morphogenetic events. This is consistent with the known spatial expression of BMP-2 and OP-1 in the developing kidney and the knowledge that BMPs play diverse roles during the development of nonrenal tissues. Further studies aimed at determining the cellular targets of these growth factors in vivo during metanephric development will further define their role in regulating collecting duct development.

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