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Identification of pleiotrophin as a mesenchymal factor involved in ureteric bud branching morphogenesis

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SUMMARY

Branching morphogenesis is central to epithelial organogenesis. In the developing kidney, the epithelial ureteric bud invades the metanephric mesenchyme, which directs the ureteric bud to undergo repeated branching. A soluble factor(s) in the conditioned medium of a metanephric mesenchyme cell line is essential for multiple branching morphogenesis of the isolated ureteric bud. The identity of this factor had proved elusive, but it appeared distinct from factors such as HGF and EGF receptor ligands that have been previously implicated in branching morphogenesis of mature epithelial cell lines. Using sequential column chromatography, we have now purified to apparent homogeneity an 18 kDa protein, pleiotrophin, from the conditioned medium of a metanephric mesenchyme cell line that induces isolated ureteric bud branching morphogenesis in the presence of glial cell-derived neurotrophic factor. Pleiotrophin alone was also found to induce the formation of branching tubules in an immortalized ureteric bud cell line cultured three-dimensionally in an extracellular matrix gel. Consistent with an important role in ureteric bud morphogenesis during kidney development, pleiotrophin was found to localize to the basement membrane of the developing ureteric bud in the embryonic kidney. We suggest that pleiotrophin could act as a key mesenchymally derived factor regulating branching morphogenesis of the ureteric bud and perhaps other embryonic epithelial structures.

Key words: Ureteric bud, Kidney development, Mesenchymal epithelial interaction, Pleiotrophin, Rat, GDNF

INTRODUCTION

Many epithelial organs such as kidney, lung and prostate undergo branching morphogenesis in the course of development. The kidney is formed by mutual induction between two precursor tissues derived from the intermediate mesoderm, the metanephric mesenchyme (MM) and the ureteric bud (UB; Grobstein, 1953). The UB induces the MM to differentiate and form the proximal nephron, while the UB undergoes dichotomous branching and elongation as it invades the MM, ultimately forming the kidney collecting system (Saxen, 1987). Although it is believed that the MM directs UB branching morphogenesis, the exact nature of the directive signal(s) is unknown.

Soluble factors that have been hypothesized to function in such a morphogenetic capacity include hepatocyte growth factor (HGF) and epidermal growth factor (EGF) receptor ligands, which have been shown to induce branching tubular structures in epithelial cells cultured in collagen gels (Barros et al., 1995; Cantley et al., 1994; Montesano et al., 1991; Sakurai et al., 1997b). However, these studies have been carried out largely on adult cell lines. In a cell culture model that employs UB cells, an epithelial cell line derived from embryonic day 11.5 (E11.5) mouse UB, neither HGF, EGF receptor ligands, or many other factors tested (alone or in combination), were able to induce UB cells to form branching tubular structures with lumens (Sakurai et al., 1997a). However, UB cells undergo branching tubulogenesis in the presence of a conditioned medium elaborated by a cell line derived from the MM also isolated from an E11.5 mouse (BSN cells; Sakurai et al., 1997a). This data suggests that other, yet to be identified, soluble factors present in BSN-CM are important for UB cell morphogenesis. These potentially novel factors that are presumably secreted by the MM may be as (or more) important for the development of the collecting system as those currently receiving attention.

This MM-derived cell conditioned medium (BSN-CM), when supplemented with glial cell-derived neurotrophic factor (GDNF), also induces the isolated rat UB (in the absence of MM) to undergo dichotomous branching reminiscent of that seen in the developing kidney (Qiao et al., 1999a). This indicates that the MM-derived cell line, presumably reflecting the MM itself, secretes soluble factors capable of inducing branching morphogenesis of the UB. This isolated UB culture system can serve as a powerful assay system since it directly assesses the effect of soluble factors on UB morphogenesis.

We have isolated a UB branching morphogenetic activity from the BSN-CM and identified it as an 18 kDa heparin binding protein, pleiotrophin. Pleiotrophin was originally discovered as a fibroblast proliferative factor (Milner et al.,

1989) and a neurite outgrowth-promoting factor (Rauvala, 1989). Outside the nervous system, pleiotrophin is generally detected in embryonic organs in which mesenchymal-epithelial interactions are thought to play an important role, such as salivary glands, lung, pancreas, and kidney (Mitsiadis et al., 1995; Vanderwinden et al., 1992). Although pleiotrophin has been shown to be mitogenic for certain epithelial cells (Li et al., 1990; Sato et al., 1999), there has been no compelling evidence for a key role for pleiotrophin during epithelial organogenesis. Here, we have shown that purified pleiotrophin induces impressive branching morphogenesis of the isolated UB (in the presence of GDNF) as well as tubule formation in a UB cell line in vitro. We suggest that pleiotrophin is a key metanephric mesenchymally derived factor that plays a critical role in branching morphogenesis of the UB during kidney development.

MATERIALS AND METHODS

Unless otherwise stated, the incubations were performed at 37°C in an atmosphere of 5% CO_2 and 100% humidity. For the immunodetection of pleiotrophin either on western blots or frozen sections of E13 mouse kidney, a goat anti-pleiotrophin antibody (R&D systems) was used.

Cell culture and conditioned medium

BSN cells were grown to confluency in DMEM/F12 supplemented with 10% fetal calf serum (FCS). The growth medium was removed and the cells were then incubated in serum-free DMEM/F12 for 3-4 days followed by collection of the conditioned medium (Qiao et al., 1999a). Swiss 3T3 cells (ATCC) were grown to confluency in DMEM with 10% FCS. Once the cells were confluent, the growth medium was replaced with DMEM supplemented with 2% FCS and the cells were cultured for an additional 3-4 days. The conditioned medium was collected and used for the experiments. UB cells were cultured in DMEM supplemented with 10% FCS at 32°C in an atmosphere of 5% CO_2 and 100% humidity.

Isolated ureteric bud and whole embryonic kidney culture

Timed pregnant female Sprague-Dawley rats at day 13 of gestation (day 0 being the day of appearance of the vaginal plug) were sacrificed and the uteri were removed. The embryos were dissected free of surrounding tissues and the kidneys were isolated. For the culture of the whole kidney rudiment, 2-3 kidneys were applied directly to the top of a polyester Transwell filter (0.4 μm pore size; Corning-Costar). The Transwells were then placed within individual wells of a 24-well tissue culture dish containing 400 μl DMEM/F12 supplemented with 10% FCS with or without purified pleiotrophin. Following 7 days in culture, the kidneys were fixed in 2% paraformaldehyde and double-stained with fluorescein-conjugated *Dolichos biflorus* lectin, which binds specifically to UB-derived structures (Laitinen et al., 1987), and rhodamine-conjugated peanut agglutinin, a lectin which binds to structures derived from the MM (Laitinen et al., 1987), as described previously (Qiao et al., 1999a). Fluorescent staining was detected using a laser-scanning confocal microscope (Zeiss).

In the case of culture of the isolated UB, the isolated kidneys were trypsinized for 15 minutes at 37°C in L-15 medium containing 2 μg/ml trypsin (Sigma). Trypsin digestion was arrested by the addition of 10% FCS and the kidneys were removed to fresh L-15 where the UBs were isolated from surrounding MM by mechanical dissection. Isolated UBs were suspended within an extracellular matrix gel [1:1 mixture of growth factor reduced Matrigel (BD) and Type 1 collagen (BD)] applied to the top of a polyester Transwell filter (0.4 μm pore size; Corning-Costar). The Transwells were placed within individual wells of a 24-well tissue culture dish containing 400 μl of either whole BSN-CM, purified fractions of BSN-CM, or DMEM/F12 which were supplemented with human recombinant fibroblast growth factor 1 (FGF1; 250 ng/ml; R&D Systems), rat recombinant GDNF (125 ng/ml; R&D Systems) and 10% FCS and cultured as previously described (Qiao et al., 1999a). Phase-contrast photomicrographs of the developing UB were taken using a RT-Slider Spot digital camera (Diagnostic Instruments Inc.) attached to a Nikon Eclipse TE300 inverted microscope.

Three-dimensional UB cell culture

Confluent monolayers of UB cells were removed from tissue culture dishes by light trypinization and the cells (20,000 cells/ml) were suspended in an extracellular matrix gel composed of 80% Type 1 collagen and 20% growth factor-reduced Matrigel (Sakurai et al., 1997a). 100 μl of the UB cell-containing gel was then aliquoted into individual wells of a 96-well tissue culture plate. After gelation, 100 μl of growth medium (DMEM/F12 with or without purified pleiotrophin) supplemented with 1% FCS was applied to each well and the cultures were incubated at 32°C in 5% CO_2 and 100% humidity. Following 4 days in culture, the percentage of cells and/or colonies processes per colony was counted as an indicator of the tubulogenic activity. Phase-contrast photomicrographs were taken as described above.

Purification of morphogenetic factor

1.5-2 l of BSN-CM collected as described above was filtered to remove extraneous cellular debris using a 0.22 μm polyethersulphone membrane filter (Corning). The BSN-CM was then concentrated approx. 40-fold using a Vivaflow 200 concentrator with a 5 kDa molecular mass cutoff (Sartorius). After adjusting the salt concentration to 0.4 M NaCl, the concentrated BSN-CM was then subjected to sequential liquid column chromatography using an AKTA purifier (Amersham-Pharmacia). Initial fractionation was performed using a heparin sepharose chromatography column (HiTrap heparin, 5 ml; Amersham-Pharmacia). The flow-through fraction was collected and individual 5 ml fractions of the heparin-bound proteins were eluted using increasing concentrations of NaCl (0.4 M-2.0 M) buffered to pH 7.2 with 50 mM Hepes. Aliquots of each fraction were subjected to buffer exchange by dia-filtration using an Ultrafree 500 spin column (Millipore) according to the manufacturer's instructions and then tested for morphogenetic activity using the isolated UB culture system.

An active fraction corresponding to the 1.2~1.4 M NaCl eluate was identified based on its ability to induce branching morphogenesis of the isolated UB. After adjusting this fraction to 1.7 M ammonium sulfate (pH 7.2) it was subjected to further fractionation using a Resource phenyl sepharose hydrophobic interaction column (1 ml; Amersham-Pharmacia). The flow-through was collected and 1 ml fractions of bound proteins were eluted with decreasing concentrations of ammonium sulfate (1.7 M-0 M). After buffer exchange, the individual fractions were again tested for their ability to induce UB branching morphogenesis.

The morphogenetically active fractions from the hydrophobic interaction column were diluted 10-fold with 50 mM Hepes and applied to a Resource S cation exchange column (1 ml; Amersham-Pharmacia). The flow-through was collected and individual 1 ml fractions of bound proteins were eluted using increasing NaCl concentrations (0 M-2.0 M) and assayed for their ability to induce branching morphogenesis.

The active fractions from the Resource S cation exchange column were subjected to further fractionation using a Superdex 200 gel filtration column (Amersham-Pharmacia). Individual 1 ml fractions were collected and assayed for morphogenetic activity. In addition, the active fractions from the Resource S cation exchange column were subjected to SDS-PAGE and the proteins were visualized using Coomassie Blue (Colloidal Coomassie; Invitrogen) staining.

Individual protein bands were cut out of the gels and submitted for microsequencing. Sequence analysis of the protein bands was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer.

RESULTS

Conditioned medium from metanephric mesenchyme-derived cells is required for isolated UB branching morphogenesis

To identify mesenchymal factors that induce branching morphogenesis of the ureteric bud (UB), we employed a metanephric mesenchyme (MM)-derived cell line (BSN cells) as a substitute for the embryonic MM (Sakurai et al., 1997a). These cells were derived from the embryonic day 11.5 (E11.5) MM from a SV40 large T-expressing transgenic mouse and have been extensively characterized. BSN cells are positive for vimentin and negative for cytokeratin, E-cadherin and ZO-1 by immunostaining, as well as negative for *Dolichos biflorus* lectin-binding. As determined by PCR the cells express WT1 and are negative for c-ret (H. S., unpublished observations), they also express mRNA for growth factors such as HGF and TGFβ as determined by northern blot (Sakurai et al., 1997a). cDNA array analysis has confirmed their non-epithelial character (Pavlova et al., 1999). Most importantly, conditioned medium elaborated by BSN cells (BSN-CM) has been shown to act similarly to the MM: it induces branching morphogenesis of cultured UB cells and the isolated UB (in the presence of GDNF).

UBs isolated from E13 rat embryos, when suspended in an extracellular matrix gel and cultured in the presence of BSN-CM (with GDNF), grew to form impressive multiply branching tubular structures comparable to those seen in in vivo kidney development (though the growth was non-directional) (Fig. 1B). As previously shown (Qiao et al., 1999a), in the absence of BSN-CM, however, the UBs failed to develop (Fig. 1C). Thus, BSN-CM apparently contains an additional soluble factor(s) necessary for epithelial cell branching morphogenesis. Using this isolated UB culture model as an assay, we attempted to purify the key morphogenetic factor present in the BSN-CM.

Pleiotrophin is a morphogenetic factor present in BSN-CM

SDS-PAGE and silver staining of BSN-CM revealed the presence of many protein bands (Fig. 2A). Liquid column chromatography was used to fractionate BSN-CM, and each fraction was tested for its ability to induce branching morphogenesis of the isolated UB. Of the multiple columns tested, a heparin sepharose column was found to adsorb most of the morphogenetic activity. Within this heparin-binding fraction, a fraction that eluted at a NaCl concentration of 1.2-1.4 M possessed particularly strong morphogenetic activity. Silver stain analysis of this fraction revealed the presence of prominent lower molecular mass (<20 kDa) protein bands (Fig. 2A). This active fraction was then applied to a Resource phenyl sepharose hydrophobic interaction column. A morphogenetic activity was eluted from this column at 1.4-1.2 M ammonium sulfate. Again, silver staining of this peak fraction revealed prominent low molecular mass protein bands (Fig. 2A). This active fraction was diluted 10-fold with 50 mM Hepes (pH 7.2) buffer and applied to a Resource S cation exchange column. The Resource S column chromatogram is shown in Fig. 2B. Each 1 ml fraction of the Resource S eluate was substituted for whole BSN-CM in the isolated UB culture and compared with BSN-CM itself. Of the 8 fractions eluted from the column, only fraction 4, the peak protein fraction, induced significant UB morphogenesis (Fig. 2C, panel 4). SDS-PAGE analysis and silver staining of this peak fraction revealed the presence of a single protein band with an approximate molecular mass of 18 kDa (Fig. 2D, lane 4). This protein band was subjected to in-gel digestion followed by tandem mass spectrometry and was identified as pleiotrophin. (This type of experiment was done 3 times during different purifications, and pleiotrophin was always detected by mass spectrometry). The presence of pleiotrophin in the active fraction (fraction 4) was confirmed by immunoblot analysis using anti-pleiotrophin antibodies (Fig. 2E). The morphogenetic activity of individual fractions corresponded to the presence of pleiotrophin in that fraction. In a similar fashion, further purification of the peak fraction from the Resource S column was accomplished by applying the active fraction to a Superdex 200 gel filtration column. A single protein peak eluted at 15.93 ml (Fig. 3A), corresponding to a protein with a molecular mass of approximately 18 kDa, and was positively identified as pleiotrophin by immunoblotting (Fig. 3B). This fraction induced isolated UB branching morphogenesis (Fig. 3C). Taken together, these results identify pleiotrophin as a morphogenetic factor present in BSN-CM.

Previous studies have found that pleiotrophin can be isolated to homogeneity from a conditioned medium elaborated by

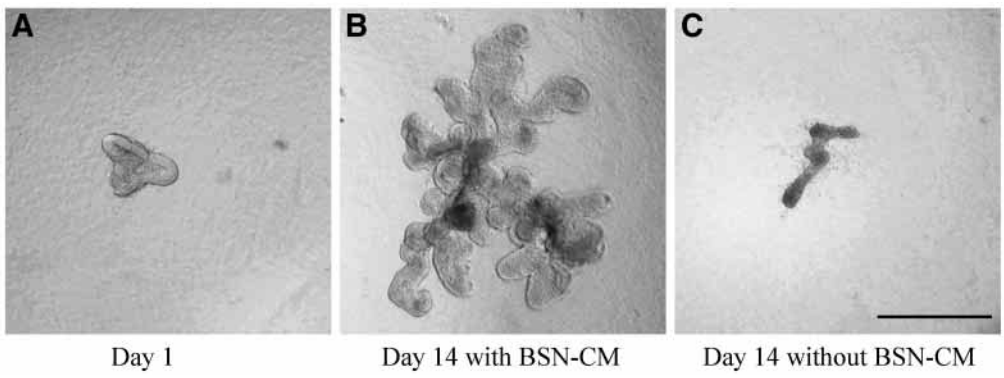

Fig. 1. BSN-CM is necessary for isolated ureteric bud branching morphogenesis. Phase contrast photomicrographs of isolated ureteric buds cultured for 14 days in the presence (B) or absence (C) of BSN-CM in the presence of 10% FCS, 125 ng/ml GDNF, and 250 ng/ml FGF1. In the presence of BSN-CM, the T-shaped ureteric bud (A) underwent extensive branching morphogenesis (B). In the absence of BSN-CM, no significant growth/morphogenesis was observed (C). Scale bar, 500 μm.

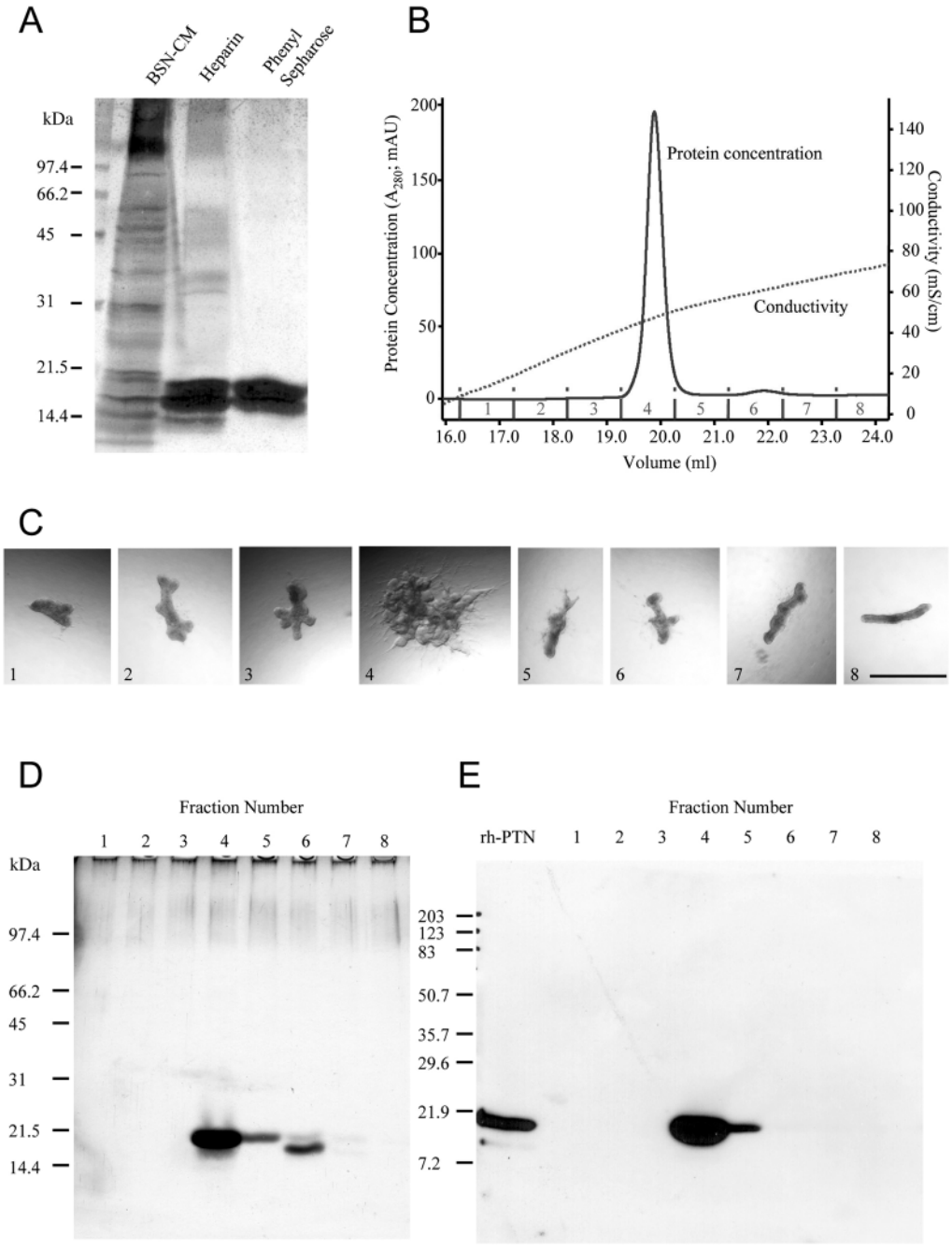

Fig. 2. Purification of the morphogenetic factor. (A) Silver-stained SDS-PAGE gel of active fractions from column chromatography of BSN-CM. Lane 1, whole BSN-CM; lane 2, active fraction from heparin sepharose column; lane 3, active fraction from the Resource phenyl sepharose hydrophobic interaction column. (B) Elution profile from the Resource S cation exchange column of the active fraction from Resource phenyl sepharose column. A single, sharp protein peak was eluted at 0.4-0.6 M NaCl. Each of the individual 1 ml fractions eluted from the column are indicated by the numbers (1-8) above the x axis. (C) Phase-contrast photomicrographs of isolated ureteric buds cultured for 7 days in the presence of each 1 ml fraction from the Resource S cation exchange column (1-8 in B) supplemented with 10% FCS, 125 ng/ml GDNF and 250 ng/ml FGF1. Fraction 4, which corresponded to the protein peak on the elution profile (B) exhibited potent morphogenetic activity. Scale Bar, 500 μm. (D) Silver-stained SDS-PAGE gel of each fraction (1-8) eluted from the Resource S cation exchange column (B). Fraction 4, which possessed potent morphogenetic activity (C) contained a single low molecular mass band, which was identified as pleiotrophin by mass spectrometry. (E) Immunoblot analysis of the individual fractions eluted from the Resource S cation exchange column (1-8 in B). The blot was probed with anti-pleiotrophin antibodies. Rh-PTN; 250 ng of human recombinant pleiotrophin as a positive control.

Swiss 3T3 cells (Sato et al., 1999). Thus, using this alternative purification procedure, a pure fraction of pleiotrophin was isolated from 3T3 conditioned medium (3T3-CM), as confirmed by silver staining, immunoblot analysis (Fig. 4A,B) and mass spectrometry. Like the pleiotrophin purified from BSN cells, this pure pleiotrophin was capable of inducing impressive branching morphogenesis of the isolated UB (Fig. 4C, left panel). Thus, pleiotrophin purified from two different cell lines gave the same results. Interestingly, as we have reported previously (Qiao et al., 1999a), approx. 10× concentrated whole 3T3-CM failed to induce branching morphogenesis of the isolated UB (data not shown), suggesting that 3T3-CM may contain an inhibitory factor.

Nevertheless, to provide further confirmation that pleiotrophin is the factor inducing the morphogenetic changes observed in the isolated UB culture, we took advantage of the documented ability of polyA-sepharose to adsorb pleiotrophin (Corbley, 1997). As seen in Fig. 4A,B, treatment of purified pleiotrophin with polyA-sepharose beads results in the loss of detectable pleiotrophin, both by silver staining and immunoblot analysis. Importantly, this bead-depleted fraction was no longer capable of inducing UB branching morphogenesis (Fig. 4C, right panel), providing further evidence that pleiotrophin is a morphogenetic factor for UB branching morphogenesis. It is worth adding here that insect cell-derived recombinant human pleiotrophin is incapable of

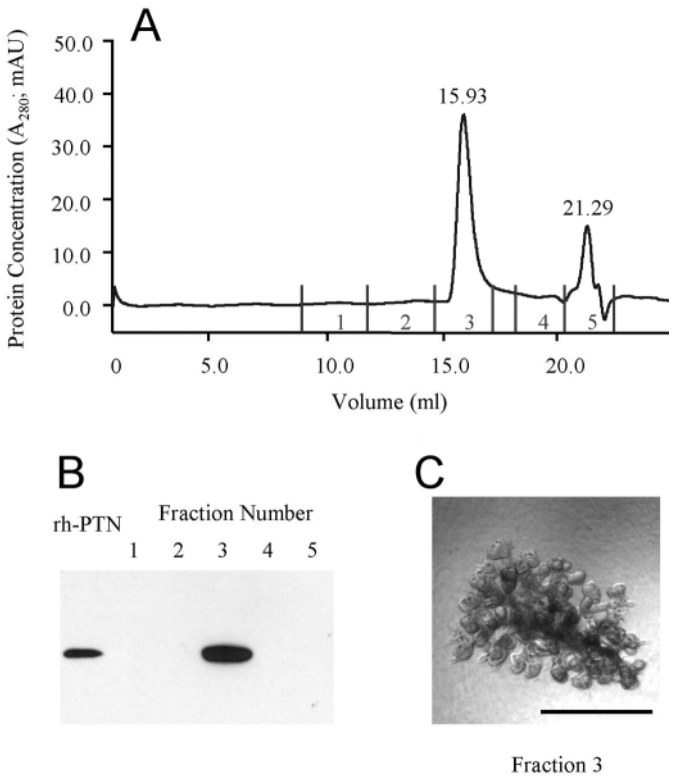

Fig. 3. Gel filtration chromatography of the eluate from the Resource S cation exchange column. (A) Elution profile from a Superdex 200 gel filtration column of the peak fraction from the Resource S cation exchange column (Fig. 2B, fraction 4). A single protein peak was eluted at 15.93 ml, which corresponds to a relative molecular mass of 18 kDa. Each of the individual 1 ml fractions are indicated by the numbers (1-5) along the x axis. (B) Immunoblot analysis of fraction 3 (A) from the gel filtration column demonstrated the presence of pleiotrophin. rh-PTN, human recombinant pleiotrophin used as a positive control. (C) Phase contrast photomicrograph of isolated ureteric bud grown for 7 days in the presence of fraction 3 supplemented with 10% FCS, 125 ng/ml GDNF and 250 ng/ml of FGF1. Scale bar, 500 μm.

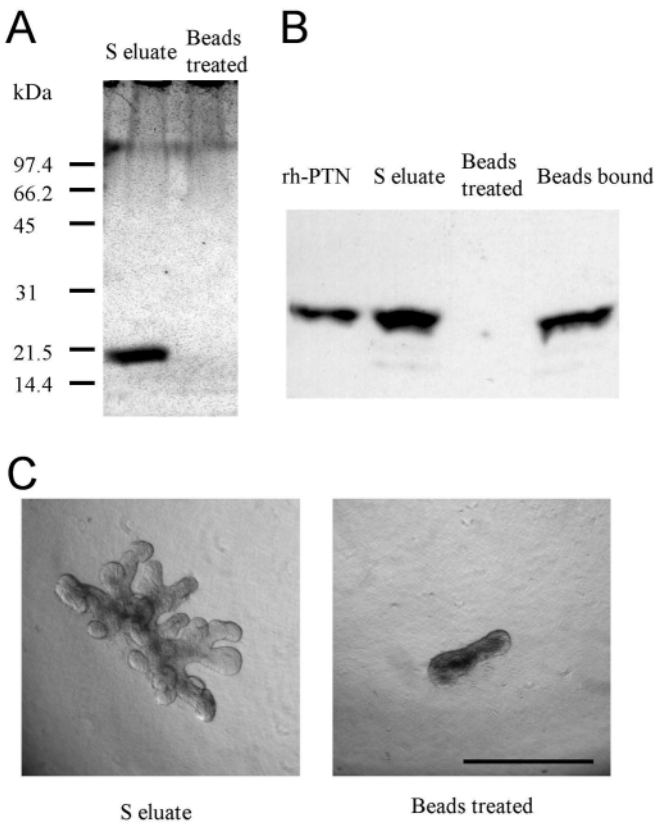

Fig. 4. Adsorption of pleiotrophin abolishes morphogenetic activity. (A) Silver-stained SDS-PAGE gel of morphogenetically active fraction from Resource S cation exchange column. Lane 1, whole fraction; Lane 2, fraction incubated with polyA-sepharose beads. The protein band at 18 kDa was not detected following treatment with polyA-sepharose beads. (B) Immunoblot analysis of the morphogenetically active fraction from Resource S cation exchange column. Lane 1, recombinant human pleiotrophin (positive control); Lane 2, active fraction; Lane 3, active fraction treated with polyA-sepharose beads; Lane 4, protein bound to beads. The blot was probed with anti-pleiotrophin antibodies. PolyA-sepharose beads adsorb pleiotrophin present in the fraction eluted from the Resource S cation exchange column. (C) Phase contrast photomicrographs of isolated ureteric buds grown for 7 days in the morphogenetically active fraction eluted from the Resource S cation exchange column with or without exposure to polyA-sepharose beads. In either case, the fraction was supplemented with 10% FCS, 125 ng/ml GDNF and 250 ng/ml FGF1. Scale bar, 500 μm.

inducing proliferation (Kurtz et al., 1995; Souttou et al., 1997; Zhang and Deuel, 1999), and in our experiments recombinant human pleiotrophin produced in the insect cell line (R&D systems) was also unable to induce UB branching morphogenesis (data not shown).

The pattern of pleiotrophin induced UB morphogenesis depends upon its concentration

During the course of purification, we observed differences in the morphology of the branching UB, depending upon the amount of pleiotrophin present in the fraction (detected by immunoblotting). This was examined more carefully using the purified protein in which the pleiotrophin concentration was determined by immunoblotting using recombinant human pleiotrophin as a standard. High concentration (≥5 μg/ml) pleiotrophin resulted in robust proliferation with less elongation, while lower concentrations of pleiotrophin (156 ng/ml-2.5 μg/ml) induced dichotomous branching and elongation of the stalk (Fig. 5A), similar to that seen with whole BSN-CM.

Pleiotrophin and GDNF are required and sufficient to induce UB branching morphogenesis.

In the course of purification, variation in the inductive capacity of whole BSN-CM on UB branching was encountered. It was found that the addition of an FGF could potentiate the activity of the BSN-CM (K. T. B., J. Qiao, D. L. Steer, R. O. Stuart, H. S. and S. K. N., unpublished), although alone or in combination with GDNF it was not sufficient to induce isolated UB branching morphogenesis (Fig. 1C). Based on this finding, the growth media (either BSN-CM or individual fractions) used in the culture of the isolated UB was supplemented with 250 ng/ml of FGF1. However, it was found that purified pleiotrophin supplemented with GDNF was capable of inducing UB branching morphogenesis in the absence of

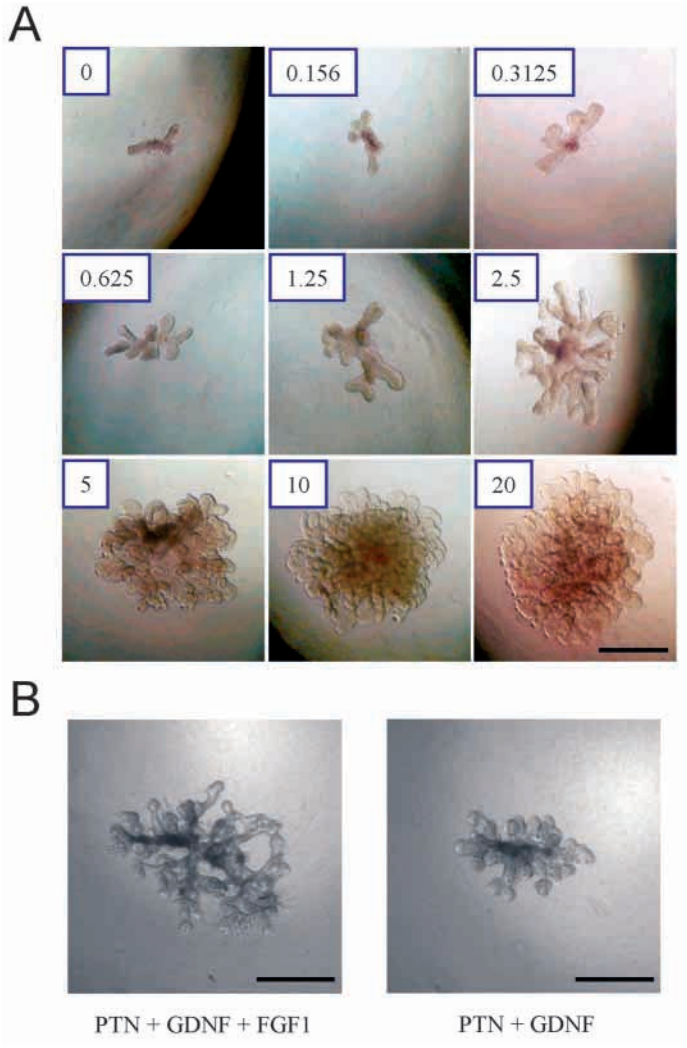

Fig. 5. Pleiotrophin-mediated UB branching morphogenesis is concentration dependent. (A) Phase contrast photomicrographs of isolated ureteric buds grown for 7 days in DMEM/F12 supplemented with increasing concentration of purified pleiotrophin. In each case, the growth medium was also supplemented with 10% FCS, 125 ng/ml GDNF and 250 ng/ml FGF1. The numbers in the upper-left-hand corner of each picture indicate the concentration of pleiotrophin in μg/ml. Clear differences in the phenotype depending on the concentration of pleiotrophin are exhibited. (B) Phase contrast photomicrographs of isolated ureteric buds grown for 11 days in the absence or presence of 250 ng/ml FGF1 diluted in DMEM/F12 supplemented with 2.5-5 μg/ml pleiotrophin (PTN), 10% FCS, and 125 ng/ml GDNF. Scale bar, 500 μm.

FGF1, although the UB grew faster when FGF1 was added to the culture (Fig. 5B). This result suggests that pleiotrophin and GDNF alone are necessary and sufficient for the observed branching morphogenesis of the isolated UB, though a FGF-like activity could play a facilitory role in the process.

Pleiotrophin also induces branching morphogenesis of UB cells in three-dimensional culture

As discussed previously, it has been shown that E11.5 mouse UB-derived cells (UB cells) develop into branching tubular structures with lumens in the presence of BSN-CM. DNA array, PCR analysis and immunostaining have confirmed the epithelial and UB-like characteristics of these cells (Barasch et al., 1996; Pavlova et al., 1999; Sakurai et al., 1997a). Using this model for UB branching morphogenesis, pleiotrophin was also capable of inducing the formation of branching structures of UB cells at concentrations of 200 ng/ml and above. As in the isolated UB culture model, the extent of UB branching morphogenesis was found to be concentration dependent, with higher concentrations resulting in more extensive growth and branching (Fig. 6A). Morphologically, the structures were similar to those induced by whole BSN-CM (Fig. 6B), although there was a higher fraction of 'spiny cysts'.

Pleiotrophin is expressed in the embryonic kidney and secreted from MM-derived cells but not UB-derived cells

By immunoblot, pleiotrophin was found in an extract of whole E13 rat kidney (Fig. 7Aa). To determine whether epithelial cells or mesenchymal cells secrete pleiotrophin, conditioned medium derived from the UB cell line and the BSN cell line were compared. Only BSN-CM contained pleiotrophin (Fig. 7Ab). This is consistent with a previous in situ hybridization study (Vanderwinden et al., 1992), which showed that the

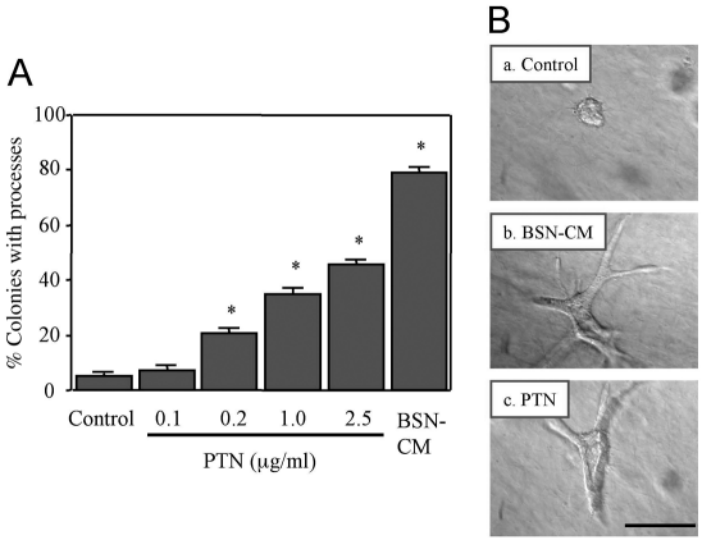

Fig. 6. Pleiotrophin induced UB cell tubulogenesis in vitro. (A) Bar graph demonstrating the morphogenetic effects of pleiotrophin on UB cells grown in three-dimensional extracellular matrix gels. UB cells were suspended in 20% Matrigel, 80% collagen gel mixture and grown for 4 days in the absence (control) or presence of purified pleiotrophin (PTN; 0.1-2.5 μg/ml). Whole BSN-CM served as a positive control. All conditions were supplemented with 1% FCS. The percentage of cells and/or colonies with processes was counted as an indicator for tubulogenic activity. 20 cells and/or colonies were counted in three randomly selected fields for each condition. Data is presented as mean ± s.e.m., $*P<0.05$ (by unpaired Student's *t*-test). (B) Phase contrast photomicrographs of UB cells grown for 8 days in DMEM/F12 supplemented with 1% FCS (a, control) and either BSN-CM (b) or purified pleiotrophin (c). BSN-CM and pleiotrophin induced the formation of branching tubules with lumens (compare b and c). Scale bar, 50 μm.

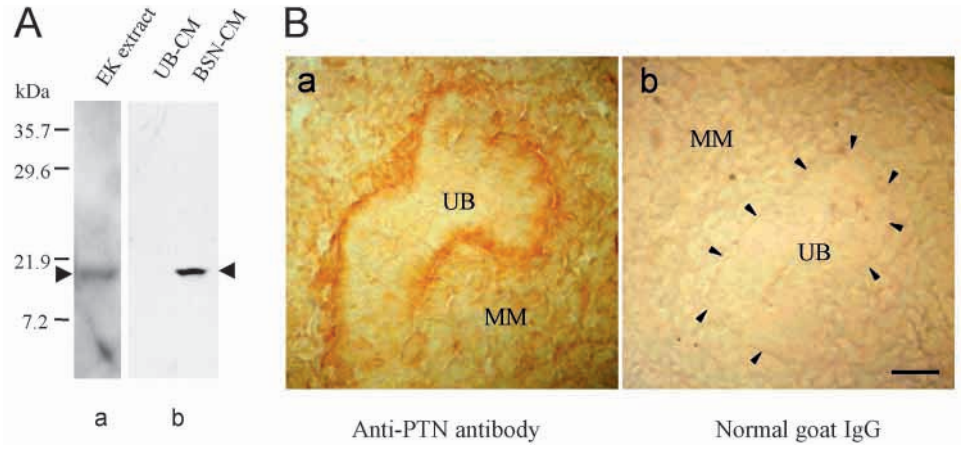

Fig. 7. Pleiotrophin expression in the embryonic kidney. (A) Immunoblot detection of pleiotrophin. Lane 1, extract of whole E13 rat kidney; lane 2, conditioned medium collected from UB cells; lane 3, conditioned medium from BSN cells. Whole kidney and BSN-CM were positive for pleiotrophin (arrowheads). (B) Frozen sections of E13 mouse kidney stained with anti-pleiotrophin antibody. Pleiotrophin is present at the basement membrane of developing UB (a). Treatment with normal goat IgG did not result in significant staining (b). Scale bar, 100 μm.

developing rat kidney mesenchyme (as early as E13 of development) expresses pleiotrophin mRNA, but the ureteric bud does not. Another study suggested the presence of pleiotrophin in the basement membrane of epithelial tubules in the developing kidney of E13 mouse embryos (Mitsiadis et al., 1995). When we examined frozen sections of mouse E13 kidneys stained with anti-pleiotrophin antibodies, a strong signal was observed in the basement membrane of the UB with weak staining in the surrounding MM (Fig. 7B). Since the MM expresses pleiotrophin mRNA at the earliest stages of kidney development (Vanderwinden et al., 1992), the data presented here suggest that pleiotrophin is secreted by the MM and binds to the basement membrane of the UB where it can exert its morphogenetic effect.

Exogenous pleiotrophin affects UB morphology in embryonic kidney organ culture

While the spatiotemporal expression pattern and in vitro data from the isolated UB and the UB cell culture model strongly support a direct role for pleiotrophin in UB morphogenesis, it was also important to determine its effect in a system that more closely approximates the intact developing kidney. Thus, we applied pleiotrophin to whole embryonic kidney organ culture. Exogenously added pleiotrophin disproportionately stimulated growth of the UB (Fig. 8). Pleiotrophin-treated kidneys exhibited an expanded UB area in a concentration-dependent manner similar to that seen in the isolated UB culture (compare Figs 5A and 8). Furthermore, the central area of UB expansion became more prominent at higher concentrations of pleiotrophin. The whole kidney also appeared slightly larger following pleiotrophin treatment. Nephron induction visualized with PNA lectin appeared to be normal even in the presence of high concentrations of pleiotrophin (data not shown). Thus, not only isolated UB, but also the UB in the whole embryonic kidney responded to pleiotrophin, supporting the notion that the UB is the target for pleiotrophin action in the developing kidney.

DISCUSSION

Early studies suggested an essential role for direct contact between the MM and the UB during metanephrogenesis. Induction of the isolated MM was reported to be inhibited by the placement of a filter with <0.1 μm pore size between an inducer and the MM, suggesting an absolute requirement for cell contact between the MM and an inducer (Saxen, 1987). Recently, however, it has been demonstrated that a combination of soluble factors elaborated by an immortalized UB cell line supplemented with either FGF2, or a combination of FGF2 and transforming growth factor α are sufficient, in the absence of direct contact between the UB and MM, to induce the mesenchymal-epithelial transition and differentiation of the proximal nephron in cultures of isolated MM (Barasch et al., 1999; Karavanova et al., 1996). Likewise, it has recently been found that soluble factors produced by a MM cell line (BSN cells) supplemented with GDNF are necessary and sufficient to induce extensive branching morphogenesis of the UB (Qiao et al., 1999a). Thus, soluble factors play a key role in both aspects of the mesenchymal-epithelial interaction leading to the formation of a functionally mature kidney. This constitutes an important revision in thinking relating to kidney organogenesis.

The identification of specific MM-derived soluble factors

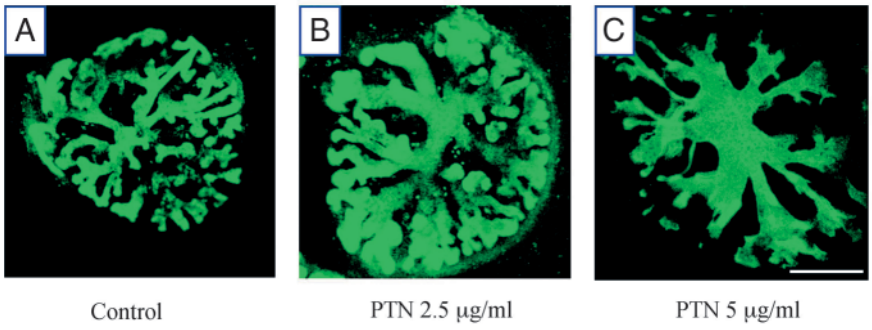

Fig. 8. Effect of exogenous pleiotrophin on UB morphology in whole kidney organ culture. Fluorescence photomicrographs of E13 rat kidneys cultured for 7 days in DMEM/F12 supplemented with 10% FCS in the absence (A, control) or presence of pleiotrophin (B, 2.5 μg/ml; C, 5 μg/ml). The UB was visualized E13 mouse with FITC-conjugated lectin from *Dolichos biflorus*. Scale bar, 500 μm.

mediating UB branching morphogenesis remains a central question in this field. HGF has been shown to induce the formation of branching tubular structures with lumens in three-dimensional cultures of epithelial cell lines derived from adult kidneys (i.e., MDCK and mIMCD cells; Barros et al., 1995; Cantley et al., 1994; Montesano et al., 1991; Santos et al., 1993). However, incubation of three-dimensional cultures of an embryonic cell line derived from the UB (UB cells) with HGF had only a slight morphogenetic effect, and the formation of branching tubular structures with lumens was not observed (Sakurai et al., 1997a). Furthermore, HGF, alone or in the presence of GDNF, does not induce branching morphogenesis of the isolated UB (unlike that seen with the MM cell conditioned medium; Qiao et al., 1999a). These findings suggest that HGF is not an essential factor for early branching morphogenesis of the embryonic UB, though it may play a facilitory role. This notion is supported by the fact that genetic deletion of *hgf* or its receptor (c-*met*) apparently has little, if any, effect on kidney development (Bladt et al., 1995; Schmidt et al., 1995).

Another group of soluble factors implicated in branching morphogenesis of epithelial cells are the family of EGF receptor ligands. EGF receptor ligands are capable of inducing the formation of branching tubular structures with lumens in three-dimensional cultures of mIMCD cells, a kidney cell line derived from adult collecting duct cells (Barros et al., 1995; Sakurai et al., 1997b). However, as is the case with HGF, EGF receptor ligands are not capable of inducing the formation of branching tubular structures in three-dimensional cultures of the embryonically derived UB cells (Sakurai et al., 1997a), nor are they capable of inducing branching morphogenesis of the isolated UB (Qiao et al., 1999a). Deletion of the EGF receptor gene results in cystic dilation of collecting ducts in mice of certain genetic backgrounds, perhaps suggesting a role in final maturation of these structures (Threadgill et al., 1995). However, as with HGF, most experimental evidence indicates that the EGF receptor ligands are not essential for early steps in UB branching morphogenesis.

In fact, among many growth factors hypothesized to play a role in kidney development, no single factor, or combination of factors, has been shown to induce UB cells to form branching tubular structures. Only the conditioned medium elaborated by the MM-derived cell line, BSN-CM, consistently induced UB cells in three-dimensional culture to form branching tubular structures with clearly distinguishable lumens (Sakurai et al., 1997a). Likewise, in the isolated UB culture system (in the presence of GDNF), no growth factor, alone or in combination, could induce the extensive branching morphogenesis observed when the isolated UB was cultured with BSN-CM (Qiao et al., 1999a).

An essential role for GDNF in UB development is supported by a number of studies, including gene knockouts. For example, null mutations of *gdnf*, its receptor, *c-ret*, or its co-receptor, *gfrα*, result in abnormal kidney development, although variable phenotypes have been reported in the *gdnf* and *c-ret* knockout mice (Enomoto et al., 1998; Moore et al., 1996; Schuchardt et al., 1996). Moreover, although the proliferative effect of GDNF on UB cells has been debated (Pepicelli et al., 1997; Sainio et al., 1997), it has been shown to initiate UB growth (Sainio et al., 1997), and it is required for branching morphogenesis of the isolated UB (Qiao et al., 1999a). Nevertheless, GDNF is not sufficient to induce branching morphogenesis of either the isolated UB (Qiao et al., 1999a) or cultured UB cells (Sakurai et al., 1997a), again consistent with the view that there are additional factors in BSN-CM that are critical to the branching morphogenesis of the UB.

Studies in the developing mammalian lung and *Drosophila* trachea indicate that members of the FGF family function in branching morphogenesis of epithelial tissues (Hogan, 1999; Metzger and Krasnow, 1999; Zelzer and Shilo, 2000). Furthermore, null mutations of either *fgf7* or *fgf10* have also been reported to affect kidney development (Ohuchi et al., 2000; Qiao et al., 1999b), although in both cases the kidneys appear to be mildly affected. For example, in *fgf7*-null kidneys, there is a 30% reduction in the number of nephrons, and the kidneys appear to function normally (Qiao et al., 1999b). Moreover, since FGF7 is detected in the developing kidney only after several iterations of UB branching have already occurred, it is likely that other factors are necessary for the early steps of the branching program. In the case of FGF10, the defect appears similar, although the phenotype has yet to be investigated in detail since the embryos die at birth due to severe lung defects (Ohuchi et al., 2000). Nevertheless, by potentiating the effect of an essential branching morphogen produced by the MM, certain FGFs may play a facilitory role in early UB branching morphogenesis (see below).

In this study, serial liquid column chromatographic fractionation of BSN-CM lead to the isolation of an active morphogenetic fraction that contained an apparent single protein (capable of inducing branching morphogenesis comparable to whole BSN-CM). This protein was identified as pleiotrophin (Fig. 2). Immunoblot analysis of BSN-CM (Fig. 7A) as well as in situ hybridization data of developing kidney (Vanderwinden et al., 1992), clearly demonstrated that the embryonic MM is a source of pleiotrophin. In addition to its ability to induce branching morphogenesis in the isolated UB, pleiotrophin also induced a UB cell line to form branching tubular structures with lumens, and is thus the only soluble factor so far identified with this capability (Fig. 6). Based on these in vitro studies with the isolated UB as well as the UB cell line, we propose that pleiotrophin could act as a UB morphogenetic factor produced by the MM.

Pleiotrophin and another heparin binding growth factor, midkine, make up a distinct growth factor family. These proteins share about 50% sequence homology (Rauvala, 1989), both are expressed widely during organogenesis (Mitsiadis et al., 1995), and are highly conserved among species (Kurtz et al., 1995). Both have been implicated in neurite outgrowth (Li et al., 1990; Rauvala et al., 1994), a phenomenon that has some similarity to branching morphogenesis (particularly as it occurs in cultured cells), and they exhibit a spatiotemporal expression pattern in other developing organ systems that suggests a role in mesenchymal-epithelial interactions (Mitsiadis et al., 1995). However, other than the finding that pleiotrophin enhances bone formation (Imai et al., 1998) and limb cartilage differentiation (Dreyfus et al., 1998), little is known about the role of pleiotrophin in organogenesis. It will be important to confirm an in vivo role for pleiotrophin in branching morphogenesis during epithelial organogenesis. To our knowledge, a pleiotrophin gene knockout has not been

reported. However, an in vivo study, which utilized dominant-negative mutant chimera mice, did suggest a role for pleiotrophin in spermatogenesis, although other organs including brain, kidney and bone appear normal in these mice (Zhang et al., 1999). There has also been some question about the mitogenic activity of pleiotrophin (Hampton et al., 1992; Souttou et al., 1997; Szabat and Rauvala, 1996), which seems to be affected by the source and purification method (reviewed by Zhang and Deuel, 1999). Nevertheless, in our studies, pleiotrophin freshly purified to apparent homogeneity from either BSN cells or 3T3 cells induced impressive growth and branching morphogenesis of the isolated UB.

A wide range of concentrations of pleiotrophin has been reported to exhibit biological activity (up to 50 μg/ml) in various systems (Imai et al., 1998; Li et al., 1990; Rauvala et al., 1994; Souttou et al., 1997). Pleiotrophin binds to the extracellular matrix, which may explain why concentrations of 200-600 ng/ml were required for morphogenetic activity in the systems employed in our study (Figs 5A and 6). The UB cells and isolated UB were cultured within basement membrane Matrigel, which could conceivably bind a large fraction of the pleiotrophin. It seems improbable, though not inconceivable, that another protein could have co-purified with pleiotrophin through 4 very different chromatography steps and not been detected by silver staining and microsequencing. If such a protein were there, it would have to possess morphogenetic activity in the subnanogram to nanogram range and have physical properties (i.e. size, charge, hydrophobicity) very similar to pleiotrophin.

To date, several glycoproteins, including brain-specific proteoglycans, the receptor type tyrosine phosphatase beta (Maeda and Noda, 1998; Meng et al., 2000) and syndecan 3 (Raulo et al., 1994) have been postulated to function as receptors for pleiotrophin. The UB has been shown to express syndecan 1 (Vainio et al., 1989), and while pleiotrophin is capable of binding to the syndecan 1 (Mitsiadis et al., 1995), it remains to be determined whether syndecan 1 mediates pleiotrophin binding and signal transduction during UB branching morphogenesis. Whether proteoglycans serve as co-receptors for pleiotrophin, as is the case for FGF signaling (Schlessinger et al., 1995), or whether they directly transduce the pleiotrophin signal is presently unclear.

The possible involvement of proteoglycans in pleiotrophin-mediated branching morphogenesis of the UB is particularly interesting in light of several studies demonstrating the importance of proteoglycans in kidney development (Bullock et al., 1998; Davies et al., 1995; Kispert et al., 1996). In these studies of whole kidney, chemical or genetic depletion of sulfated proteoglycans inhibits UB branching morphogenesis, and this is accompanied by decreased GDNF expression, and loss of c-Ret at the UB tips (Bullock et al., 1998; Kispert et al., 1996). Even at early time points, when c-Ret expression is still preserved, addition of exogenous GDNF alone does not completely restore UB branching morphogenesis (Sainio et al., 1997), suggesting that other molecules are required in this process. One possibility is that depletion of sulfated proteoglycans also affects pleiotrophin-mediated signaling or binding.

Together, our results suggest that pleiotrophin functions as a MM-derived morphogen acting upon the UB. Moreover, the results support the idea that UB branching morphogenesis is likely to be regulated by more than a single factor. At least two soluble factors, GDNF and pleiotrophin are necessary for the morphogenetic changes. GDNF may initiate the UB outgrowth (Sainio et al., 1997), and pleiotrophin may induce proliferation and/or facilitate branching (Figs 5 and 8). Whether pleiotrophin acts primarily through control of epithelial proliferation, survival, or elongation/branching requires further study. In vivo loss-of-function studies could potentially clarify the role of pleiotrophin in UB branching morphogenesis. In addition, a FGF family member may play a facilitory role, since FGF1 potentiates the effects of purified pleiotrophin on UB branching morphogenesis, though by itself (with GDNF present) exerts little if any morphogenetic activity (K. T. B., J. Qiao, D. L. Steer, R. O. Stuart, H. S. and S. K. N., unpublished). There may also be a similar set of inhibitory factors, which may include members of the transforming factor-beta family (Sakurai and Nigam, 1997). As previously argued (Nigam, 1995), gradients of positive and negative factors, most of which are matrix-bound, may exist in the mesenchyme and stroma. By regulating proliferation, apoptosis and the expression of morphogenetic molecules at branch tips, branch points and stalks, the global and local balance of these stimulatory and inhibitory factors could be a crucial determinant of branching patterns during collecting system development. In addition, it is likely that sulfated proteoglycans must also be present either to maintain expression of these soluble factors or to secure their binding sites. At later stages, other soluble factors such as HGF and/or EGF receptor ligands may play supplementary roles, either during branching (particularly in the later stages) or shaping/maturation of tubular structures.

Lastly, it should be noted that the concentration-dependent morphogenetic changes induced by pleiotrophin in the UB (Fig. 5A), raise the possibility that pleiotrophin represents a classical “morphogen”, in the sense of activin in early *Xenopus* development (Green and Smith, 1990). Such a molecule might be expected to produce different phenotypic changes in the responding tissue depending upon its concentration. In this regard, the basement membrane of the developing UB, to which pleiotrophin is localized, could potentially act as a “reservoir”. Release of pleiotrophin from the basement membrane at the UB tips, perhaps through digestion by matrix degrading proteases, could produce a local concentration gradient, resulting in increased growth and proliferation of tips, while lower amounts of pleiotrophin along the length of the stalk would appear to induce elongation of the forming tubule. Such a concentration gradient of pleiotrophin could provide a basis for modulating the shape and directionality of the developing UB.

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