

Branching morphogenesis independent of mesenchymal–epithelial contact in the developing kidney

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ABSTRACT Whether mesenchymal–epithelial interactions leading to branching morphogenesis in developing epithelial tissues such as the kidney require direct cell–cell contact or are due to soluble mediators elaborated by the inducing tissue has been the subject of much debate. Here we demonstrate that ureteric bud (UB) epithelium, from which the kidney collecting system and upper urinary tract are derived, can undergo impressive three-dimensional branching morphogenesis when cultured in the appropriate extracellular matrix context in the absence of direct contact with mesenchymal tissue, indicating that the program for branching morphogenesis is inherent to the UB. Both a soluble factor in BSN cell-conditioned medium (BSN-CM) derived from an immortalized cell line thought to originate in the early metanephric mesenchyme and glial cell line-derived neurotrophic factor (GDNF) were required for early and later events in branching morphogenesis. In the absence of BSN-CM, the isolated UB did not survive; a similar result was obtained in the presence of neutralizing antibodies against glial cell line-derived neurotrophic factor. Preliminary analysis of key activity present in BSN-CM indicates that it is a heat-sensitive, heparin-binding factor with a probable molecular mass greater than 100 kDa. When the *in vitro* cultured UB was recombined with freshly isolated metanephric mesenchyme, nephric units were induced in the mesenchyme, and the UB branches underwent elongation. Our data suggest that, although UB branching morphogenesis *per se* does not require direct mesenchymal contact, such contact may play a key role in regulating branch elongation and establishing the pattern of branching. The results also suggest an approach to *in vitro* engineering of nephron.

Branching tubulogenesis (ductogenesis) is a key mechanism by which epithelial tissues such as kidney, salivary gland, and prostate develop. Largely based on the classical studies of Grobstein and coworkers, direct interactions between mesenchymal and epithelial components of embryonic tissue have been thought to be crucial for branching morphogenesis in most epithelial tissues (1–3). During kidney development, for example, direct cell–cell interactions between the metanephric mesenchyme and the epithelial component, the ureteric bud (UB), are believed to be essential for branching morphogenesis of the latter (4–6). This view is based on the fact that it had not been possible, in many previous studies, to observe proliferation and branching of the UB in the absence of direct contact with the metanephric mesenchyme or another inducing tissue, suggesting that the developmental program necessary for branching depended on direct contact between surface proteins of the UB with surface proteins of the metanephric mesenchyme. Furthermore, no known soluble factor or set of factors had been able to induce UB branching morphogenesis *in vitro* (7). This view has gained additional support from

knockout experiments in which absent expression of a variety of individual soluble growth factors held to be important in kidney development, based on previous organ culture experiments, fail to show defective branching morphogenesis of the UB (8, 9). Nevertheless, recent studies also have shown that glial cell line-derived neurotrophic factor (GDNF) is necessary for early UB outgrowth (10–15), but (as we demonstrate here), on its own, it fails to promote proliferation and branching morphogenesis of isolated UB *in vitro*. These results left open the possibility that some unknown soluble factor, or combination of factors, derived from the metanephric mesenchyme might be sufficient to induce epithelial branching morphogenesis.

We now have established a novel system in which it has been possible to demonstrate that a combination of soluble factors, which include a high-molecular-mass, heat-sensitive activity in the conditioned medium from a cell line derived from metanephric mesenchyme (BSN cells) and GDNF, are able, in the appropriate matrix context, to induce extensive branching morphogenesis of the UB. Thus, branching morphogenesis of the UB can occur *in vitro* in the absence of contact with mesenchymal tissue, indicating that the early developmental program for branching morphogenesis in the embryonic kidney is inherent to the UB and, even in tissues where mesenchymal–epithelial interactions are intimate, epithelial branching morphogenesis can occur independent of direct contact with mesenchymal cells.

MATERIALS AND METHODS

Isolation of UB Epithelium and UB Culture. Kidney rudiments were dissected from timed pregnant Sprague–Dawley rats at gestation day 13. (The plug day was designated as day 0.) The UB was isolated from mesenchyme by incubating kidney rudiments in 0.1% trypsin in the presence of 50 units/ml of DNase at 37°C for 15 min and by mechanical separation with two fine-tipped minutia pins. For culture, Transwell tissue culture plates and a polycarbonate membrane insert with 3- μ m pore size were used. The extracellular matrix (ECM) gel (a mixture of type I collagen and Matrigel) was applied on top of the Transwell insert. Isolated UB was suspended in the ECM gel and cultured at the interface of air and medium. All cultures were carried out at 37°C with 5% CO₂ and 100% humidity in DMEM/F12 supplemented with 10% FCS. Growth factors were added as indicated elsewhere. Culture media were changed weekly if necessary.

Cells and Conditioned Medium. The BSN cell line was derived from day 11.5 mouse embryonic kidney metanephric

Abbreviations: UB, ureteric bud; GDNF, glial cell line-derived neurotrophic factor; ECM, extracellular matrix; DB, *Dolichos biflorus*; EGF, epidermal growth factor; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; BSN-CM, BSN cell-conditioned medium.

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mesenchyme originally obtained from a mouse line transgenic for the early region of simian virus 40 (SV40)/large T antigen. As described elsewhere, the BSN cells express the mesenchymal protein marker vimentin, but not classic epithelial marker proteins such as cytokeratin, ZO-1, and E-cadherin (16). Differences in the expression patterns of 588 genes in BSN cells have been analyzed on commercially available cDNA grids (33), and confirmed the largely nonepithelial character of BSN cells, though it remains to be determined whether they are mesenchymal or stromal or have characteristics of both cell types. The SV40/large T antigen-transformed UB cell line and murine inner medulla collecting duct (mIMCD) cells have been characterized extensively (16–19). To obtain conditioned medium, a confluent cell monolayer was washed with serum-free medium and then cultured in serum-free medium for another 2–4 days. Various conditioned media were harvested after low-speed centrifugation to remove cell debris and then concentrated 10-fold with a Centricon filter with an 8-kDa nominal molecular mass cutoff (Millipore). For preliminary size fractionation, Centricon filters with 50- and 100-kDa nominal molecular mass cutoff also were used. According to the manufacturer, the 14-kDa protein lysozyme is retained 0–5%, the 67-kDa protein BSA is retained 15–20%, and the 156-kDa protein goat IgG is retained 90–95% after using the Centricon filter with a 100-kDa nominal molecular mass cutoff. In addition, BSN-CM was subfractionated on a heparin-Sepharose affinity column (Hitrap Heparin; Pharmacia). Concentrated BSN-CM ($\approx 10\times$) was applied to a heparin column. After washing the column with Hanks' balanced buffer solu-

tion, the heparin-bound fraction was eluted with 2 M NaCl in Hanks' balanced buffer solution. After desalting with a PD-10 column (Pharmacia), the heparin-bound fraction's final volume was adjusted to the starting volume. The heparin flowthrough fraction was collected and its volume was adjusted to the starting volume by using a Centricon filter (8-kDa cutoff). The partially purified fractions were assayed for their effect on UB morphogenesis in the presence of GDNF.

The ECM Gel Mix. The ECM gel mix was composed of 50% type I collagen (Collaborative Biomedical Product) and 50% growth factor-reduced Matrigel (Collaborative Biomedical Product). The procedure for gelation has been described previously in detail (16).

Induction of Nephrogenesis by Cultured UB. Isolated UBs first were cultured for 7–10 days as already described. Then, the cultured UB was isolated from the ECM gel by incubation with collagenase (1 mg/ml) and dispase (2 ml/ml) at 37°C for 30 min, followed by mechanical separation with fine-tipped minutia pins. The UB then was recombined with freshly isolated E-13 rat metanephric mesenchyme and cocultured on a transfilter for another 5 days in DMEM/F12 plus 10% FCS.

Lectin Staining. *Dolichos biflorus* (DB) lectin. Tissues were fixed with 2% paraformaldehyde for 30 min at 4°C, permeabilized with 0.1% Saponin, and then incubated with fluorescent-conjugated DB (50 $\mu\text{g/ml}$, Vector) in a moisturized chamber for 60 min at 37°C. After extensive washing, tissues were postfixed in 2% paraformaldehyde again for 5 min and viewed by using a laser-scanning confocal microscope. The specificity of DB lectin binding has been demonstrated previously (20).

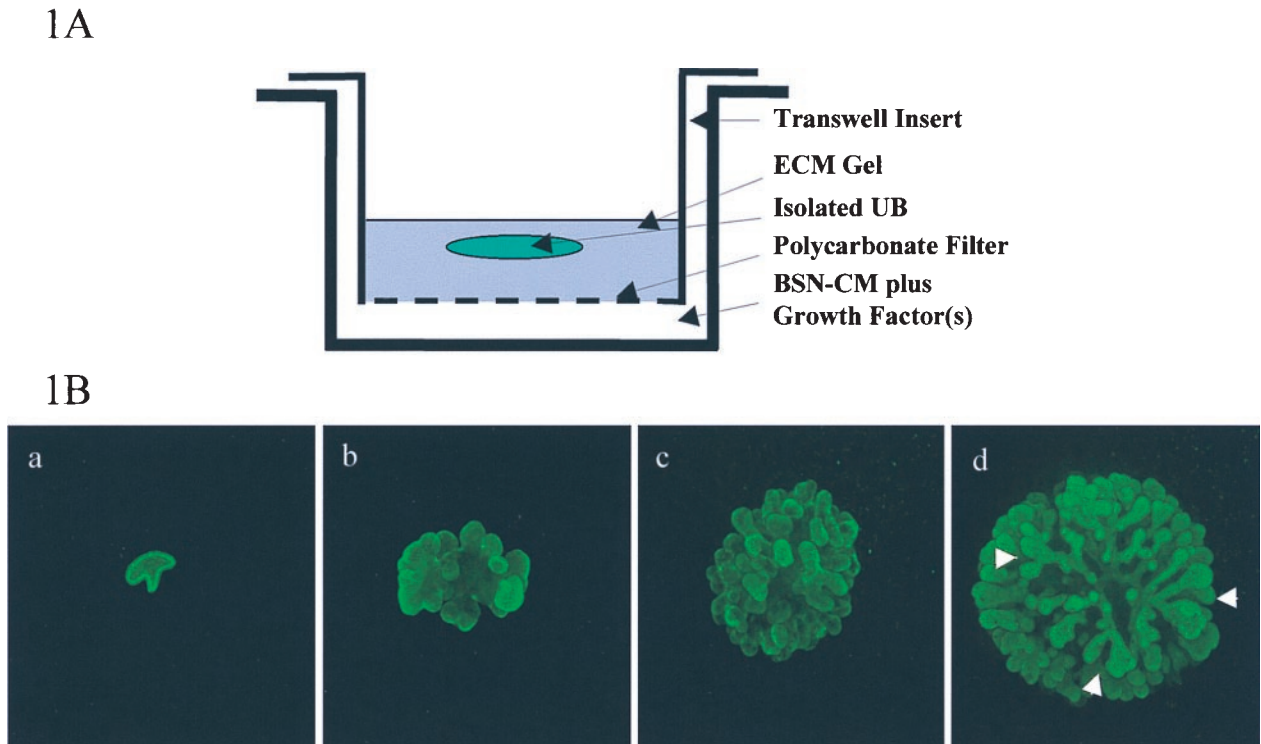


FIG. 1. A novel system for *in vitro* branching morphogenesis of the UB. (A) The culture system. UBs free from mesenchyme were microdissected from E-13 rat kidney rudiments and placed in an ECM gel suspension composed of type I collagen and growth factor-reduced Matrigel and cultured in BSN-CM supplemented with 10% FCS and growth factors. Details are given elsewhere in the text. The cultured UB was monitored daily by microscopy. (B) The UB undergoes branching morphogenesis *in vitro* and develops three-dimensional tubular structures in the absence of mesenchyme. E-13 rat UB was isolated and cultured as described. After culture, UBs were fixed at different time points and processed for DB lectin staining. Three-dimensional reconstructions of confocal images are shown. (a) A freshly isolated UB from an E-13 rat embryonic kidney with a single, branched tubular structure. (b) The same UB shown in (a) cultured for 3 days. The tissue has proliferated and small protrusions have formed. (c) The same UB shown in (a) cultured for 6 days. More protrusions have formed, and the protrusions have started to elongate and branch dichotomously. (d) The same UB shown in (a) cultured for 12 days. The protrusions have undergone further elongation and repeated dichotomous branching to form a structure resembling the developing collecting system of the kidney. The white arrows indicate branch points. At higher power, the structures formed in this *in vitro* culture system exhibited lumens. Phase microscopic examination and staining for markers revealed no evidence for contamination by other tissue or cells.

Peanut agglutinin (PNA) lectin. Tissues were fixed with 2% paraformaldehyde for 30 min at 4°C, blocked with 50 mM NH₄Cl overnight at 4°C, and followed by an incubation with 1% gelatin in 0.075% Saponin for 30 min at 37°C (21). After two washes with Neuraminidase buffer (150 mM NaCl/50 mM sodium acetate, pH 5.5), tissues were incubated with Neuraminidase (1 unit/ml) for 4 hr at 37°C and then with rhodamine-conjugated PNA (50 μg/ml) for 60 min at 37°C. Tissues were postfixed with 2% paraformaldehyde and viewed with a laser-scanning confocal microscope.

Immunocytochemistry. Tissues were fixed with either 2% paraformaldehyde at 4°C or 100% methanol at -20°C. Tissues were permeabilized with 0.1% Saponin and nonspecific binding was blocked with 100% FCS. The incubations with primary and secondary antibodies were carried out for 60 min at 37°C. The staining with FITC or tetramethylrhodamine B isothiocyanate (TRITC)-conjugated antibodies was viewed with a laser-scanning confocal microscope.

Confocal Analysis. Confocal images were collected with a laser-scanning confocal microscope (Bio-Rad MRC 1024). Each three-dimensional picture was reconstructed from a set of 10-μm serial sections that spanned the tissue. Images were processed with LASER SHARP (Bio-Rad) and PHOTOSHOP (Adobe, Systems, Mountain View, CA) software.

RESULTS AND DISCUSSION

Immortalized UB cells are known to undergo impressive morphogenesis in the presence of soluble factors when seeded in extracellular matrix gels containing type I collagen mixed with growth factor-depleted Matrigel, a basement membrane extract derived from Engelbreth-Holm-Swarm sarcoma cells (16). A conditioned medium elaborated by BSN cells (BSN-CM), an immortalized cell line derived from the early metanephric mesenchyme, has been shown to induce the formation of branching tubular structures, some of which have apparent lumens (16); the key activity in BSN-CM was shown to be distinct from a number of growth factors known to induce morphogenesis in mature kidney epithelial cell lines. The results from these cell culture studies suggest that the program for branching morphogenesis exists within UB cells and does not require direct contact with metanephric mesenchymal cells. Reasoning that the conditions for branching morphogenesis of isolated UB tissue might be similar to this *in vitro* cell culture system employing a UB cell line, we separated embryonic rat kidney UB from the metanephric mesenchyme before induction and cultured the isolated UB (free from mesenchyme) in a mixture of collagen and growth factor-depleted Matrigel (Fig. 1A). After trying many different conditions, dichotomous-branching morphogenesis resembling the structures of the developing embryonic kidney was achieved when the isolated UB was cultured in the presence of a combination of BSN-CM and a mixture of growth factors [epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), fibroblast growth factor 2 (FGF-2), and GDNF] (Fig. 1B). The growth factor mixture was chosen based on the effects of individual factors on *in vitro* morphogenesis of cultured UB and mIMCD cells (16, 22–24); HGF and EGF induce complex morphogenetic changes in UB and mIMCD cells, whereas IGF and FGF-2 induce some morphogenetic changes in UB cells. Because of strong genetic and cell culture data supporting the role of GDNF/cRET in early UB morphogenesis and survival of UB-derived cells (11, 12, 25), GDNF also was added to the mixture.

At gestational day 13, rat UB is a “T”-shaped epithelial tubule (Fig. 1B, a). *In vivo*, this single-branched epithelial tubule undergoes repeated dichotomous branching and forms the “tree”-shaped collecting system through interactions with metanephric mesenchyme (26). This epithelial-mesenchymal interaction is thought to be required for the tubular/ductal

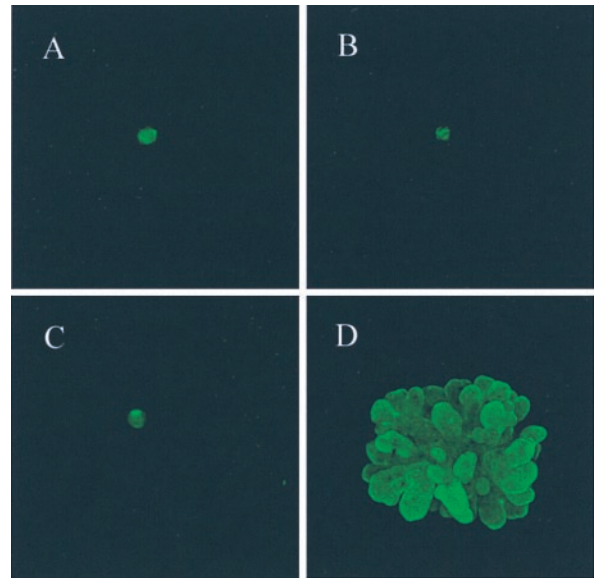


Fig. 2. BSN-CM and at least one soluble growth factor are required for branching morphogenesis of the isolated UB. (A) The UB cultured in the absence of BSN-CM and growth factors. (B) The UB cultured with the mixture of growth factors (including EGF, IGF, HGF, FGF-2, and GDNF) but no BSN-CM. (C) The UB cultured in the presence of BSN-CM alone. (D) The UB cultured in the presence of both BSN-CM and the mixture of growth factors. All cultures were carried out for about 1 week and then processed for DB lectin staining. Shown is the three-dimensional reconstruction of confocal images. The isolated UB exhibits branching morphogenesis only in the presence of both BSN-CM and the mixture of growth factors.

development of several organ systems, such as kidney, lung, pancreas, and mammary gland (27–29). In our system, isolated UB (free from metanephric mesenchyme) can be cultured and induced to undergo branching morphogenesis *in vitro*. The cultured UB branched dichotomously with formation of struc-

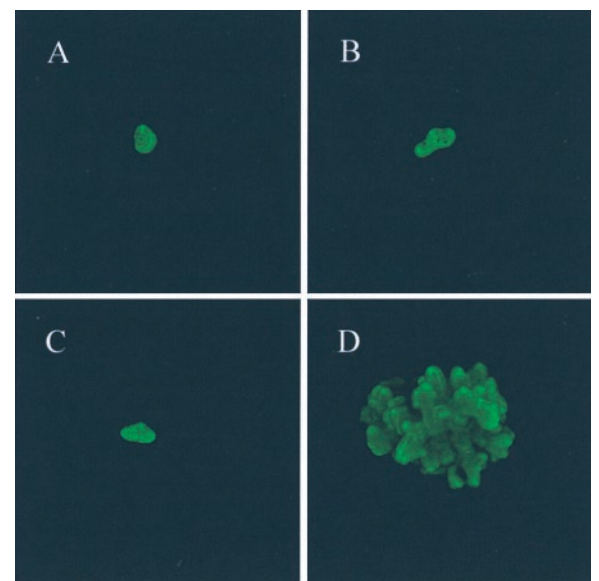


Fig. 3. BSN-CM contains unique soluble factor(s) for branching morphogenesis of the isolated UB. The UBs were cultured in the presence of the key growth factor (GDNF; see Fig. 5) but with different cell-conditioned media. (A) 3T3 fibroblast cell-conditioned medium. (B) Immortalized UB cell-conditioned medium. (C) mIMCD cell-conditioned medium. (D) BSN cell-conditioned medium. After culture, the UBs were fixed and processed for DB lectin staining. Only BSN-CM could promote branching morphogenesis of the isolated UB.

Table 1. Preliminary fractionation of BSN-CM indicates at least one morphogenetic activity is heparin-binding and likely to be greater than 100 kDa

Fraction of BSN-CM	Isolated UB morphogenesis
8-kDa Centricon-retained fraction	Yes
50-kDa Centricon-retained fraction	Yes
100-kDa Centricon-retained fraction	Yes
Heparin-bound fraction	Yes
Heparin flowthrough fraction	No

First three rows show the UB cultured in the presence of GDNF and BSN-CM fractionated with different nominal molecular mass-cutoff Centricon filters. Last two rows show the UB cultured in the presence of GDNF and either the heparin-bound BSN-CM fraction (2 M salt eluate) or heparin flowthrough fraction. After 1 week of culture, all samples were stained with DB lectin (FITC) and tubular morphogenesis of UB was analyzed.

tures that had apparent lumens. Each branch had both tubular and ampullary portions (Fig. 1 *B*, b–d). Staining with lectins and antibodies indicated that the tubular structures remained UB-derived and epithelial in character. Both cell proliferation and branching morphogenesis appeared to occur simultaneously. In most cases, after 48 hr of culture, UB epithelial tissues started to increase in size and developed small protrusions from the T-shaped ureteric bud. After 3–4 days of culture, those protrusions started to elongate, and the tips of the elongated structure started to branch dichotomously. The structures formed from the cultured UB revealed no staining with vimentin antibodies and peanut lectin (PNA), markers for mesenchymally derived elements, further supporting the notion that, in the appropriate milieu of soluble factors, complex branching of the UB can occur in the absence of direct contact with the metanephric mesenchyme. Moreover, growth of isolated UB was observed for up to 3–4 weeks, with many generations of branching.

BSN-CM played a critical role in this morphogenetic process (Fig. 2). In the absence of BSN-CM, growth factors had no effect on proliferation and branching morphogenesis of the UB (Fig. 2*B*). Although most of these growth factors (except GDNF, which was from rat) were in human recombinant form, they seem to be capable of inducing morphogenesis in mouse-derived UB cells (16). (Nevertheless, we cannot completely exclude interspecific differences in growth factor potency.) Only when BSN-CM was present did the UB develop into a three-dimensional tubular structure (Figs. 2*D* and 3*D*). To examine whether BSN-CM contained unique factors for the branching morphogenesis of the UB, conditioned media from different cell lines were compared. Neither conditioned medium derived from Swiss 3T3 fibroblasts (an inducer of MDCK cell branching tubulogenesis in type I collagen) nor that from UB cells or mIMCD3 cells was capable of substituting for BSN-CM (Fig. 3*A–C*), suggesting that the BSN cells retain the

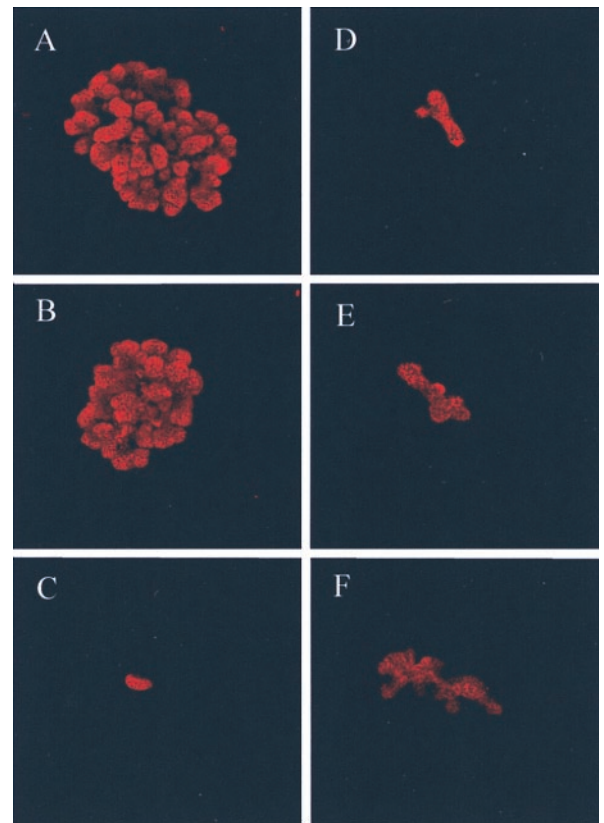


FIG. 5. GDNF is required for both early- and late-branching morphogenesis *in vitro*. (*A–C*) The antibodies against GDNF are neutralizing antibodies. (*A*) UB was cultured in the presence of BSN-CM and GDNF without antibodies. (*B*) Same as *A*, but normal goat IgG antibody were added. (*C*) Same as *A*, but antibodies against GDNF were added. (*D–F*) GDNF is required for branching morphogenesis. The UBs initially were cultured in the presence of BSN-CM and GDNF, and then the cultures were washed to remove GDNF at different time points; the UBs then were cultured continuously in BSN-CM without GDNF. To ensure neutralization of residual GDNF in the culture, antibodies against GDNF were added after removal and washing of GDNF from the culture medium. (*D*) The UB was cultured as in *A*, but GDNF was removed and antibodies against GDNF were added on the first day of culture. (*E*) Same as *D*, but the GDNF was removed and antibodies against GDNF were added on the second day of culture. (*F*) Same as *D*, but the GDNF was removed and antibodies against GDNF were added on the third day of culture (compare with structures in Fig. 1*B*). All cultures were carried out until the fifth day and processed with DB lectin staining. Whenever GDNF is depleted, UB growth and branching morphogenesis are aborted, indicating that GDNF is required for both early- and late-branching morphogenesis *in vitro*.

ability to secrete a relatively unique factor, or set of factors, made by the metanephric mesenchyme and required for UB

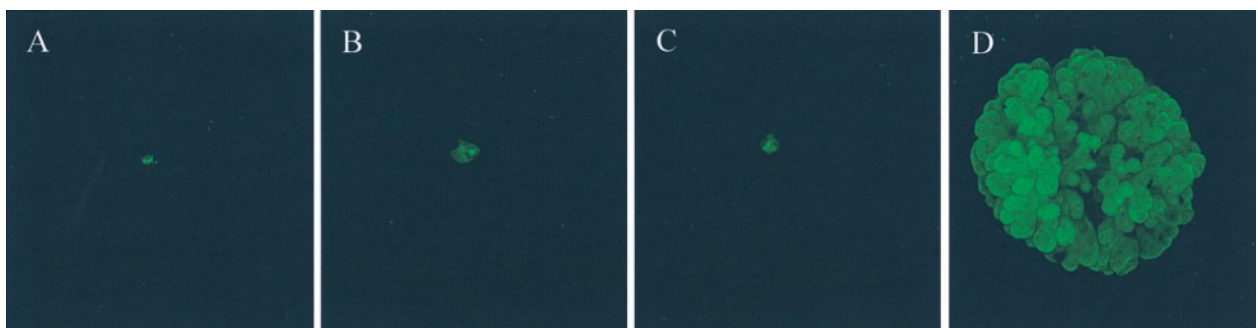


FIG. 4. GDNF plus BSN-CM is required for branching morphogenesis. The UBs were cultured in the presence of BSN-CM as in Fig. 2 but with each of single growth factors present in the growth factor mixture. Several examples are shown: with EGF alone (*A*); with FGF-2 alone (*B*); with HGF alone (*C*); and with GDNF alone (*D*). Only GDNF combined with BSN-CM could promote branching morphogenesis of the isolated UB.

branching morphogenesis (Fig. 3D). This activity was heat-sensitive. More recent experiments aimed at size fractionation by using Centricon filters revealed that the >100-kDa retained fraction exhibited activity. Thus, our data suggest that one activity necessary for UB morphogenesis is more than 100 kDa (Table 1). Furthermore, when BSN-CM was fractionated over a heparin-Sepharose column, only the heparin-bound fraction exhibited morphogenetic activity (Table 1). These preliminary data indicate that a heparin-binding protein or set of proteins greater than ≈ 100 kDa is one of the key morphogenetic activities in BSN-CM.

Nevertheless, the factor (or a set of factors) in BSN-CM was not sufficient to induce UB branching morphogenesis. In the absence of the growth factor mixture, the UB underwent apoptosis as determined by the TUNEL assay (data not shown). To define further conditions for *in vitro* UB branching morphogenesis, we examined whether any single growth factor present in the growth factor mixture could, in combination with BSN-CM, induce UB branching morphogenesis. The combination of BSN-CM and GDNF, but no other combination, was found to be sufficient to induce the formation of three-dimensional branching structures comparable to those observed with BSN-CM and the growth factor mixture (Fig. 4). Consistent with this observation, the combination of BSN-CM and GDNF prevented the UB from undergoing apoptosis and facilitated UB proliferation (data not shown). Because GDNF alone could not induce branching morphogenesis in the absence of BSN-CM, a factor or factors present in the BSN-CM must be required for the action of GDNF in the induction of UB branching morphogenesis. Although studies from others have indicated that GDNF is involved in the initial formation of the UB (30, 31), it has not been established whether GDNF is required for further branching morphogenesis of the UB. Therefore, the UB was first cultured in the presence of BSN-CM and GDNF and then in the absence of GDNF after

repeatedly washing away GDNF from the culture and then adding antibodies known to neutralize GDNF in this system (Fig. 5 A–C). Withdrawal of GDNF from the culture system blocked further UB branching morphogenesis, suggesting that GDNF is not only involved in early UB formation but also in further iterations of UB branching (Fig. 5 D–F and compare Figs. 5 E and F with 1 B, b and c). In this regard, it is interesting to note that mesenchymal cell contact or some other soluble factor may be able to partially compensate for GDNF, at least under certain conditions in whole organ culture, because c-RET antisense oligonucleotides are not strongly inhibitory of continued branching of the UB when added after induction (32).

To characterize the complex tubular structures of the *in vitro* cultured UB, expression of several markers was examined. The cultured UB structures exhibited positive staining with DB lectin and cytokeratin antibodies, but negative staining with PNA lectin and vimentin antibodies. The expression pattern of these markers confirmed that the tubular structures formed *in vitro* were UB-derived (Fig. 6 A–F). Nevertheless, a key issue was whether the cultured UB retained the capacity for induction of nephric units in the metanephric mesenchyme. When this was tested, it was confirmed that the tubular structures resulting from the cultured UB were capable of eliciting mesenchymally derived metanephric nephronal structures and of being incorporated into the nephric unit when recombined with the freshly isolated metanephric mesenchyme (Fig. 6 G–I). As shown by PNA staining, most nephrons were located at the periphery of the cultured tissue, where tips of new UB branches were forming. All formed mesenchymally derived nephronal structures appeared connected with the tubular structures of UB. In addition, the cultured UB structures continued to respond to the inductive effect of mesenchyme by elongating further into the mesenchymal tissue (Fig. 6 H and I). Together, these results indicate that the structures grown *in vitro* are UB-derived epithelial tubules and retain induction

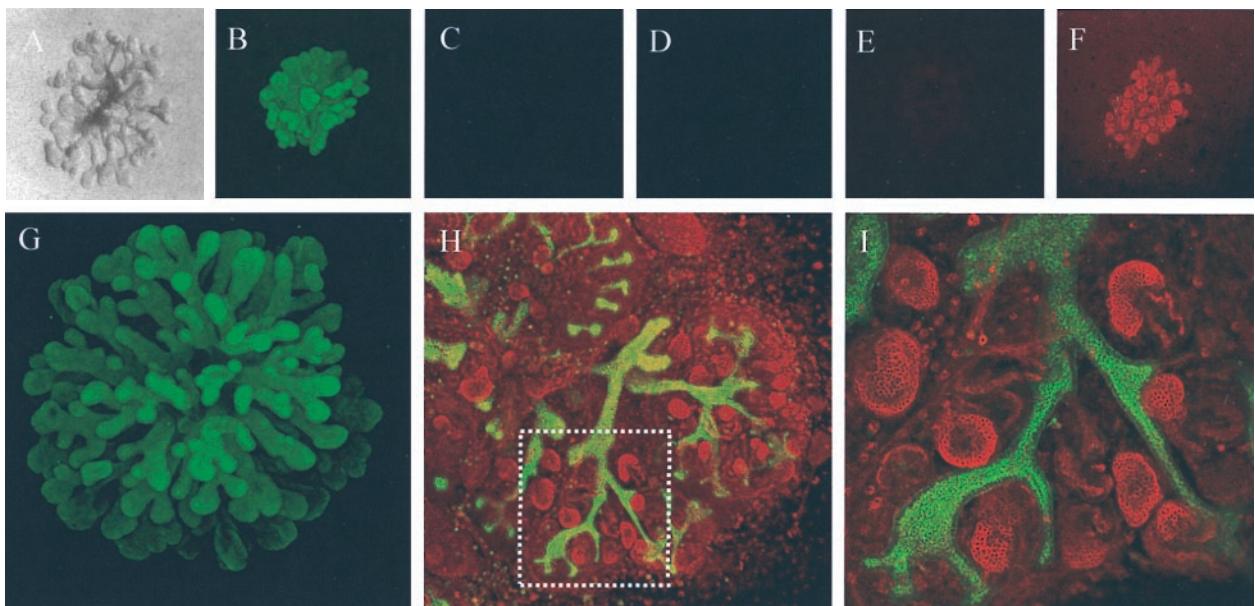


FIG. 6. The cultured three-dimensional tubular structure exhibits markers of UB epithelium and is functionally capable of inducing nephrogenesis when recombined with metanephric mesenchyme *in vitro*. (A–F) The cultured three-dimensional tubular structure exhibits markers of UB epithelium. The UBs were cultured in the presence of BSN-CM and GDNF and then stained for various markers. (A) Light microscopic photograph of cultured UB. (B) Staining with DB lectin, a ureteric bud-specific lectin that binds to the UB and its derivatives. (C) Staining for vimentin, a mesenchymal marker. (D) Staining for neural cell adhesion molecule, the early marker for mesenchymal-to-epithelial conversion in the kidney. (E) Staining with PNA lectin, a mesenchymally derived renal epithelial cell marker. (F) Staining for cytokeratin, an epithelial marker. (G–I) The cultured three-dimensional tubular structure is capable of inducing nephrogenesis when recombined with metanephric mesenchyme. The isolated UB was first cultured 7–10 days as shown in G. Then, the cultured UB was removed from the ECM gel and recombined with freshly isolated metanephric mesenchyme from E-13 rat kidneys. The recombinant was cultured on a Transwell filter for another 5 days. After cultured, the sample was double-stained with DB lectin (FITC) and PNA lectin (tetramethylrhodamine B isothiocyanate) as shown in H and in the enlarged section of H shown in I. Results indicate that the *in vitro* cultured UB-derived structures are capable of inducing nephrogenesis *in vitro*.

competence even after many days of *ex vivo* culture. The results also suggest that although the factor(s) in BSN-CM plus GDNF may be sufficient for the initial branching processes, later events in UB morphogenesis (e.g., elongation and establishing the pattern of branching) may require contact with mesenchyme.

Thus, by using this novel model system, we have found that, in contrast to the widely held view that the complex arborization of the UB during kidney development depends on direct contact between cells of the metanephric mesenchyme and cells of the UB, a substantial degree of branching morphogenesis can be mediated by soluble factors alone. Therefore, the branching program exists within the UB itself after it is formed from the Wolffian Duct, and soluble factors can trigger its initiation and continuation. No singular soluble factor, however, appears sufficient. A combination of GDNF and an activity, or set of activities, present in BSN-CM is necessary. Whether this latter activity is the same as that which induces the formation of branching tubules with lumens of UB cells in culture remains to be determined (16). It seems very likely that more direct mesenchymal interactions with the UB are important for establishing the direction of branching events because the cultured UB-derived structures lack directionality, and only when the cultured UB was recombined with metanephric mesenchyme did directionality and elongation occur (Fig. 6 *H* and *I*). Epithelial-mesenchymal cell-cell contact is probably essential for the later steps in the development of UB/collecting system. Additional mechanisms are likely to be involved in the formation of junctions between mesenchymally derived nephronal segments and collecting tubules and the development of tertiary structures of the collecting tubule, such as the formation of arcades. Moreover, contact with the mesenchyme might provide a "stop" mechanism for kidney growth because we found that the isolated UB continued to grow *in vitro* as long as soluble factors were provided.

Our data also clarify the role of GDNF in kidney development. To date, GDNF has been implicated in initial UB outgrowth and early survival, but its role in branching morphogenesis of the UB has been debated. Our data indicate that GDNF, in combination with factors in BSN-CM, supports true morphogenesis of the UB, at least *in vitro*. GDNF is required for not only the initial outgrowth but also the subsequent branching morphogenesis of the UB.

These results suggest that it is worth reevaluating the role of cell contact vs. soluble factors in a wide variety of epithelial tissues in which intimate cellular interactions between epithelial and mesenchymal tissues are thought to play a crucial inductive role, particularly with respect to branching morphogenesis. In the developing kidney and, perhaps in many of these other tissues, the role of cell contact may be facilitative rather than crucial for early branching morphogenesis *per se*, although it may be essential for the establishment of vectoriality and later events in differentiation.

Finally, the fact that the isolated UB can be induced to undergo morphogenesis *ex vivo* and incorporate itself into nephrons when recombined with mesenchyme suggests an approach to *in vitro* engineering of kidney tissue.

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