

Conditioned medium from a rat ureteric bud cell line in combination with bFGF induces complete differentiation of isolated metanephric mesenchyme

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SUMMARY

Differentiation of metanephric mesenchyme is triggered by an inductive signal(s) from the epithelial ureteric bud. As a result of this induction, most of the metanephric mesenchyme converts into epithelium of a nephron. We have developed and characterized an explant culture system, in which metanephric mesenchyme can grow and completely differentiate in vitro in the absence of an inductive tissue. When separated 13 dpc rat metanephric mesenchymes were cultured in serum-free conditioned medium from a rat ureteric bud cell line (RUB1) in the presence of bFGF and TGF α , they were induced to differentiate into nephron epithelia and glomeruli-like structures. The nephric type of differentiation was confirmed by both morphological and molecular criteria and paralleled the developmental

changes of nephron differentiation in vivo. Expression patterns of brush-border antigen as well as molecular markers of kidney differentiation *Wt1*, *Lim1*, *Hgf* and *c-met*, *c-ret*, *Shh*, *Wnt4*, *Wnt7b*, and *Wnt11* were analyzed in explants by whole mount and tissue section in situ hybridization following 1-9 days in culture. The expression of secreted patterning molecules *Bmp7* and *Wnt7b*, but not *Shh* or *Wnt11*, were demonstrated by RT-PCR and northern blot hybridization with RNA from the RUB1 cells. Our culture system lends itself to examining the relevance of these and other signaling molecules required for nephron differentiation.

Key words: kidney, induction, tubulogenesis, Wnt genes, FGF

INTRODUCTION

Kidney development is mediated by mechanisms of induction, proliferation, and patterning and as such provides a comprehensive model system for the study of embryonic organogenesis. Differentiation includes all the basic developmental processes such as epithelial-mesenchymal inductions and reciprocal interactions, epithelial growth and branching, as well as the reprogramming of mesenchyme to form epithelium. Formation of the metanephros, the permanent kidney, begins with the invasion of the epithelial ureteric bud into mesenchymal metanephric blastema. Ureteric bud induces metanephric mesenchyme to condense and convert into the epithelium of a nephron, while the mesenchyme reciprocally induces the bud to grow and branch, thus forming the collecting duct system (for review see Saxén 1987; Davies, 1993; Bard et al., 1996). A considerable advantage to the study of the renal system is that most of these well-defined morphological changes can be monitored in vitro, when separated kidney rudiments are maintained in explant culture.

Several genes have been implicated in metanephros development (reviewed by Bard et al., 1994). Transcription factors *Wt1* (Kreidberg et al., 1993), *Lim1* (Shawlot and Behringer, 1995) and *Pax2* (Dressler et al., 1993; Torres et al., 1995), the tyrosine kinase receptor *c-ret* (Schuchardt et al., 1994), and signaling molecules *Wnt4* (Stark et al., 1994) and *Bmp7*

(Dudley et al., 1995; Luo et al., 1995) are absolutely essential since the loss of function of any of these factors in transgenic mice results in severe renal deficiencies. The expression patterns of more than 50 genes have been mapped and associated with certain morphological events in the developing kidney (reviewed by Bard et al., 1994). These have provided useful markers for the forming structures, but their functions in these processes have remained largely undefined. Even less is understood about the signaling pathways involved in organogenesis. So far, it has been shown that *Pax2* is not expressed in metanephric blastema where *Wt1* expression is disrupted by targeted mutation, suggesting that the *Wt1* signal is upstream of *Pax2* in metanephric development (Kreidberg et al., 1993). Similarly, *Pax8* is not expressed in the structures lacking *Wnt4* (Stark et al., 1994).

Although transgenic mice provide invaluable information on the role of particular genes in renal development, in most cases, development is interrupted either very early or prior to the initiation of nephros development. Thus, other approaches are needed to more effectively study the gene interactions during organogenesis. We have developed an experimental system that may serve as a tool for elucidating mechanisms of signal transduction and their connection with transcription factors in metanephric kidney in vitro. We have shown previously that bFGF induces isolated metanephric mesenchyme to form condensations similar to those formed in vivo following induction

by the ureteric bud (Perantoni et al., 1995). Here we report that conditioned medium from a rat ureteric bud cell line (RUB1) in combination with bFGF provides the necessary factors not only for the first stage of induction, i.e., condensation, but for the whole range of developmental events characteristic of metanephros, including the formation of epithelial nephric tubules and differentiation of glomeruli.

MATERIALS AND METHODS

Organ cultures

Embryonic kidneys were excised from 13 days post coitum (dpc) F344 rat embryos. Metanephric mesenchymes were enzymatically separated from the ureteric buds and cultured on filters according to the method introduced by Saxén (Saxén, 1987) with modifications (Perantoni et al., 1991). Each explant culture always consisted of only one mesenchyme that was immediately placed on a type IV collagen-coated (Collaborative Biomedical Products) polycarbonate filter (0.1 µm) (Nuclepore), in serum-free Ham's F12:DMEM 1:1 (Gibco, BRL) medium with supplements as described (Ekblom et al., 1981; Perantoni et al., 1991) and with the addition of one or more of the following: 10 ng/ml TGF α (Boehringer Mannheim), 50 ng/ml bFGF (Boehringer Mannheim), 100 ng/ml HGF (Sigma), 30 µl/ml of 50 \times concentrated conditioned medium (CM; 10K NMWT cut-off filtration unit (Filtron MacroSep)), collected from cultures of ureteric bud cell line (RUB1), which was characterized previously (Perantoni et al., 1985). For fractionation of CM, it was dialyzed in 50 mM Tris-HCl, pH 7.5, and proteins were then eluted from an HPLC heparin-affinity column (Bio-Rad) using a step-wise NaCl gradient. To test the effect of heparin on the inductive activity of CM, explants were treated with heparin (100 µg/ml) (Sigma). RUB1 cells were propagated in plastic flasks (Costar) for 2-3 days in Ham's F12:DMEM 1:1, 10 ng/ml TGF α , without serum, and were confluent at the time of collection.

Immunocytochemistry

Whole-mount indirect immunofluorescence was done according to Sariola (Sariola et al., 1988). Briefly, whole mesenchymal or kidney rudiment cultured explants were fixed for 6 minutes in cold 100% methanol at -20°C, washed in several changes of phosphate-buffered saline (PBS) for at least 2 hours, and incubated overnight with polyclonal antibodies against the brush-border antigen (BB) (kindly provided by Dr Sariola; Miettinen et al., 1976) or with 50 µg/ml of the Dolichos Biflorus (DB) FITC-labeled lectin (Sigma). Incubation with secondary FITC-labeled anti-rabbit antibodies (Sigma) was done overnight after 8-10 hours of washes. The explants were analyzed by laser scanning confocal microscopy (Zeiss, LSM 310).

In situ hybridization

Explants were fixed with 4% paraformaldehyde in PBS after 1-9 days in culture and processed for in situ hybridization as whole mounts or tissue sections. In situ hybridization to sections was done with ³⁵S-radiolabeled or DIG-labeled probes (Boehringer Mannheim) according to Wilkinson (Wilkinson and Green, 1990). Whole-mount in situ hybridizations were done with DIG-labeled probes (Boehringer Mannheim) according to Conlon and Herrmann (1993).

Probes

Probes for *Wt1* and *c-met* were used as previously described (Perantoni et al., 1995). An *Hgf* probe was kindly provided by Dr Nakamura (Tashiro et al., 1990), a *Wnt4* probe by Dr Vainio (Stark et al., 1994) and a *Lim1* probe by Dr Chin.

Probes for *Bmp7* (Ozkaynak et al., 1991), *c-ret* (Iwamoto et al., 1993), *Sonic hedgehog* (Echelard et al., 1993), *Wnt7b* (Gavin et al., 1990), and *Wnt11* (Christiansen et al., 1995) were generated from 19 dpc rat embryonic kidney RNA by RT-PCR, cloned into the pCR-

Script SK(+) (Stratagene) vector, and sequenced to confirm homology with published mouse or rat sequences.

Northern blotting

Cultured RUB1 cells were lysed in a tissue culture flask (162 cm) with TRIzol (Gibco, BRL), total RNA was purified according to the manufacturer's protocol, and poly(A) RNA was selected on oligo-dT cellulose columns (Pharmacia). Poly(A) RNA (8 µg) was electrophoresed in a formaldehyde-agarose gel and blotted onto Gene-Screen Plus membranes (Biotechnology Systems). The filters were hybridized with a ³²P-labeled probe in QuickHyb according to the manufacturer's protocol (Stratagene).

RT-PCR

For RT-PCR, random hexamer primers were used to generate the cDNA from total RNA. Gene-specific primers were then used to produce PCR fragments. Primers and the fragment sizes were as follows: for *Bmp7* (S) AGACGCCAAAGAACCAAGAG, (AS) GCTGTCGTCGAAGTAGAGGA, 323 bp; for *Sonic hedgehog* (S) AGTGACCGAGGGCTGGGATG, (AS) TTGTGCGGCGCCACGAAGAG, 462 bp; for *Wnt7b*, (S) TGCCTTCACCTATGCCATCA, (AS) CGTCTCCTCGCAGTAGTTG, 533 bp; and for *Wnt11*, (S) TGC GGCCCCGTCCCAGGTGA, (AS) GTAGCCGCGCC-CACAGCACA, 598 bp. RT reactions were performed according to manufacturer's instructions (Gibco, BRL) with modifications. Incubation mixtures included 100 units MMLV RT (Boehringer Mannheim), 2 µg total RNA, 40 units RNase inhibitor (Boehringer Mannheim), 1 mM dNTPs, and 10 µM random hexamers. PCR reactions were performed with the following additions to the RT reactions: 50 µl 1 \times PCR buffer, 1 mM dNTPs, and 50 ng 20-mer primers. Incubation conditions consisted of 30 two-step cycles (30 seconds at 94°C and 60 seconds at T_m - 10°C).

RESULTS

Morphology and immunocytochemistry of the explant cultures

In an effort to characterize an inductive signal for the metanephric mesenchyme, we studied the inducing activity of conditioned medium (CM) from the RUB1 cell line, which is derived from the natural inducer of mesenchyme, on uninduced separated metanephric mesenchymes in vitro. Filtered and concentrated CM supplemented with TGF α , which was also used for propagation of the RUB1 cells, induced condensations after 24 hours of culturing, but these condensations failed to convert into tubules (Fig. 1B). This is very similar to the effect of bFGF. As we have shown previously, bFGF can induce condensation and proliferation in separated metanephric mesenchyme in vitro in the absence of an inductive tissue (Perantoni et al., 1995), but it is not sufficient for tubule formation. Without bFGF or CM, mesenchymes failed to condense, flattened, and degenerated after 3-4 days regardless of the addition of TGF α (Fig. 1A).

When separated mesenchymes were cultured in CM supplemented with bFGF and TGF α (CM/bFGF/TGF α), the differentiation of nephric epithelia was induced, similar to the differentiation which is induced in the metanephric mesenchyme by recombination with spinal cord (Fig. 1C,D). One day of exposure to these conditions was enough to induce tubule formation. Explants that were after 24 hours transferred to medium without CM and bFGF developed tubules, though the size of the explants and the extent of tubule formation was significantly reduced. The morphological changes in explants

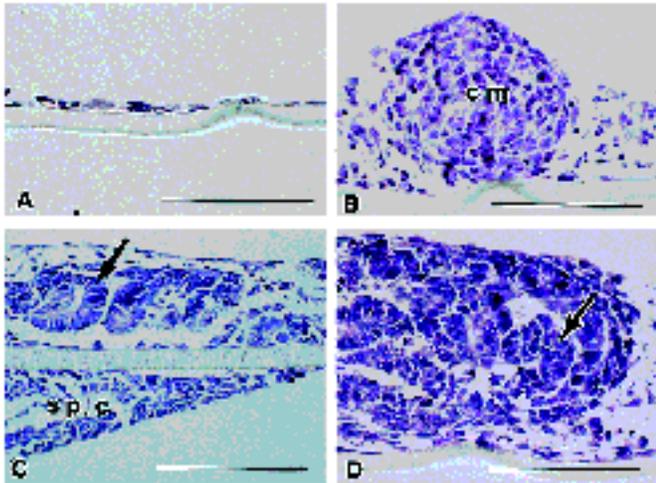


Fig. 1. Comparison of hematoxylin-eosin stained sections of explanted metanephric mesenchymes cultured for 3 days in medium with various supplements. (A) In medium with $TGF\alpha$, mesenchyme is flattened and no morphogenesis is induced; (B) in medium with $CM/TGF\alpha$, condensations are formed without signs of tubulogenesis; (C) when separated mesenchyme is recombined with spinal cord, a primitive epithelium is formed (arrow); (D) in medium with $CM/bFGF/TGF\alpha$, a primitive epithelium including S-shaped bodies (arrow) is formed. sp.c., spinal cord; cm, condensed mesenchyme. Bar, 0.05 mm.

cultured in $CM/bFGF/TGF\alpha$ were studied in detail after 1, 3, 6, and 9 days of culturing.

After 24 hours in culture, condensations formed in the explanted mesenchymes (Figs 2A, 3A). Epithelial morphogenesis subsequently began in these condensations in a manner similar to *in vivo* mesenchymal-epithelial transitions. The condensed mesenchyme in explants gradually converted into epithelium, through comma-, and S-shaped configurations, that were apparent in the sections of explants after 3 days (Figs 1D, 3B). Formation of newly condensed areas continued throughout culturing, giving rise to new epithelia, in the same manner as occurs *in vivo*. After 4 days in culture, the explants consisted largely of tubules (Figs 2B, 3C). Whole-mount immunostaining showed that portions of these tubules were positive for the brush-border antigen (Miettinen and Linder, 1976; Ekblom et al., 1980), which localizes only to proximal tubules (Fig. 4A-D). To eliminate the possibility that the newly formed epithelium might arise from ureteric bud contamination or from mesenchymal differentiation along a collecting duct pathway, as has been suggested (Qiao et al., 1995), binding assays in whole

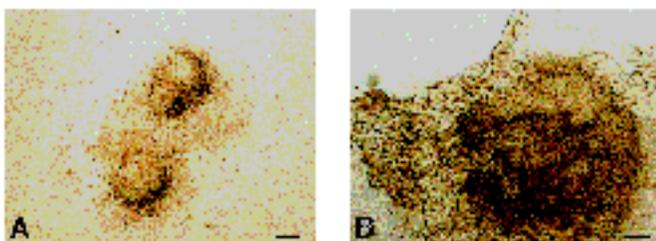


Fig. 2. Metanephric mesenchyme explants cultured in $CM/bFGF/TGF\alpha$ for (A) 1 day and (B) 6 days. Extensive tubule formation is apparent after 6 days. Bar, 0.1 mm.

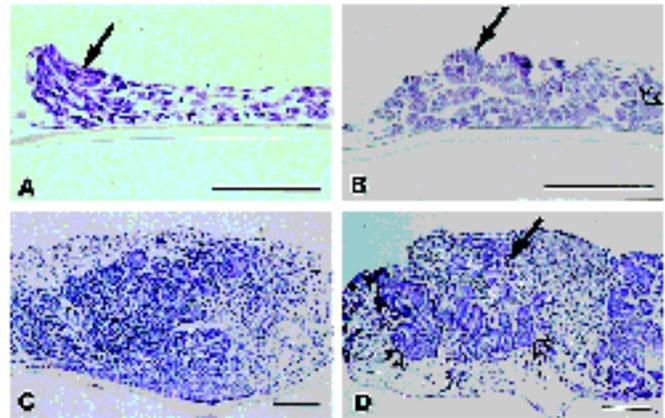


Fig. 3. Hematoxylin-eosin stained sections of explants cultured in $CM/bFGF/TGF\alpha$ for 1, 3, 6, or 9 days. (A) After 1 day, condensed mesenchyme is formed (arrow); (B) after 3 days, primitive epithelium is apparent (arrow points to the S-shaped body); (C) after 6 days tubules develop; (D) after 9 days, the explant is filled with tubules, late S-shaped bodies (hollow arrows), and glomeruli-like structures (arrow). Bar, 0.05 mm.

mounts with the collecting duct-specific FITC-labeled DB lectin (Lehtonen et al., 1987) were done. As a positive control, cultured whole kidney rudiments were used. By confocal microscopy, the epithelium in the mesenchymal explants was not labeled, while collecting ducts in whole kidneys showed strong binding by the lectin (Fig. 4E-H). A higher magnification showed weak non-specific binding to the basement membrane of the nephric tubules in kidney rudiments (Fig. 4H). The same weak binding was observed in the basement membrane of most tubules in the mesenchymal explants, supporting the nephric nature of the forming tubules (Fig. 4F). After 7 days in culture, glomeruli-like bodies were found; however, their formation was very irregular in three dimensions and the structures were often squeezed or disproportionately enlarged (Fig. 3D). The predominant structures were tubules (partially proximal). Control explants in $CM/bFGF$ without $TGF\alpha$ showed the same tubule development, but the accompanying stromal cell mass was significantly reduced. These results are summarized in Table 1.

Molecular markers of metanephric differentiation in the explants grown in $CM/bFGF/TGF\alpha$

To characterize further the structures induced by $CM/bFGF/TGF\alpha$ in mesenchymal explants, we studied the expression of several genes that were used as markers of the metanephric differentiation. *Wtl* transcription factor was the only marker used that is expressed exclusively in the metanephric mesenchyme and its derivatives, but not in the collecting ducts (Pelletier et al., 1991). By whole mount and tissue section *in situ* hybridization, expression was detectable in condensed mesenchyme and in most primitive tubules that appeared after 3-4 days in culture, suggesting that they corresponded to comma- and S-shaped bodies *in vivo*. Most of the more advanced tubules were negative, while the glomeruli-like structures expressed *Wtl* (Fig. 5E).

In vivo, *Lim1* transcription factor is the only marker of the metanephric mesenchyme that is not expressed in the con-

Table 1. The effect of various culture supplements on the differentiation of isolated metanephric mesenchymes

Supplements in the culture medium	Condensations	Tubules in 3-5 days	Tubules after 9 days	Glomeruli-like structures*
TGF α	5/200	do not survive	–	–
bFGF+TGF α	38/38	0/38	2/38	n/d
CM+TGF α	41/41	0/41	do not survive	–
CM+bFGF	485/485	370/460	245/250	after 6d 7/11
+TGF α				after 9d 5/5
CM+bFGF (without TGF α)	10/10	10/10	n/d	n/d

Each entry denotes the number of individually cultured mesenchymal explants which developed a particular structure/the total number of mesenchymal explants analyzed for this type of morphological change.

*The appearance of glomeruli-like structures was analyzed only in histological sections. n/d, not done.

densifying mesenchyme around the tips of the ureteric bud. It appears later in primary vesicles, comma- and S-shaped bodies, and tubules but not in glomeruli (Fujii et al., 1994). The condensing areas in explanted mesenchymes were negative for *Lim1*, while it was expressed in primitive epithelium and tubules. Glomeruli-like structures were negative as in vivo (Fig. 5B). *Wnt4* encodes a secreted molecule which is a putative early marker for epithelial conversion. In vivo it appears in the condensing mesenchyme, persists in primary vesicles and comma-shaped bodies, but is down regulated in S-shaped bodies. The epithelium of the nephron does not express it (Stark et al., 1994). In CM/bFGF/TGF α explants, *Wnt4* expression was consistent with its in vivo expression, i.e., condensations were positive, while most of the tubules and all glomeruli-like structures were negative (Fig. 5C).

Metanephric blastema consists of uniform mesenchymal cells, which after induction differentiate into condensed cells that convert into epithelium or into 'loose' stromal cells. *Hgf* is a marker for the differentiated 'loose' stromal mesenchyme of the cortical layer (Sonnenberg et al., 1993; Woolf et al., 1995). The *Hgf* receptor *c-met* is expressed in vivo in the condensing mesenchyme and all subsequent epithelia of the nephron (as well as in the collecting ducts). Spindle-shaped stromal cells in the explants were *Hgf* positive, while its receptor was expressed reciprocally in all of the epithelial and condensed areas but not 'loose' stromal cells (Fig. 6).

Absence of markers of the collecting duct epithelium: *c-ret*, *Wnt7b*, *Wnt11* and *Sonic hedgehog*, in the explants grown in CM/bFGF/TGF α

In vivo, the *c-ret* tyrosine kinase receptor is expressed only in the tips of the ureteric bud and never in the mesenchyme or its derivatives (Pachnis et al., 1993). The secreted molecules *Wnt7b* and *Shh* are also markers specific to the collecting duct epithelium and are never expressed in metanephric mesenchyme or its derivatives in vivo (Vainio et al., 1994; Bitgood et al., 1995). By in situ hybridization of tissue sections and whole mounts, explants were invariably negative for *c-ret*, *Shh* and *Wnt7b* expression after 1-9 days in culture (the absence of expression is shown for *Wnt7b* only; Fig. 7E,F), though kidney sections that were used as positive controls showed specific hybridization with each probe (Fig. 7A-D). The fact that the explants were always negative for *c-ret*, *Wnt7b*, and *Shh*

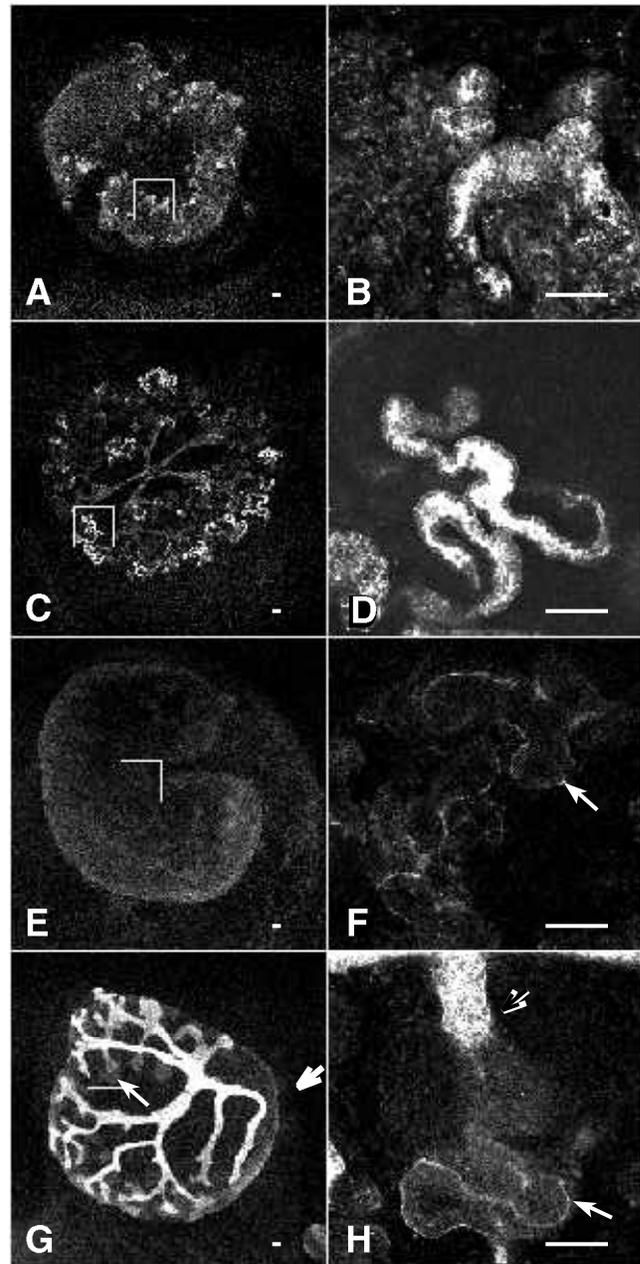


Fig. 4. Whole-mount immunostaining of mesenchymal explants in CM/bFGF/TGF α or kidney rudiments cultured for 6 days. (A-D) Staining with a polyclonal antibody against a brush border antigen. (A) Many tubules in the whole mesenchymal explant show positive staining. (B) The enlarged area indicated by the box in A shows the proximal-type tubule with intense staining in the center (compare with proximal tubule in D). (C) Proximal tubules in a cultured whole kidney rudiment. (D) Proximal tubules in the enlarged area indicated by the box in C. (E-H) Binding of FITC-labeled DB lectin. (E) None of the tubules in an entire mesenchymal explant bind the DB lectin (compare with binding to collecting ducts in the kidney rudiment in G). (F) Enlarged area indicated by the box in E shows weak non-specific binding of the lectin to basement membrane of the tubules (arrow) (compare with the same pattern in nephric tubules in the kidney rudiment in H). (G) Collecting ducts in kidney rudiments bind the lectin (hollow arrow); (H) the enlarged area indicated in 'G' shows that strong binding of the lectin to collecting ducts (hollow arrow) is different from the weak non-specific binding to basement membranes of nephric tubules (arrow). Bar, 0.06 mm.

indicates that the forming epithelium originated from the metanephric mesenchyme, and not from contaminating ureteric bud. It also shows that the mesenchyme differentiated into nephric epithelium and not collecting ducts. In vivo, RNA for the *Wnt11* secreted protein is expressed in the tips of the ureteric bud (Vainio et al., 1994), and, as we observed, in the stromal mesenchymal cells, surrounding collecting ducts in the medullar area of the kidney after 15 dpc (Fig. 7B). It is absent from any type of mesenchymal cells in the cortical layer. In whole mounts and tissue sections, explants at all stages showed no transcripts. Even the loose stromal cells surrounding condensates and tubules were negative for *Wnt11*.

Expression of potential signal molecules in the RUB1 cell line

Molecules that are involved in induction and epithelial-mesenchymal interactions show striking similarity in all organisms and are represented by a relatively small number of conserved families of signaling molecules. Since in our system we are presumably dealing with a soluble factor(s) produced by the RUB1 cells, which are derived from the natural renal inductive tissue, secreted molecules such as *Bmp7*, *Shh*, *Wnt7b* and *Wnt11* are likely candidates for this factor. Therefore, we have performed RT-PCR with specific primers for these gene sequences using total RNA from cultured RUB1 cells or 19 dpc rat kidneys, which served as a positive control for each sequence. While appropriately sized RT-PCR fragments were amplified from the 19 dpc kidney, only *Wnt7b* and *Bmp7* were generated from RUB1 cell RNA (Fig. 8A). The specificity of the PCR amplification, including two bands that had been amplified with primers for *Wnt11*, was confirmed by hybridization of blotted fragments with specific ^{32}P -labeled DNA probes for each gene (Fig. 8B). The multiple bands hybridizing with the *Wnt11* probe suggest that there are different splice variants, recognized by the

probe. These results were confirmed by northern blot hybridization with ^{32}P -labeled probes for *Bmp7*, *Shh*, *Wnt7b*, and *Wnt11*. Purified mRNA from RUB1 cells or 13 dpc whole embryos, as a positive control, was run in parallel for each probe. The *Bmp7* probe hybridized to 4 transcripts, two of which approximated its estimated 2.0 kb transcript, and the other two, its 4.0 kb transcript. *Wnt7b* showed one abundant transcript of the estimated 4.0 kb in both control and bud cells. *Shh* and *Wnt11* were not expressed by RUB1 cells (data not shown), which is consistent with RT-PCR studies (Fig. 8C).

Preliminary characterization of RUB1-secreted inductive factor(s)

To establish the nature of the inductive factor(s) released by RUB1 cells, CM was manipulated in various ways and then examined for inductive activity in the metanephric explant

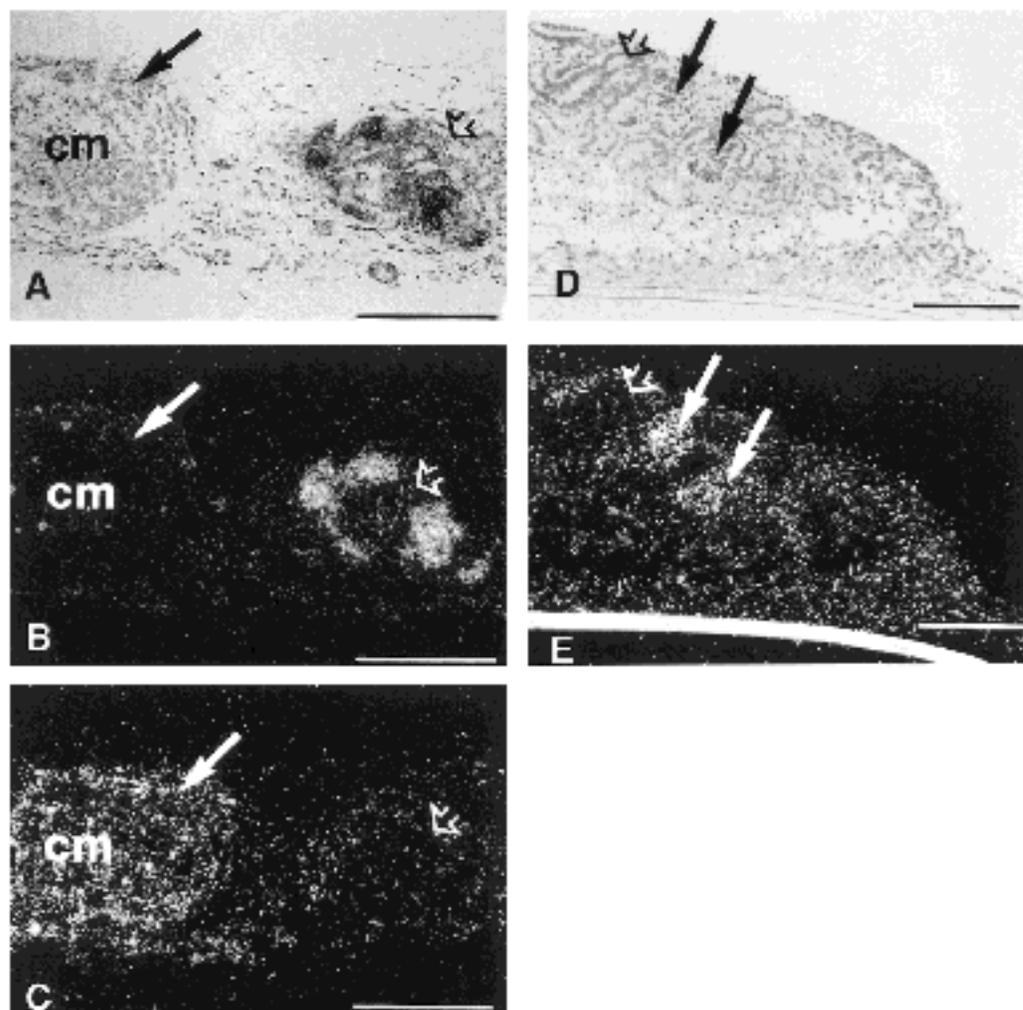


Fig. 5. In situ hybridization for *Lim1*, *Wnt4*, or *Wt1* expression in tissue sections from mesenchymal explants cultured in CM/bFGF/TGF α . (A) Bright-field image of B and C. (B) *Lim1* or (C) *Wnt4* in consecutive sections of explant, cultured for 6 days. *Lim1* is not expressed in condensed mesenchyme (arrows), but strong hybridization is found in tubules that differentiate from induced mesenchyme (hollow arrows). *Wnt4* is expressed in condensed mesenchyme, but tubules are negative (hollow arrows). (D) Bright-field image of E. (E) *Wt1* probe in a section of explant cultured for 9 days. Glomeruli-like structures (arrows) and condensed areas are positive, while loose mesenchyme and most tubules (hollow arrows) are negative. cm, condensed mesenchyme. Bar, 0.1 mm.

culture system. From these studies, the factor(s) was stable at 60°C for 30 minutes but unstable at 100°C for 10 minutes. It maintained activity despite exposure (30 minutes) to weak acids (acetic acid, pH 4.5) but was inactivated by exposures to strong acids (trifluoroacetic acid, pH 2.0). It was retained by a 10 NMWT-cutoff ultrafiltration membrane, was precipitated by ammonium sulfate (40-60% saturation), and was weakly adherent to a heparin-affinity column (eluted with 0.15 M NaCl). Furthermore, heparin, which inhibits tubule formation in tissue-induced renal mesenchymes (Ekblom et al., 1978), completely blocked tubule formation in explant culture. Finally, since HGF activity has been associated with renal epithelial conversion (Tsarfaty et al., 1994) and tubule formation (Montesano et al., 1991), we assessed the ability of commercial preparations of HGF to induce morphogenesis in our metanephric explants. In these studies, HGF was incapable either in the presence (0/6 mesenchymes) or absence (0/6 mesenchymes) of bFGF of mediating tubule formation.

DISCUSSION

We have developed and characterized an explant culture system, in which separated metanephric mesenchyme can grow and completely differentiate in vitro in the absence of an inductive tissue. Our results indicate that the isolated metanephric mesenchyme is induced to differentiate into epithelia of a nephron, including glomeruli-like structures, when cultured in CM from a ureteric bud cell line supplemented with bFGF and TGF α in serum-free medium. Morphologically, tubules and glomeruli-like structures formed in our explants, were similar to those that develop in vitro when metanephric mesenchymes are induced by spinal cord (Saxén, 1987) or *Wnt1*-expressing cells (Herzlinger et al., 1994). Furthermore, we have shown, by both morphological and molecular criteria, that the developmental changes are comparable to those in vivo.

The first morphological event of metanephric induction is condensation of mesenchymal cells around the tips of the ureteric bud. *Wt1*, *Wnt4*, and *c-met* are expressed in vivo in this condensed mesenchyme (Pelletier et al., 1991; Stark et al., 1994; Woolf et al., 1995), and their expression was evident

in explant condensations, beginning from the first day in culture and extending to the ninth day. Furthermore, *Lim1* RNA, which is not found around the tips of the ureteric bud in vivo, was not expressed in the condensates in vitro.

After 3-4 days in culture, the explants were replete with tubules. By in situ hybridization it was shown that tubules expressed markers such as *Lim1*, *c-met*, *Wt1*, and *Wnt4*, which are characteristic of the epithelial structures derived from the metanephric mesenchyme (Fujii et al., 1994; Woolf et al., 1995; Pelletier et al., 1991; Stark et al., 1994). The expression of *Wt1* and *Wnt4* was found only in a small percentage of tubules, which corresponds to the situation in vivo. There, *Wt1* is expressed in the comma-shaped bodies and the region of the S-shaped bodies that will become the podocytes. In contrast, *Wnt4* expression is obvious in comma-shaped bodies, is down-regulated in S-shaped bodies, and persists only in small portions of the proximal tubules which fuse with the collecting ducts. As immunostaining for proximal tubules, i.e., brush-

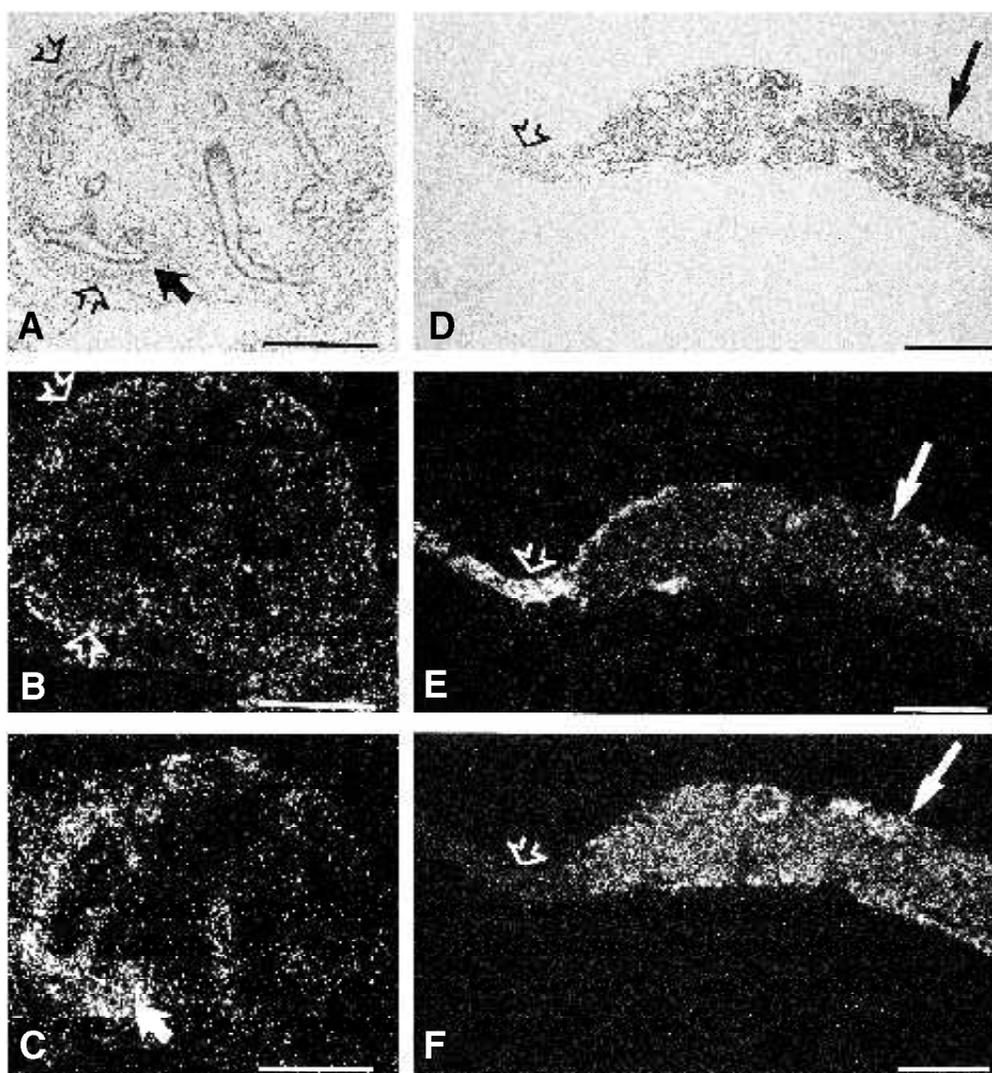


Fig. 6. *Hgf* (B,E) and *c-met* (C,F) in situ hybridization with consecutive sections of 15 dpc rat kidney (A-C) or explanted mesenchyme (D-F), cultured for 9 days in CM/bFGF/TGF α . In vivo and in vitro, *Hgf* is expressed in loose mesenchymal cells (hollow arrows), while the receptor *c-met* is expressed in condensed areas and tubules (arrows). Bar, 0.1 mm.

border antigen shows, part of the developing tubules were of the proximal-type.

All epithelial structures in our system belonged to nephric-type epithelium, and not the collecting duct type. Whole mount and tissue sections were invariably negative for the binding of DB lectin as well as the expression of *c-ret*, *Wnt7b*, or *Shh*, markers of collecting duct epithelium. The absence of these markers shows that (a) tubules in the explants are not the result of contamination by the ureteric bud, and (b) the only epithelium that differentiates from metanephric mesenchyme in our system is the epithelium of a nephron and not of the collecting duct, as was suggested recently (Qiao et al., 1995). The difference might be explained by technical differences in the systems used. We used a ureteric bud cell line that had gone through about 10 passages and not primary cultures, used only conditioned medium from those cells, and did not coculture bud cells with the explants. Additionally, our medium was chemically defined and serum-free, while Qiao et al. used serum, which introduces many undefined factors into the system. However, our findings are in agreement with the classical view of two distinct origins for renal epithelia: metanephric mesenchyme for the epithelium of a nephron and ureteric bud for the collecting duct epithelium (reviewed by Saxén 1987).

Those mesenchymal cells in the explants that remained loose and formed the surrounding stroma expressed *Hgf*, suggesting that they corresponded to cortical-type stroma (Sonnenberg et al., 1993; Woolf et al., 1995). Interestingly, we did not detect *Wnt11* or *Wnt4* in the loose stromal cells in our explants. We have found that in vivo, these markers, in addition to the described localizations in the tips of the ureteric bud (*Wnt11*) and early stages of epithelial conversion (*Wnt4*), are also expressed in the stromal cells that surround collecting ducts in the medullar region of the metanephric kidney, but are never seen in stromal cells of the cortical layer.

Our culture medium

was sufficient to support the differentiation of isolated metanephric mesenchyme up to the final step of nephron differentiation, formation of glomeruli. Similar to the in vivo pattern, glomeruli-like structures in the explants were deficient for all of the markers analyzed, except *Wt1*. Hence, we conclude that our medium contained all of the factors needed for differentiation of the nephron, both by morphological and molecular criteria.

We have shown that metanephric mesenchyme can be induced to differentiate without contact with an inductive tissue and that these processes can apparently be triggered by soluble factors in the filtered medium. Prior indication that the inductive signal is soluble was shown using pituitary extract that induced tubule formation in the metanephric mesenchyme (Perantoni et al., 1991). The accepted theory regarding the requirement of inductive tissue for renal differentiation was mainly based on experiments in which recombined mesenchyme and the inducing tissue were cocultured on opposite

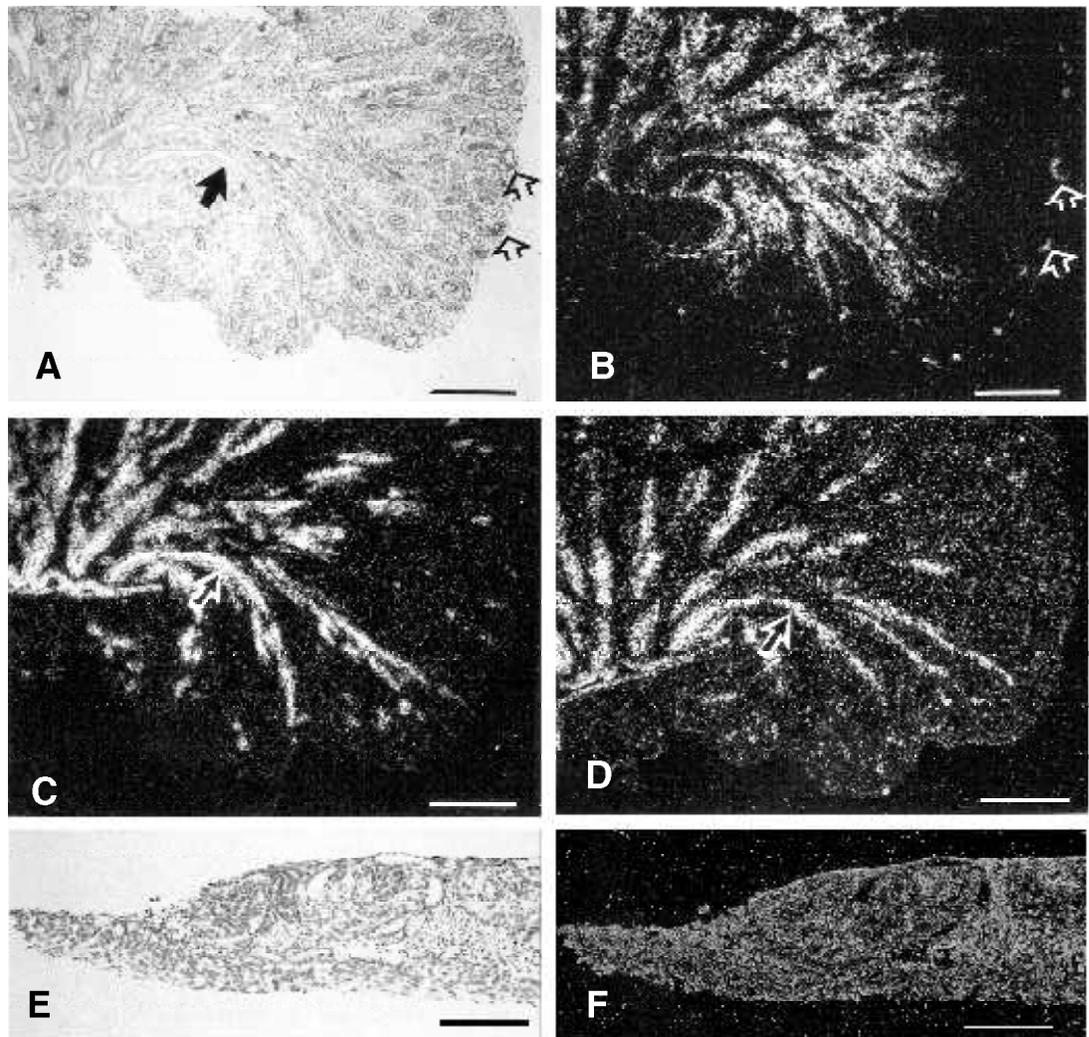


Fig. 7. (A) Bright-field image for B, C and D. (B-D) Consecutive sections of 19 dpc rat kidney hybridized with *Wnt11* (B), *Wnt7b* (C), or *Shh* (D) and a section of a mesenchymal explant cultured for 9 days in CM/bFGF/TGF α and hybridized with *Wnt7b* (E,F). *Wnt11* is expressed in the tips of the ureteric bud (hollow arrows) and in mesenchyme surrounding the collecting ducts. *Wnt7b* and *Shh* are expressed only in collecting ducts (arrows). (E) Bright-field image of F. (F) There is no hybridization with a *Wnt7b* probe in the explant. Bar, 0.1 mm.

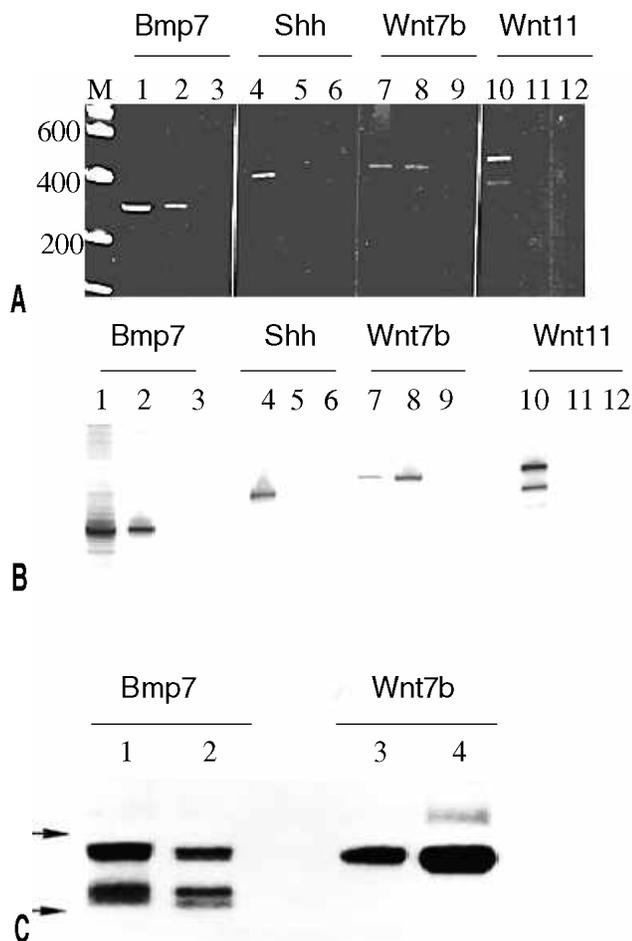


Fig. 8. (A) Expression of *Bmp7*, *Shh*, *Wnt7b*, or *Wnt11* in 19 dpc rat kidney (lanes 1, 4, 7, 10), RUB1 (lanes 2, 5, 8, 11), or no-template controls (lanes 3, 6, 9, 12) shown by RT-PCR. Only *Bmp7* and *Wnt7b* are expressed in RUB1. (B) The same PCR gel blotted and hybridized with *Bmp7* (lanes 1-3), *Shh* (lanes 4-6); *Wnt7b* (lanes 7-9); *Wnt11* (lanes 10-12) ³²P-labeled probes. Three bands in lane 10 hybridized with the *Wnt11* probe. (C) Northern blot analysis of mRNA from 13 dpc rat whole embryos (lanes 1, 3) or ureteric bud cells (lanes 2, 4) hybridized with *Bmp7* (lanes 1, 2) or *Wnt7b* (lanes 3, 4). Arrows indicate the positions of 18S and 28S ribosomal RNA.

sides of filters with different pore sizes. It was shown that a pore size of $<0.1 \mu\text{m}$ inhibited the extension of cellular micro-processes into the filter as well as induction (Grobstein 1956; Wartiovaara et al., 1974; Saxén et al., 1978). However, the block in induction might also be explained by the fact that filters with such small pore sizes do not provide sufficient support for the mesenchymes. In our study, $0.1 \mu\text{m}$ pore-size filters were coated with collagen, which provided good attachment and enhanced differentiation. Davies and Bard (personal communication) have found that the inductive signal from spinal cord can pass through a set of four Nucleopore filters, at least one of which had $0.1 \mu\text{m}$ pores through which cell processes were unable to pass. These results suggest that the inductive signal does not require contact for its effect. Similarly, when metanephric mesenchyme was induced by *Wnt1*-expressing cells (Herzlinger et al., 1994), though the cells were grown on opposite sides of the filter, the existence

and necessity of cell processes was not demonstrated, leaving open the possibility that the inducing factor is soluble.

Since bFGF has been shown to play a critical role in the early stages of renal development by inducing condensation of the mesenchyme (Perantoni et al., 1995), one might predict that the bud cells would produce this factor as well and question why our explant cultures required supplementation. Indeed, we have found by both RT-PCR of RUB1 cell RNA and immunoblotting of CM that bFGF is produced by these cells (unpublished observation). However, the bFGF levels observed by immunoblotting are small relative to the levels added in vitro, indicating that it is sufficiently diluted in vitro to require supplementation.

We have done a preliminary assessment of possible secreted factor(s). By RT-PCR and northern blot hybridization the expression of secreted factors *Bmp7*, *Shh*, *Wnt7b*, and *Wnt11* was examined in RUB1 cells. These are potential candidates for inductive signals based on their expression patterns in the developing kidney in vivo and the role that these patterning factors play in cell-cell signaling in other organs (Lyons et al., 1995; reviewed by Blair, 1995; reviewed by Johnson and Tabin, 1995; reviewed by Nusse and Varmus, 1992). Among these, only *Bmp7* and *Wnt7b* were expressed in RUB1. *Bmp7* has been shown to be more of a maintenance and survival factor, since mice deficient for *Bmp7* could still develop a few differentiated nephrons, though the size and architecture of the kidney were highly abnormal (Dudley et al., 1995; Luo et al., 1995). *Wnt7b* is perhaps a more likely candidate. The induction of metanephric mesenchyme by *Wnt1*-expressing cells (Herzlinger et al., 1994) suggests that, though *Wnt1* is not normally expressed in kidney, there may be another *Wnt*-family member which substitutes for *Wnt1*. *Wnt7b* is expressed in the ureteric bud as soon as it grows into the metanephric blastema and thus may be a natural inducer. However, at later stages of metanephric development, *Wnt7b* is not expressed in the tips of the collecting ducts, where the new nephrons are continuously induced. This could suggest that other *Wnt*-family genes may be found, which are expressed in the tips of the ureteric bud (in our cell line).

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