

## Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium

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### SUMMARY

The shapes of different organs can be explained largely by two fundamental characteristics of their epithelial rudiments – the pattern of branching and the rate of proliferation. Glial-cell-line-derived neurotrophic factor (GDNF) has recently been implicated in the development of metanephric ureteric epithelium (Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B.J., Sariola, H. and Westphal, H. (1996). *Nature* 382, 73-76; Sánchez, M.P., Silos-Santiago, I., Frisé, J., He, B., Lira, S.A. and Barbacid, M. (1996). *Nature* 382, 70-73; Vega, Q.C., Worby, C.A., Lechner, M.S., Dixon, J.E. and Dressler, G.R. (1996). *Proc. Nat. Acad. Sci. USA* 93, 10657-10661). We have analysed the target cells of GDNF and the manner in which it controls ureteric development, and have compared it with other growth factors that have been associated with the regulation of branching morphogenesis, namely hepa-

toocyte growth factor (HGF) and transforming growth factor- $\beta$ 1 (TGF $\beta$ 1). We show that GDNF binds directly to the tips of ureteric bud branches, and that it has the ability to promote primary ureteric buds from various segments of Wolffian duct and to attract ureteric branches towards the source of GDNF. It increases cell adhesion, but is not obviously mitogenic for ureteric cells. The data indicate that GDNF is required primarily for bud initiation. Comparison of GDNF, HGF and TGF $\beta$ 1 suggests that the latter act later than GDNF, and may represent a partially redundant set of mesenchyme-derived growth factors that control ureteric development. Thus, GDNF is the first defined inducer in the embryonic metanephric kidney.

Key words: Glial-cell-line-derived neurotrophic factor, kidney morphogenesis, ureteric bud growth, branching morphogenesis, cell adhesion

### INTRODUCTION

Development of the ureter and urinary collecting duct system of the metanephric kidney is first triggered by a signal from the nephrogenic mesenchyme. This signal induces the nearby Wolffian duct to produce an outgrowth, the ureteric bud, which then elongates, invades the mesenchyme, and undergoes dichotomous divisions. Its tips induce condensation and epithelial conversion of the mesenchyme into excretory tubules (reviewed by Saxén, 1987). Kidney tubule induction and ureteric morphogenesis are regulated reciprocally (Grobstein, 1953; 1955). Attempts to identify the signals involved have traditionally concentrated on the induction of epithelial differentiation of kidney tubules, and some interesting candidate molecules have been identified (Kreidberg et al., 1993; Stark et al., 1994; Dudley et al., 1995; Luo et al., 1995; Perantoni et al., 1995; Torres et al., 1995; Vukicevic et al., 1996). Less is known about the control of ureteric bud growth and differentiation, although some growth factors, such as hepatocyte growth factor/scatter factor (HGF)

(Santos et al., 1994; Woolf et al., 1995), transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) (Ritvos et al., 1995), and extracellular matrix molecules (Davies et al., 1995) have been implicated in the regulation of its growth and branching. Moreover, HGF has been shown to regulate branching morphogenesis of kidney-derived Madin-Darby canine kidney (MDCK) epithelial cells in collagen-matrix cultures (Montesano et al., 1991a).

Recent data have shown that glial-cell-line-derived neurotrophic factor (GDNF) is expressed in the condensing mesenchyme that surrounds the developing ureteric system of kidneys (Hellmich et al., 1996; Suvanto et al., 1996). GDNF is a distant member of the TGF $\beta$  superfamily (Lin et al., 1993) and maintains dopaminergic, noradrenergic and motor neurones of the central nervous system (Lin et al., 1993; Tomac et al., 1995; Arenas et al., 1995; Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) as well as various sub-populations of the peripheral sensory and sympathetic neurones (Henderson et al., 1994; Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995).

One known receptor for GDNF is the cRet receptor tyrosine kinase (Takahashi et al., 1988; Trupp et al., 1996; Durbec et al., 1996), which is expressed in several tissues adjacent to sites of GDNF synthesis, and it is autophosphorylated upon GDNF binding. The functional receptor complex of GDNF and cRet additionally includes novel types of glycosylphosphatidylinositol-linked (GPI) cell surface receptors, GDNFR- $\alpha$  (Jing et al., 1996; Treanor et al., 1996) or GDNFR- $\beta$  (Suvanto et al., 1997; also named TGF- $\beta$ -related neurotrophic factor receptor, TrnR2; Baloh et al., 1997). Comparative analysis of GDNFR- $\alpha$ , GDNFR- $\beta$  and cRet expression suggests that multiple receptor complexes exist in vivo (Baloh et al., 1997; Suvanto et al., 1997). The ligand specificities of GDNFR- $\alpha$  and GDNFR- $\beta$  have not yet been fully resolved, but they bind both GDNF and its novel homologue neurturin (Kotzbauer et al., 1996), and both these GPI-linked receptors can mediate growth factor signaling via cRet (Baloh et al., 1997).

Transgenic mice deficient for GDNF, and those deficient for cRet, show remarkably similar phenotypes that are characterised by a severe defect in intestinal innervation, and renal aplasia or hypodysplasia (Pichel et al., 1996; Schuchardt et al., 1994; 1996). This observation, together with those from antibody inhibition experiments (Vega et al., 1996), suggests strongly that GDNF and cRet play a major role in development of renal epithelia. We have therefore investigated the target cell types and developmental functions of GDNF in kidney morphogenesis. We show that GDNF binds to ureteric bud tips, that it is required for bud initiation, and that it may function through increasing cell adhesion rather than cell proliferation. Comparison of GDNF, HGF and TGF $\beta$ 1 in different experimental assays suggests that none of these growth factors is directly mitogenic upon the ureteric epithelium, and that additional, growth-promoting effectors are also required, if a mesenchyme is to support ureteric development.

## MATERIALS AND METHODS

### Animals

Sprague-Dawley and Wistar rat embryos, of various stages of gestation, were used throughout the study. Rats were mated overnight and the next day was defined as embryonic day 0 (E<sub>r</sub>0). The gestation stage was further estimated by the size of the limb buds, and the stage of the kidney was verified visually under a stereo-microscope after dissection. In desulphation experiments, E<sub>m</sub>11 mouse kidney rudiments were also used (Davies et al., 1995). Generation of the transgenic mice deficient for cRet has been described by Schuchardt et al. (1994, 1996).

### Organ culture

Microdissection was used to isolate the following tissues: early mouse E<sub>m</sub>11 bud-negative metanephric mesenchymes with adjacent Wolffian ducts, mouse E<sub>m</sub>11 bud-stage kidney rudiments; rat E<sub>r</sub>13 bud- and T-bud-stage metanephric kidneys; and E<sub>r</sub>13 whole urogenital blocks (including metanephric kidney, Wolffian duct, genital ridge and mesonephros). These tissues were then cultured in Trowell-type dishes either intact or as separated nephrogenic mesenchymes and ureteric buds, separation being performed using 0.25% pancreatin-trypsin. Two types of culture media were used: (1) MEM (Eagle's Minimum Essential Medium; Gibco) supplemented with 5-10% fetal bovine serum (FBS) (Bioclear) and (2) I-MEM (Improved Eagle's Minimum Essential Medium; Gibco), originally based on MEM modified by Richter et al. (1972) supplemented with 50  $\mu$ g/ml iron-loaded transferrin instead of serum (Ekblom et al., 1983). For the

tissue recombination cultures, different mesenchymes (lung, salivary gland, limb and gut) from E<sub>r</sub>13 to E<sub>r</sub>14 rats and tooth mesenchyme from E<sub>m</sub>11 mouse were separated with pancreatin-trypsin from their respective epithelia, and cultured in recombination with isolated ureteric buds. Organs were cultured on Nuclepore filters (pore-size 1  $\mu$ m or 0.1  $\mu$ m; Costar) placed on top of a metal grid in a Trowell-type organ culture. Separated ureteric buds were also cultured in 30  $\mu$ l drops of medium hanging from a lid of a Petri dish (Nunc), the bottom of the dish being filled with sterile phosphate-buffered saline (PBS). The microdissection and tissue culture techniques have been described in detail by Saxén and Lehtonen (1987).

### Growth factors

Human recombinant GDNF was provided by PeproTech Inc. and Promega. GDNF stock was dissolved in sterile PBS at 100 ng/ $\mu$ l; concentrations ranging from 1 ng/ml to 100 ng/ml were tested in tissue recombination and hanging drop cultures. TGF $\beta$ 1, a kind gift of Dr Marikki Laiho (University of Helsinki), was dissolved to make a 50 ng/ $\mu$ l stock, and concentrations ranging from 1 pg/ml to 100 ng/ml were tested in cultures. Human recombinant HGF (Sigma or Collaborative Biomedical) was kept as a 50 ng/ $\mu$ l stock, and concentrations from 1 ng/ml to 100 ng/ml were used in culture experiments. All growth factor stocks were stored at -70°C until used. Culture medium was changed every second day.

### Immunohistochemistry

#### Antibodies

Primary antibodies included polyclonal antibodies to EHS-tumour laminin (Gibco), monoclonal antibodies to cytokeratin-8 (Amersham), polyclonal antibodies to mouse L1 neural cell adhesion molecule (Rathjen and Schachner, 1984), and polyclonal antibodies to rat brush border epitopes (Ekblom et al., 1980). Secondary antibodies comprised rhodamine-conjugated goat-anti-mouse IgG and fluorescein-conjugated donkey-anti-rabbit IgG (Jackson Immuno-Research Lab.). E<sub>m</sub>11 mouse kidneys were stained using the collecting-duct-specific stain anti-calbindin-D-28k (Davies, 1994) or with monoclonal antibodies to cytokeratin-18 (Virtanen et al., 1985). In some experiments, ureteric epithelium was visualised by rhodamine-conjugated *Dolichos biflorus*-agglutinin (Vector) at 25  $\mu$ g/ml concentration.

#### Whole-mount staining

Organ rudiments, from embryos and cultures, were stained by the whole-mount immunocytochemical technique described by Sariola et al. (1988) with some modifications. Briefly, tissue explants were fixed in ice-cold methanol for 5 minutes, washed in PBS containing 11% sucrose and 1% bovine serum albumin (BSA), and incubated overnight in primary antibodies. The samples were washed extensively in PBS and incubated overnight in secondary antibodies, washed three times for 2 hours each in PBS, and mounted in Immumount (Shandon). All antibody incubations were done in Eppendorf tubes at +4°C. Hanging drop cultures were stained using a similar method but, immediately before fixation, they were attached to filters by either Matrigel (Becton Dickinson) or 2% agarose in MEM.

### In situ hybridisation and probes

Single-stranded antisense and sense cRNA probes were synthesised and labelled with <sup>35</sup>S-UTP (Amersham) using appropriate RNA polymerases. Rat GDNFR- $\alpha$  probe was cloned by PCR as described by Suvanto et al. (1997). The primers used for GDNFR- $\alpha$  probe were: forward (nucleotides 294-313) 5' GCG GCA CCA TGT TCC TAG CC 3' and reverse (nucleotides 1020-1039) 5' CAG ACT CAG GCA GTT GGG CC 3'. The identity of the cloned fragment was verified by direct sequencing with a Pharmacia A.L.F. automatic DNA sequencer. The cRet probe spanned the tyrosine kinase domain of mouse cRet (nucleotides 2534-3217; Pachnis et al., 1993). The cloning of rat GDNF probe for in situ hybridisation has been described by Suvanto et al. (1996).

In situ hybridisation for sections was performed according to Wilkinson and Green (1990) with some modifications. Briefly, whole rat E<sub>r</sub>13 to E<sub>r</sub>17 embryos or dissected kidneys were fixed in fresh, neutral-buffered 4% paraformaldehyde (PFA) for 2 hours at room temperature or overnight at +4°C, rinsed in PBS and processed for paraffin sectioning at 7 µm on silanized slides. Slides were deparaffinised, treated with proteinase K (15–40 µg/ml, Sigma), post-fixed in 4% PFA, rinsed in PBS and hybridised overnight at +52°C with cRNA probes. The sections were washed at high stringency conditions, treated with RNase A (Boehringer Mannheim), dehydrated and air dried. For autoradiography the slides were dipped in Kodak NTB-2 emulsion, exposed for 12 days to 2 weeks, developed in D-19 (Kodak), counterstained in Harris hematoxylin (Shandon) and mounted in Mountex. Photos were taken with an Olympus AX70 Provis microscope. Hybridisation with probes in the sense orientation resulted in only low background labelling (data not shown).

### In situ binding assay

1–2 µg recombinant human GDNF was treated with Chloramine T (Serva) for 30–40 seconds in the presence of 250 µCi of <sup>125</sup>I-labelled NaI (Amersham) and the reaction was stopped by Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and NaI. Unbound <sup>125</sup>I-labelled NaI was separated by gel filtration with Sephadex G-25 PD-10 columns (Pharmacia). Specific activity, determined from trichloroacetic acid-precipitated samples of the reaction product, was approximately 100 µCi/mg. Kidneys from E<sub>r</sub>13 to E<sub>r</sub>17 rats were incubated at room temperature with 10 ng/ml of <sup>125</sup>I-labelled GDNF for 90 minutes in MEM with 120 mM Hepes (Gibco) and 0.1% BSA as described by Partanen and Thesleff (1987). Samples were washed at +4°C, first for 60 minutes in the binding medium, then four times for 30 minutes in PBS, and were fixed in fresh buffered 4% PFA, embedded in paraffin, and serially sectioned at 7 µm. Binding of <sup>125</sup>I-GDNF was competed with 250-fold excess of unlabelled GDNF, and the kidneys were processed for autoradiography as described for in situ hybridisation (see above).

### Experiments with growth factor-soaked agarose beads

For bead experiments, E<sub>r</sub>13 rat kidneys, E<sub>r</sub>13 urogenital blocks, and E<sub>m</sub>11 mouse bud-positive and bud-negative metanephric rudiments, were cultured as described above. Separated E<sub>r</sub>13 kidney ureteric buds without metanephric mesenchyme were recombined with lung mesenchyme from the same embryo. To prepare growth factor-soaked beads, agarose beads (Affi-gel Blue; BioRad) of 80–120 mesh were washed extensively in sterile PBS, then incubated in 5 µl of growth factor solutions (100 ng/µl GDNF, 50 ng/µl and 10 ng/µl GDNF, HGF and TGFβ1) or in control 1% BSA/PBS as described (Vainio et al., 1993). They were placed by a micro-capillary next to ureteric buds or Wolffian ducts of the embryos mentioned above, and also of E<sub>m</sub>11 urogenital blocks (GDNF only), from transgenic mice deficient for cRet (Schuchardt et al., 1994). Tissues were cultured for 3–5 days with the beads and then processed by whole-mount immunohistochemistry.

### Culture of ureteric buds in collagen gels

Rat tail collagen gels were prepared essentially as described by Montesano et al. (1991b). Briefly, 8 volumes of collagen stock solution was mixed with 1 volume of 10× concentrated MEM, 10% of FBS and 1 volume of sodium bicarbonate (11.76 mg/ml), the mixture being kept on ice to prevent gelation. 200 µl of the control or growth factor (50 ng/ml of GDNF or HGF)-containing mixture was added to each well of the Nunclon 24-well culture dish and separated E<sub>r</sub>13 ureteric buds without metanephric mesenchyme were pipetted on to the gel. An additional 100 µl of the collagen mixture was placed on top of the tissues, and after gelation was complete a further 100 µl of MEM with 10% FCS was added. During the 5 days of culture, tissues were photographed each day under an Olympus phase contrast ZDH10 microscope.

### Desulphation of GAGs in the developing kidney

To inhibit the sulphation of extracellular matrix sulphated GAGs, E<sub>m</sub>11

kidneys were cultured and treated with chlorate as described by Davies et al. (1995). The medium of some cultures was supplemented with one of the following: (1) 20 mM NaClO<sub>3</sub> (BDH AnaLaR 10435); (2) 50 ng/ml GDNF; (3) 20 mM NaClO<sub>3</sub> plus 50 ng/ml GDNF; (4) 20 mM NaClO<sub>3</sub> plus 100 ng/ml HGF; (5) 20 mM NaClO<sub>3</sub> plus 50 ng/ml GDNF plus 100 ng/ml HGF. Kidney rudiments were left to develop in these media for approximately 72 hours, then fixed for whole-mount immunohistochemistry and stained with antibodies against the collecting-duct-specific stain anti-calbindin-D-28k. Alternatively, E<sub>r</sub>13 dissected rat kidneys were cultured as described, and heparitinase III (Sigma) and chondroitinase ABC (Sigma) were added to the culture medium at 0.33 U/ml each as described (Davies et al., 1995). The culture medium was changed daily with fresh enzymes. At the second day of the culture, either GDNF- or BSA-soaked agarose beads were added next to ureters and Wolffian ducts, tissues were cultured for an additional 2 days, and were then processed for whole-mount immunohistochemistry and western blotting analysis.

### Western blotting analysis

Sets of 12 kidneys, grown for 55 hours in medium with or without 20 mM NaClO<sub>3</sub>, were homogenised in 100 µl of 2-mercaptoethanol-containing Laemmli sample buffer (Biorad), then their proteins were separated by SDS-PAGE (12.5% gel) and blotted onto Biorad transfer membranes. After transfer, membranes were washed in PBS, blocked in 1% blocking agent (Amersham RPN 3023) in PBS for 3 hours at room temperature, then incubated overnight at +4°C in 0.5 µg/ml primary antibody (either chick anti-GDNF; Promega, or rabbit anti cRet; Santa Cruz) in PBS. They were then washed in 1% blocking agent and incubated in 1:400 secondary antibody (fluorescein anti-rabbit or fluorescein anti-chicken; Sigma) for 3 hours at room temperature. After another wash, filters were probed with tertiary antibody (alkaline phosphatase anti-fluorescein; Amersham) in 100 mM Tris, 400 mM NaCl, pH 7.5, for 2 hours at room temperature. They were then washed extensively and developed using the NBT/BCPIP reagent from Amersham's RNA Colour Kit (RPN3300). Molecular masses were measured against pre-stained molecular mass standards (Biorad).

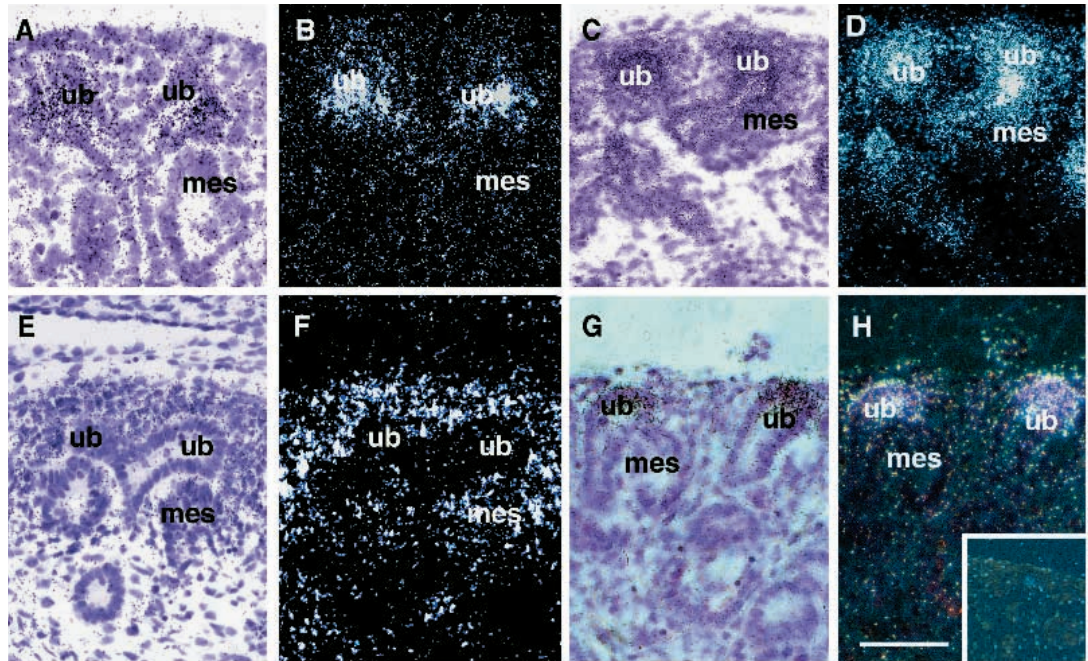
### Cell proliferation assays

To monitor the effect of GDNF and HGF on cell proliferation, separated ureteric buds were cultured in hanging drops as described above, and 5-bromo-2'-deoxyuridine (BrdU) cell-labelling reagent (Amersham) was used according to manufacturer's instructions. Briefly, two E<sub>r</sub>13 ureteric buds in each 30 µl hanging drop were cultured for 24 or 48 hours with or without 50 ng/ml GDNF in either chemically defined I-MEM or MEM supplemented with 10% FBS. The medium in the drop was then changed to fresh medium containing the BrdU-label. Cultures were incubated at +37°C for 30 minutes to 1 hour, after which they were fixed for whole-mount immunohistochemistry. Monoclonal antibodies to BrdU (Amersham) were used for double-immunofluorescence-labelling with polyclonal L1 neural cell adhesion molecule antibodies that served as an ureteric epithelial cell marker (Sainio et al., 1994). Because the isolated ureteric buds scatter cells into the medium, the BrdU data reflect the mitotic index in the bud explants but not in the cells scattered in the cultured drop.

The BrdU data were therefore verified further by counting both the enzymatically dissociated and scattered cells of separated ureteric buds either immediately after the dissection or after 24 hours in hanging drop cultures with or without ectopic growth factors (Fig. 4). Culture media with scattered cells from 10 drops containing two buds each were first pooled, collected to Eppendorf tubes and centrifuged. The scattered cells in 20 µl were then pipetted to silanised objective slides, air dried at +37°C, fixed with neutralised 10% formaldehyde, rinsed in PBS and Hoechst fluorochrome 33342, counterstained with Harris hematoxylin and mounted with Immount. The buds in each drop were collected and dissociated with 1 ml pancreatin-trypsin and trypsin-EDTA at +37°C for 20 minutes in separate Nunclon 3 cm culture dishes. The dissociated cells were collected in a small volume



**Fig. 1.** cRNA in situ hybridisation of GDNF and GDNF receptor mRNAs and GDNF binding to the E<sub>r</sub>17 metanephric kidney. (A) cRet transcripts are seen only in the tips of the branches of the ureteric tree but not in the mesenchyme. (B) Corresponding dark field image. (C) GDNFR- $\alpha$  mRNA is expressed by both the ureteric epithelium and metanephric mesenchyme. (D) Corresponding dark field image. (E) GDNF cRNA is expressed by the pretubular mesenchyme. (F) Corresponding dark field image. (G) <sup>125</sup>I-GDNF binds to the tips of ureteric epithelium, but neither to the other segments of the ureter nor medullary structures.



(H) Corresponding dark field image. Insert, 250-fold excess of unlabelled GDNF competes out the <sup>125</sup>I-GDNF-binding. mes, condensing metanephrogenic mesenchyme; ub, tip of the ureteric epithelium. Bar 200  $\mu$ m, insert 40  $\mu$ m.

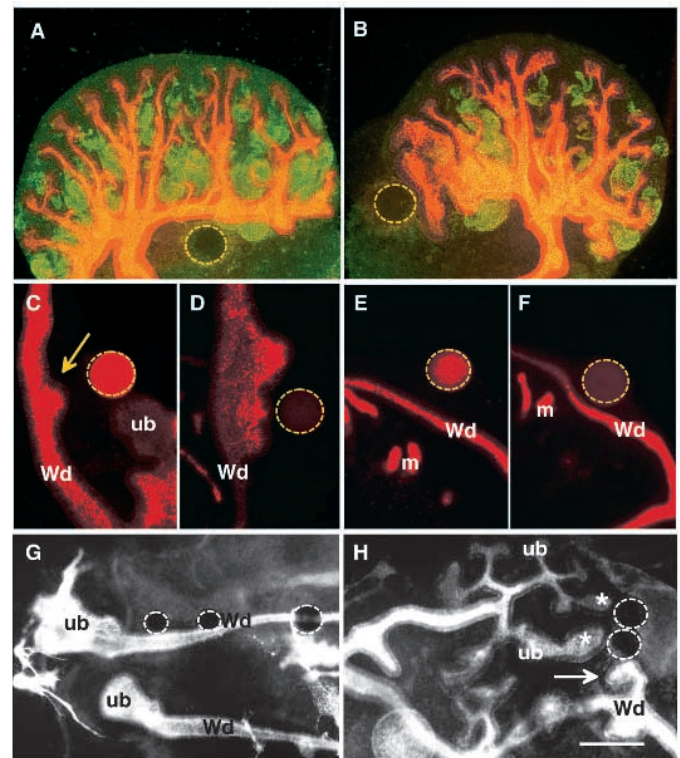
under a stereo microscope and placed on silanised objective slides, and drops were air dried, fixed and stained as described above. Cells in each slide were visualised with an Olympus AX70 Provis microscope equipped with epifluorescence and the cells on each slide were counted with ImagePro-plus program. The viability of all cells after 24 hours in culture was analysed by their ability to exclude trypan blue.

### Detection of apoptosis

Apoptosis of the ureteric epithelial cells in hanging drop cultures was

analysed by the ApopTag labelling kit (Oncor) based on the TUNEL technique (in situ terminal transferase end-labelling of fragmented DNA; Gavrieli et al., 1992) according to the manufacturer's instructions (with some modifications). Ureteric buds, after 24 hours in hanging drop culture with or without GDNF, were placed on filters and immobilised with a drop of 2% agarose. Tissues were fixed as whole mounts in 10% formalin, washed in PBS and post-fixed with

**Fig. 2.** The effect of GDNF on branching of the ureteric epithelium in metanephric kidney and urogenital explants. Cell-type markers: cytokeratin-8 (red, branches of the ureter bud and Wolffian duct) and brush border epitopes (green, secretory nephrons) (A,B), rhodamine-conjugated DB-lectin (branches of the ureter bud and Wolffian duct, C-F) and L1 neural cell adhesion molecule (branches of the ureter bud, Wolffian duct and neuronal cells, G) and cytokeratin-18 (branches of the ureter bud and Wolffian duct, H). (A) A metanephric kidney from E<sub>r</sub>13 cultured with BSA-soaked bead with a normal branch pattern after two days in culture. (B) Distortion of branching around a GDNF-soaked bead in a corresponding kidney explant. The nearby branches are distorted and show irregular branching pattern. (C) Induction of a new bud (arrow) from the Wolffian duct in the caudal mesonephric area. (D) Induction of a heterologous, abnormally broad bud from the cranial mesonephric area by a GDNF-soaked bead in an E<sub>r</sub>13 urogenital explant after 2 days in culture. (E) Failure to induce supernumerary buds by a HGF-soaked and (F) TGF $\beta$ 1-soaked bead from the E<sub>r</sub>13 Wolffian duct. (G) GDNF-soaked beads induce no supernumerary buds from Wolffian ducts in the mice deficient for cRet. (H) Corresponding wild-type mouse explant showing supernumerary buds from the Wolffian duct (arrow), similar to those seen in rat. Note that also the nearest branches from the ureteric epithelium (\*) are directed towards the beads. Ub, tip of the ureteric epithelium; Wd, Wolffian duct. Beads are surrounded with dashed lines. Bars 100  $\mu$ m (A,B), 200  $\mu$ m (C-F), 80  $\mu$ m (G,H).





95% ethanol/5% acetic acid for 30 minutes. Thereafter the manufacturer's step-by-step protocol was followed. The samples were double-stained with fluorescein-conjugated goat anti-digoxigenin (Boehringer Mannheim) and monoclonal antibodies against cytokeratin-8, followed by rhodamine-conjugated anti-mouse IgG antibodies (Jackson Laboratories).

### Electron microscopy

For electron microscopy, isolated ureteric buds, grown for 24 hours in hanging drop cultures and thereafter glued on Nuclepore filters with agarose, and freshly isolated E<sub>r</sub> 13 kidneys, were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. After ethanol dehydration, the samples were embedded in LX-112 resin (Ladd Research Industries Inc.), sectioned and examined in a Jeol 1200 EX electron microscope.

## RESULTS

### GDNF binds selectively to the tips of the ureteric bud epithelium

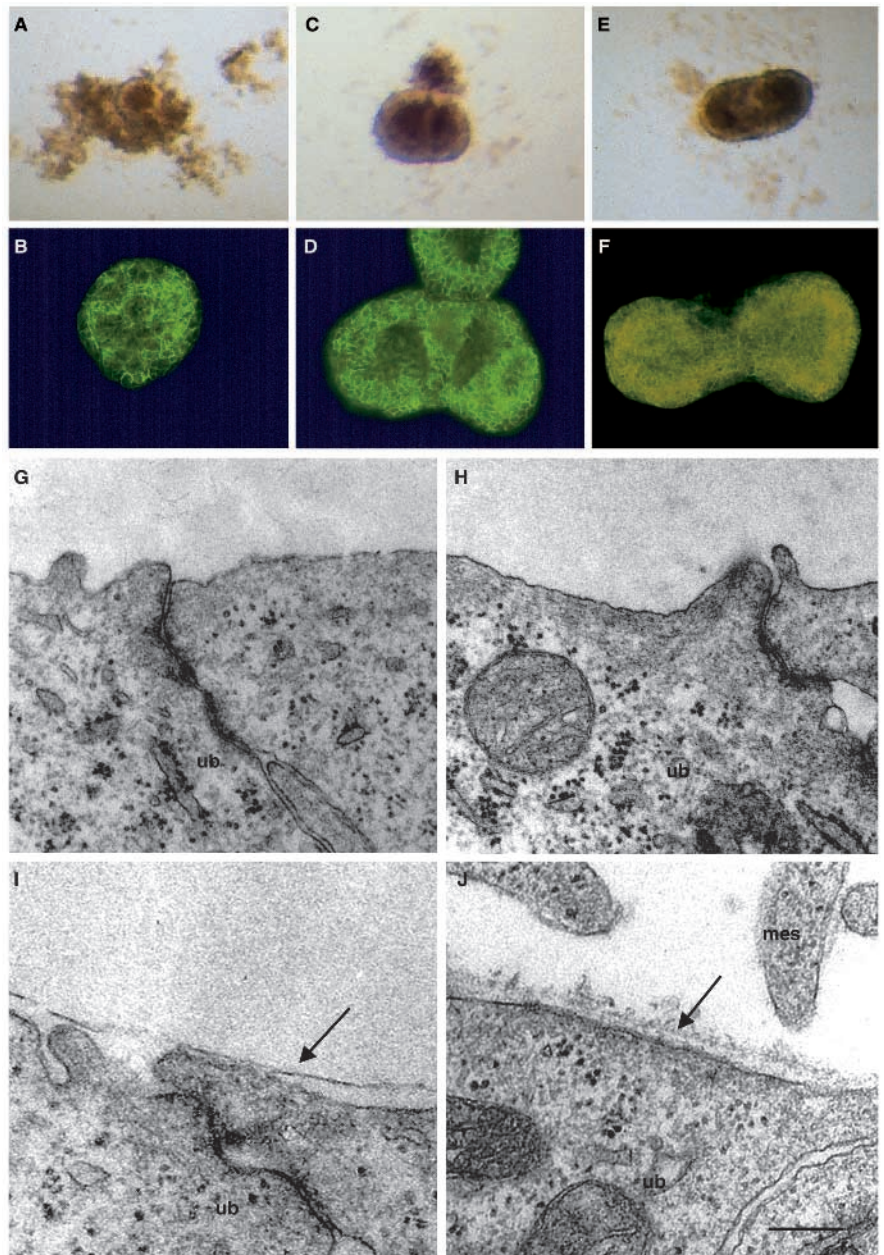
Because the published data contain some contradictory conclusions on the distribution of GDNF receptors in the embryonic kidney, we compared the expression patterns of cRet, GDNFR- $\alpha$  and GDNF mRNAs by *in situ* hybridisation (Fig. 1). cRet mRNA was located to the tips of the branches of the ureteric bud (Fig. 1A,B; Pachnis et al., 1993), but was not expressed by the nephrogenic mesenchyme or by its derivatives, as reported by Liu et al. (1996). The  $\alpha$ -receptor (Fig. 1C,D) showed an expression pattern that overlapped both GDNF (Fig. 1E,F; see also Hellmich et al., 1996; Suvanto et al., 1996) and cRet, being expressed in condensing pretubular mesenchyme, in early secretory nephrons and in the tips of the ureteric epithelium.

<sup>125</sup>I-GDNF bound selectively to the tips of the ureteric bud branches (Fig. 1G,H), and the binding could be competed out completely by a 250-fold excess of unlabelled GDNF (Fig. 1H, insert). Very little binding was detected in the condensing nephrogenic mesenchyme and none in other regions of the kidney, for instance in subcapsular uninduced mesenchyme and smooth muscle layer of ureteric pelvis, where GDNFR- $\beta$  is expressed (see Suvanto et al., 1997).

### GDNF promotes ureteric budding from the Wolffian duct and affects ureteric branching

Growth factor-soaked agarose beads were placed as follows: (1) next to early E<sub>m</sub>11 metanephric rudiments prior to ureteric budding from the Wolffian ducts; (2) next to E<sub>m</sub>11 and E<sub>r</sub>13 kidney rudiments that had already formed a small ureteric bud from the

Wolffian duct; (3) next to corresponding whole urogenital explants. In embryonic rat kidneys already possessing a branching ureteric bud, the GDNF-beads, soaked in concen-



**Fig. 3.** The effect of GDNF on the epithelial morphology and adhesiveness of two ureteric buds growing in hanging drop culture (at 24 hours). Stereomicroscopic images (A,C,E), whole-mount immunohistochemistry for L1 neural cell adhesion molecule (B,D,F) and electron microscopic analysis (G-J). Note that A and B depict only one unfused bud from the control culture, C and D two fusing buds growing with 50 ng/ml of GDNF, and E and F two fusing buds growing with 50 ng/ml HGF. (A) Two ureteric buds without exogenous growth factors are shedding cells. (B) L1 staining shows the disorganised pattern of a bud remnant without exogenous growth factors. (C,D) The two buds growing in GDNF or (E,F) in HGF, maintain their epithelial, balloon-shaped morphology and regularly fuse in the hanging drop. (G) Transmission electron microscopic analysis of ureteric buds growing without exogenous growth factors or (H) with 50 ng/ml of HGF, shows tight junctions but no basal lamina. (I) In 50 ng/ml of GDNF the bud is surrounded by a thin basal lamina (arrows), (J) resembling lamina densa at the tip of a normal ureteric bud. ub, ureter bud. Bars 200  $\mu$ m (A,C,E) 400  $\mu$ m (B, D,F), 200 nm (G-J).

trations of 50 to 100 ng/ $\mu$ l of GDNF, distorted the branch pattern by expanding the diameter of the nearby branches (Fig. 2A,B). They also induced the formation of supernumerary buds from the metanephric (prelocal) segment of the Wolffian duct (average number of buds 2,  $n=50$ ) (Fig. 2C) and from the segments outside the metanephric field (the cranial mesonephric segment), where the Wolffian duct is not normally budding (Fig. 2D; see Sainio et al., 1997 for the morphology of Wolffian duct in mesonephros region). The average number of heterologous buds in the mesonephric segment was three ( $n=50$ ). Most (85%) of the supernumerary buds were directed towards the GDNF-soaked beads. Only occasional, small supernumerary buds were induced by beads soaked in 10 ng/ $\mu$ l of GDNF. Beads soaked in 1% BSA ( $n=41$ ), or 1, 10 or 100 ng/ $\mu$ l of TGF $\beta$ 1 ( $n=15$ ) or HGF ( $n=15$ ) induced neither supernumerary budding from the Wolffian duct nor affected the number of branches in late embryonic kidneys (Fig. 2E,F), but beads soaked above concentrations of 10 ng/ $\mu$ l of TGF $\beta$ 1 and HGF did increase the length of the normal ureteric branches, as already described (data not shown; see Ritvos et al., 1995; Woolf et al., 1995; Davies et al., 1995). When urogenital blocks from mouse embryos deficient for cRet (Schuchardt et al., 1994) were cultured with GDNF-soaked beads, the Wolffian duct failed to show supernumerary budding in any segment (Fig. 2G,H), and the occasional ureteric buds present in these embryos showed no distortion of their branches by GDNF.

We next tested the effect of GDNF on isolated nephrogenic mesenchymes. 10–50 ng/ml of GDNF, in a chemically defined or serum-supplemented culture medium, induced neither epithelial differentiation nor any other morphological change (data not shown).

#### GDNF increases cell adhesion, but does not act as a mitogen upon ureteric cells

To determine the immediate effect of GDNF on ureteric epithelium, pairs of isolated ureteric buds were grown in hanging drop cultures with or without GDNF, HGF or TGF $\beta$ 1. Under these conditions without a supportive matrix, ureteric buds did not form branches in response to any of the growth factors. Control buds remained small and shed cells so that their epithelial morphology was disrupted (Fig. 3A,B). However, in the presence of GDNF, the two buds in one hanging drop soon fused together, shed only a few cells and retained their epithelial morphology (Fig. 3C,D). The effect of HGF in hanging drops was similar to that of GDNF (Fig. 3E,F). The percentage of scattered cells in the bud cultures was 60, 10 and 10% in control, GDNF and HGF cultures, respectively. TGF $\beta$ 1 response was characterised by complete dissociation and extensive death of the cells, if TGF $\beta$ 1 was applied at concentrations above of 1 ng/ml (data not shown). Below that concentration, TGF $\beta$ 1 did not have any effect on bud fusion, bud morphology or scattering of cells. TUNEL-labelling of the ureteric buds in GDNF- or HGF-supplemented hanging drop cultures showed a decrease in the number of apoptotic cells as compared to buds grown in control medium or buds supplemented with TGF $\beta$ 1 (data not shown).

The morphology of separated buds grown in hanging drop cultures with or without GDNF or HGF was further analysed by electron microscopy (Fig. 3G–I). The buds, grown in control medium, showed only few tight junctions and no basal lamina (Fig. 3G). In the buds grown with HGF or GDNF, the cells

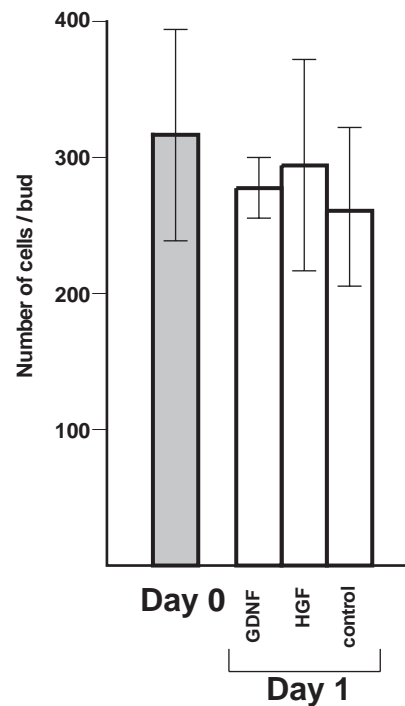
showed well-developed tight junctions (Fig. 3H,I), but only with GDNF, a thin basal lamina (Fig. 3I), which resembled the lamina densa of the basement membrane at the tip of normal ureteric buds (Fig. 3J).

BrdU-labelling of the epithelial cell clusters remained low with or without GDNF (data not shown), indicating that GDNF does not primarily act as a mitogen upon the ureteric epithelial cells. This result was verified by counting the number of bud cells immediately after the microdissection from the embryo and after 24 hours of hanging drop culture with or without GDNF or HGF (Fig. 4). Most cells in control, GDNF- and HGF-supplemented cultures were viable after 24 hours and excluded trypan blue. Cells from TGF $\beta$ 1 cultures were not counted because of the total dissociation and extensive death of cells seen with TGF $\beta$ 1. The results in Fig. 4 show that, without a proper mesenchymal support, neither GDNF nor HGF promoted proliferation of ureteric cells.

A well-known culture model for epithelial morphogenesis is the branching growth of MDCK cell cysts in collagen matrices enriched with HGF (Montesano et al., 1991a). To compare the behaviour of normal ureteric buds with that of the MDCK cells, we cultured isolated ureteric buds with or without GDNF and HGF in rat tail collagen gel, as described for MDCK epithelial cells (Montesano et al., 1991b). Neither of these growth factors induced branching of the ureteric epithelium under these conditions (data not shown).

#### GDNF expression and ureteric branching are downregulated after desulphation of extracellular matrix glycosaminoglycans

Present and previous work on ureteric bud development indicate that the processes of growth and branching may be



**Fig. 4.** The number of cells in a ureteric bud from E<sub>13</sub> kidney (day 0,  $n=24$ ) grown for 24 hours (day 1) with or without 50 ng/ml of GDNF or HGF ( $n=20$  in each media). No difference is seen in the cell number in any media.

controlled separately (Davies et al., 1995). Both can be inhibited completely when kidney rudiments are deprived of sulphated glycosaminoglycans (S-GAGs), by treatment with either chlorate ions (inhibitors of sulphation) or degradative enzymes such as heparitinase and chondroitinase, and they can be rescued apparently independently when these S-GAG-deprived rudiments are treated with exogenous factors. HGF will restore growth but not branching and, while no growth factor that can rescue branching has yet been reported, treatment of S-GAG-deprived kidneys with the phorbol ester, PMA, will activate branching but not growth (Davies et al., 1995). We used the protocols of the experiments described above to determine whether GDNF can activate ureteric bud growth, branching or both in S-GAG-deprived kidneys.

GDNF and HGF was added either to the medium or applied by growth factor-soaked beads to the cultures. Kidney rudiments were cultured for approximately 72 hours in normal medium or in two types of media designed to cause S-GAG deprivation (20 mM sodium chlorate or heparitinase-chondroitinase ABC in normal culture medium). GDNF, but not cRet, was downregulated in desulphated kidneys as shown by western blotting analysis (Fig. 5A). Ureteric epithelia of kidneys deprived of S-GAGs, through growth in desulphating media, showed neither extensive growth nor branching (Fig. 5B). Treatment of S-GAG-deprived kidney rudiments with 50 ng/ml HGF stimulated ureteric growth but not branching, as shown earlier (Davies et al., 1995). Addition of GDNF partially restored the morphogenesis of ureteric buds, causing branching but only little growth (Fig. 5C). Simultaneous treatment of S-GAG-deprived rudiments with 50 ng/ml GDNF and 100 ng/ml HGF did not extend significantly the effect that was seen with GDNF alone (Fig. 5D). When the GDNF-containing beads were used in desulphated kidneys, branching was slightly more extensive than with GDNF added in the medium and the branches were often directed towards the bead (Fig. 5E). Beads soaked in 1% BSA showed no effect (data not shown).

### Branching of early ureter bud in recombination cultures is dependent on mesenchyme

Thus far, the only embryonic mesenchymes that have been reported to support growth and branching of ureteric bud epithelium have been metanephrogenic (Grobstein, 1955; Saxén, 1987) and lung mesenchymes (Kispert et al., 1996). To discover if this specificity is mediated by GDNF, we tested the effects of GDNF on early bud-stage E<sub>r</sub>13 rat ureteric epithelium that was recombined with isochronic heterologous mesenchymes from embryonic mouse or rat lung, salivary gland, limb bud, tooth or gut.

When recombined with lung mesenchyme, which expresses GDNF mRNA when tested by RT-PCR (data not shown) and GDNFR- $\alpha$  by in situ hybridisation (data not shown, Suvanto et al., 1997), the ureteric bud branched in a manner that correlated with the dose of exogenous GDNF (Table 1, Fig. 6A). Also, exogenous TGF $\beta$ 1 and HGF added to the culture medium of ureteric bud-lung mesenchyme recombinations triggered branching (Table 1, Fig. 6B,C). If the ureteric epithelium was microdissected later, at the T-bud-stage when the first two ureteric branches were already present, and recombined with heterochronic E<sub>r</sub>13 lung mesenchyme, ureteric branching was regularly promoted without any exogenous growth factors (see Kispert et al., 1996).

**Table 1. Branches produced as a dose response to GDNF, TGF $\beta$ 1 and HGF in lung mesenchyme-ureteric bud recombination cultures**

	Concentration of growth factor (ng/ml)				
	1	10	25	50	100
GDNF	2	3	5	5	7
HGF	0	3	3	5	5
TGF $\beta$ 1	1	3	4	2	0

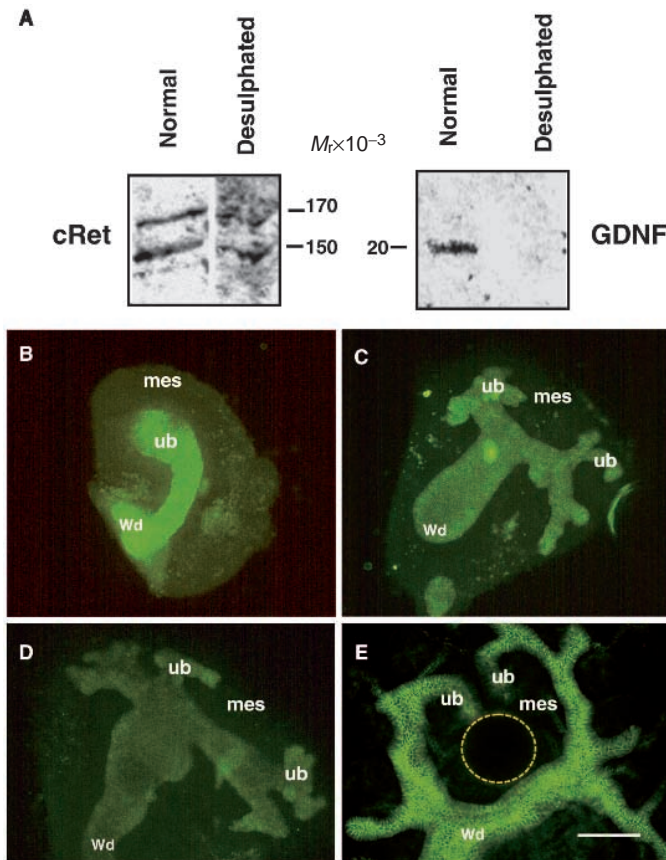
Values are average numbers of branches; the number of samples at each dose is 8.

When E<sub>r</sub>13 ureteric buds were recombined with isochronic mesenchymes from limb or tooth, the buds did not branch even if treated with GDNF, TGF $\beta$ 1 or HGF. In limb mesenchyme recombinations the ureteric bud did not form branches, with or without exogenous growth factors (Fig. 6D). In salivary gland mesenchyme recombinations, the ureteric buds maintained a rudimentary epithelial shape but did not elongate (Fig. 6E). Because gut is a rich source of GDNF (Hellmich et al., 1996; Suvanto et al., 1996), various segments and sizes of gut mesenchyme were recombined with ureteric buds, but they did not promote ureteric bud branching, not even if exogenous GDNF up to 50 ng/ml was added. All these mesenchymes express GDNFR- $\alpha$  mRNA by in situ hybridisation (data not shown, Suvanto et al., 1997). Branching of the late T-bud-stage ureteric epithelium was not supported by these heterologous mesenchymes either (data not shown).

## DISCUSSION

We have used various experimental approaches to analyse the target cell types and mode of action of GDNF in the metanephric kidney. Our data show first, that GDNF is preferentially or solely bound to the tips of the ureteric buds; second, that GDNF can induce ureteric bud formation from the Wolffian duct not only in the metanephric area, but also from the Wolffian duct segments outside the metanephric zone. The promotion of epithelial budding and branching morphogenesis requires cRet, and it does not take place in kidney cultures from cRet-deficient mice by GDNF-beads; third, that GDNF is a mesenchyme-derived signal that acts in a dosage-dependent manner on the epithelial target tissue; fourth, that the primary response to GDNF is not mitogenic and, in the hanging drop culture, it is characterised by decreased apoptosis, increased adhesiveness, secretion of basal lamina and maintenance of the polarisation of the ureteric cells. Although the maintenance and cell adhesion responses are directly mediated by GDNF, the branching response to GDNF is completely dependent on unknown mesenchyme-derived effector(s), possibly on mesenchyme-derived extracellular matrix molecules or growth factors. These growth-promoting signals are also provided to some extent by lung mesenchyme, but not the other mesenchymes tested. The tissue recombination data show further that the ureteric branching can be triggered by TGF $\beta$ 1 and HGF, which implies redundancy in the regulation of ureteric differentiation. However, unlike GDNF, these growth factors



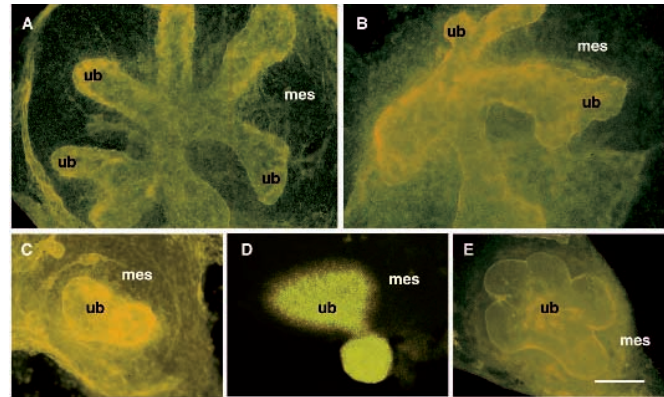


**Fig. 5.** Induction of branching by GDNF in desulphated kidneys where normal branching is disrupted. All explants were cultured for 3 days. Markers for ureteric buds and Wolffian duct: calbindin-D-28k (B-D) and L1 neural cell adhesion molecule (E). (A) Western blotting analysis with antibodies against mouse cRet and GDNF shows that GDNF (right), but not cRet (left), is down-regulated in desulphated kidneys treated in chlorate. (B) With desulphation ureteric branching morphogenesis is disrupted. (C) 50 ng/ml GDNF promotes short branches in desulphated kidneys. (D) A kidney with desulphation and 50 ng/ml GDNF and 100 ng/ml HGF. (E) A desulphated kidney grown with GDNF-soaked agarose bead. Note that two nearby branches are directed towards the bead. ub, ureteric bud; mes, metanephric mesenchyme; Wd, Wolffian duct. Bar, 250  $\mu$ m.

do not direct the growth of the branches and do not promote budding from Wolffian ducts.

Thus, GDNF promotes ureteric morphogenesis by priming the Wolffian duct for bud initiation, perhaps by increasing cell adhesion in the target tissue and inducing wnt-11 expression that is, besides GDNF and cRet, also required for ureteric branching morphogenesis (Kispert et al., 1996). Thereafter, bud elongation may be promoted by other mesenchyme-derived effectors that may include HGF, TGF $\beta$ 1 and extracellular matrix molecules. The developmental sequence of GDNF and wnt-11 remains to be solved.

In developing kidneys, GDNF is expressed exclusively by the pretubular metanephric mesenchyme. It becomes bound to its target tissue, the tips of the ureteric epithelium, where new branches of the collecting ducts are continuously being formed. Although one of its receptors, the GPI-linked protein GDNFR-



**Fig. 6.** The effects of GDNF and HGF on ureteric branching in E<sub>13</sub> ureteric bud-heterologous mesenchyme explants. Cell-type markers: laminin (A, B, C and E) and DB-lectin (D). (A) Lung mesenchyme-ureteric epithelium recombination after 48 hours in culture with 50 ng/ml GDNF shows extensive ureteric branching. Note that no secondary branches are seen. (B) Similar explant with 50 ng/ml HGF. (C) Lung mesenchyme-ureter bud recombination without exogenous growth factors shows no branches. (D) Limb mesenchyme-ureteric bud recombination with 50 ng/ml GDNF. (E) Salivary mesenchyme-ureteric bud recombination with 50 ng/ml GDNF. ub, ureteric bud; mes, mesenchyme. Bar, 200  $\mu$ m.

$\alpha$  (Jing et al., 1996; Treanor et al., 1996), is expressed in both metanephric mesenchyme and ureteric bud, we could verify GDNF binding only to the tips of the ureteric branches where the cRet binding site is expressed. Furthermore, metanephric kidneys of mice deficient for cRet (Schuchardt et al., 1994, 1996) did not respond to GDNF. These data show that the ureteric epithelium is the main target cell type of GDNF in the embryonic kidney.

Previous studies with GDNF-deficient mice (Pichel et al., 1996; Treanor et al., 1996) and with neutralising antibodies to GDNF (Vega et al., 1996) have shown that GDNF is necessary for development of the ureteric bud. In our experiments, supernumerary budding from the Wolffian duct was induced by GDNF-soaked agarose beads, initially suggesting that GDNF could act as a mitogen upon the epithelial cells. However, isolated ureteric buds did not respond to GDNF by increasing cell proliferation, but by maintaining their epithelial morphology, showing increased adhesiveness and extracellular matrix synthesis. This observation is supported by the data by Liu et al. (1996), who showed that cRet modulates extracellular matrix synthesis by ureteric cells. Still, the mechanism and mediators of the increased cell adhesion remain to be elucidated. They may include wnt-11, a member of wnt family of signal transducing molecules that could be upregulated by GDNF in the Wolffian duct. Interestingly, wnts regulate cell adhesion and signal transduction through cadherins and catenins (reviewed by Miller and Moon, 1996) and cRet has a cadherin-like domain in its extracellular part (reviewed by Takeichi, 1993) that might be involved in the cell adhesion/signal transduction response.

Thus far the only mesenchymes that have been shown to support ureteric growth and branching are metanephrogenic and lung mesenchymes (Grobstein, 1955; Saxén, 1987; Kispert et al., 1996). We have recombined various heterologous mesenchymes with isolated early ureteric buds and added GDNF.



None of the mesenchymes tested supported the development of early ureteric buds without exogenous GDNF. The lung mesenchymes supplemented by GDNF-containing medium or beads supported the branching of the early ureteric buds, showing that GDNF can promote branching when combined with a competent heterologous mesenchyme. It has been shown that lung mesenchyme induces ureteric branching and maintains *wnt-11* expression of the tips of the ureteric bud (Kispert et al., 1996). We have repeated these experiments and show that in our culture system the branching response is critically dependent on the stage of the bud. Only late, T-shaped buds undergo branching without exogenous GDNF.

The competence of heterologous mesenchymes to support the GDNF action turned out to be very restricted. Tooth, salivary and limb mesenchymes did not support ureteric branching even with GDNF (although limb mesenchymes, for instance, show endogenous GDNF expression; Wright and Snider, 1996). Gut mesenchyme, a rich source of GDNF, did not support ureteric branching. These findings suggest that either gut, salivary gland, tooth and limb mesenchymes all lack a factor essential for ureteric branching or that they inhibit the GDNF response, for example by competing out GDNF from the ureteric binding. The latter alternative is unlikely, because GDNF tested up to the concentration of 50 ng/ml did not promote branching in the gut mesenchyme recombinations. The most plausible explanation is that these mesenchymes lack effectors, so far unidentified, for bud elongation. These molecules may not only represent the GPI-linked GDNF receptors, because all mesenchymes tested in the recombination assays express either GDNFR $\alpha$  or GDNFR $\beta$  (Treanor et al., 1996; Baloh et al., 1997; Suvanto et al., 1997). Our data underline further the necessity for many simultaneously acting effectors to promote normal branching morphogenesis.

In ureteric bud-lung mesenchyme recombination cultures, the branching response of ureteric bud was promoted not only by GDNF, but also by TGF $\beta$ 1 and HGF. However, unlike mice lacking GDNF (Pichel et al., 1996; Sánchez et al., 1996), mutant mice lacking TGF $\beta$ 1 or HGF (Shull et al., 1992; Schmidt et al., 1995) show no apparent defects in kidney morphogenesis, suggesting that these molecules are not necessary to, or redundant in, kidney development in vivo. Tissue culture studies (Ritvos et al., 1995; Woolf et al., 1995), antibody inhibition experiments (Woolf et al., 1995) and studies with desulphated kidneys (Davies et al., 1995) have indicated roles for these growth factors in regulation of ureteric growth. Our data suggest that they act later than GDNF, because they did not, unlike GDNF, initiate buds from the Wolffian duct inside or outside the normal nephrogenic area.

Sulphated GAGs of the extracellular matrix are important to ureteric bud growth and branching. The ureteric buds of kidneys deprived of sulphated GAGs show neither growth nor branching. HGF has been reported to elongate ureteric epithelium in desulphated kidneys (Davies et al., 1995) and antibodies to HGF inhibit kidney morphogenesis in organ culture (Woolf et al., 1995). We therefore tested the ability of GDNF and HGF to restore ureteric morphogenesis to kidney rudiments deprived of sulphated GAGs. Ectopic GDNF does indeed initiate ureteric budding in kidneys deprived of sulphated GAGs, but new branches grew only very little. Because HGF did not significantly elongate the branches when

added together with GDNF, unidentified growth-promoting molecules may act between GDNF and HGF responses.

Accordingly, we could not induce branching of the isolated ureteric buds, deprived of mesenchymal support in hanging drop or collagen gel cultures, with GDNF, HGF or TGF $\beta$ 1. This further suggests that additional growth factors or sulphated GAGs from the mesenchymal cells are needed to complete ureteric morphogenesis. Sulphated GAGs in the extracellular matrix may be needed for GDNF synthesis or to attach GDNF to the matrix, since in desulphated kidneys GDNF protein levels were downregulated, although the mesenchyme is induced and differentiates (Davies et al., 1995).

Activation of the cRet receptor tyrosine kinase is mitogenic for some cells (Santoro et al., 1994). In neuroblastoma cell lines, for example, cRet utilises the MAP-kinase signalling pathway to activate cell proliferation (Worby et al., 1996). We evaluated the possible mitogenic effects of GDNF on ureteric epithelial cells growing without a supportive matrix or mesenchyme. In these hanging drop cultures GDNF was not mitogenic, but it did enhance survival, cell polarisation and adhesion of the bud cells. Presumably, the mitogenic effects of GDNF in the kidney, described earlier by Vega et al. (1996), are indirect and due to other mesenchyme-derived effectors acting together with or downstream to GDNF.

There is an interesting precedent for branching processes that do not depend on cell proliferation. The tracheal network and Malpighian tubules of developing *Drosophila* embryos are epithelial structures that undergo tube formation and branching morphogenesis analogous to mammalian kidney morphogenesis. In these organs bud formation and branching do not require cell proliferation, but rather they are based on cell migration, elongation and cell adhesion. *Drosophila* E-cadherin has been associated to tracheal and Malpighian tubule formation (Uemura et al., 1996). *Drosophila* fibroblast growth factor (DFGF) and its receptor *breathless* (Reichman-Fried and Shilo, 1995), as well as a TGF $\beta$  superfamily member *decapentaplegic* (Affolter et al., 1994), guide the migration of tracheal cells during branching morphogenesis. Our present data suggest that the central events taking place during the initiation of ureteric branching morphogenesis might be similar to those of *Drosophila* tracheal network and Malpighian tubule initiation.

Several GPI-anchored proteins are known to mediate signals for cell adhesion. Since the GDNF-binding data did not reveal GDNFR- $\alpha$  or GDNFR- $\beta$  as major GDNF-binding proteins in the kidney, they might have other functions unrelated to growth factor binding. One GPI-linked protein, F3, interacts in mouse cerebellum with neural cell adhesion molecule L1 (Olive et al., 1995), a molecule that is also expressed on the ureteric epithelium (Sainio et al., 1994). The possibility that GDNFR- $\alpha$  or GDNFR- $\beta$  might have similar functions can only increase interest in the roles of GDNF receptors in cell adhesion. We would like to stress, however, that our results do not contradict the suggested interplay model between cRet and GDNFR- $\alpha$  (Jing et al., 1996; Treanor et al., 1996) or GDNFR- $\beta$  (Baloh et al., 1997; Suvanto et al., 1997) in GDNF signalling.

Although GDNF did not act primarily as a mitogen on isolated bud cells, the ureteric epithelial cells were mostly directed towards the beads in explant cultures. GDNF seems to determine the direction of growth from the target epithelia. This response may be mediated by a GDNF gradient from the pretubular mesenchyme acting most efficiently on the nearby

epithelial cells. This would require a clear dosage-dependent mode of action of GDNF. We could verify it in both tissue recombination and urogenital block cultures, in which all other conditions, except the concentration of GDNF, remained the same. In urogenital block cultures, the directed growth was observed not only in the metanephric region but also in the more cranial segments of the Wolffian duct. These data suggest that, if also other mesenchyme-derived factors would also determine the orientation of buds, they should be general and permissive in nature, such as mitogenic growth factors or extracellular matrix molecules.

In summary, GDNF fulfils the criteria for being an important kidney inducer that acts early in the initiation of ureteric bud development. The growth factor shows expression and binding patterns compatible with its apparent role in the kidney, and it has a clearly defined function in the ureteric morphogenesis. HGF and TGF $\beta$ 1 show, in part, similar biological effects on ureteric epithelium, but they lack some specific characteristics of GDNF. They do neither induce bud formation from the Wolffian duct nor promote the basal lamina synthesis by the ureteric bud. These differences suggest that they act downstream to GDNF and represent the expanding set of mesenchyme-derived growth factors with overlapping, partially redundant developmental functions.

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### Note added to proof

A new nomenclature for GPI-linked receptors for the GDNF ligand family is agreed by GFR $\alpha$  Nomenclature Committee chaired by Carlos F. Ibáñez and is published in *Neuron* 19, September issue, 1997. According to the new nomenclature the GDNF- $\alpha$  and GDNF- $\beta$  are renamed GDNF Family Receptor Alpha-1 and GDNF Family Receptor Alpha-2, respectively.

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