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Proteoglycans are required for maintenance of *Wnt-11* expression in the ureter tips

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SUMMARY

Development of the metanephric kidney requires the concerted interaction of two tissues, the epithelium of the ureteric duct and the metanephric mesenchyme. Signals from the ureter induce the metanephric mesenchyme to condense and proliferate around the ureter tip, reciprocal signals from the mesenchyme induce the ureter tip to grow and to branch. *Wnt* genes encode secreted glycoproteins, which are candidate mediators of these signaling events. We have identified three *Wnt* genes with specific, non-overlapping expression patterns in the metanephric kidney, *Wnt-4, Wnt-7b* and *Wnt-11*. *Wnt-4* is expressed in the condensing mesenchyme and the comma- and S-shaped bodies. *Wnt-7b* is expressed in the collecting duct epithelium from 13.5 days post coitum onward. *Wnt-11* is first expressed in the nephric duct adjacent to the metanephric blastema prior to the outgrowth of the ureteric bud. *Wnt-11* expression in *Danforth's short-tail* mice suggests that signaling from the mesenchyme may regulate *Wnt-11* activation. During metanephric development, *Wnt-11* expression is confined to the tips of the branching ureter. Maintenance of this expression is independent of Wnt-4 signaling and mature mesenchymal elements in the kidney. Moreover, *Wnt-11* expression is maintained in recombinants between ureter and lung mesenchyme suggesting that branching morphogenesis and maintenance of *Wnt-11* expression are independent of metanephric mesenchyme-specific factors. Interference with proteoglycan synthesis leads to loss of *Wnt-11* expression in the ureter tip. We suggest that Wnt-11 acts as an autocrine factor within the ureter epithelium and that its expression is regulated at least in part by proteoglycans.

Key words: Wnt, kidney development, metanephros, proteoglycans, Danforth's short-tail, mouse

INTRODUCTION

The development of vertebrate organs requires intricate cell-cell communications to assure the concerted program of cell growth, differentiation and morphogenesis. Renal development is characterized by an interaction between an epithelial and a mesenchymal compartment: the nephric (Wolffian) duct and the nephrogenic mesenchyme, both derived from the intermediate mesoderm. In mammals, this interaction results in the formation of three embryonic kidneys, the pronephros, the mesonephros and the metanephros, the latter of which is responsible for removal of nitrogenous waste and physiological control of salt balance.

Metanephric development in the mouse begins when the ureteric bud, an outgrowth of the nephric duct, contacts the undifferentiated metanephric blastema around 10.5 days post coitum (days p.c.). Signals from the ureter induce the metanephric mesenchyme to condense and proliferate around the ureter tip, whilst reciprocal signals from the mesenchyme induce the ureter tip to grow and branch (Grobstein, 1953, 1955). The induced mesenchyme then aggregates and undergoes an epithelial transformation. Cytodifferentiation and interaction with the vasculature results in the formation of the glomerular and tubular epithelia of the nephron. In turn, the ureter gives rise to the collecting duct system of the kidney (for reviews see Saxen, 1987; Bard et al., 1994; Patterson and Dressler, 1994).

The development of transfilter culture techniques using isolated metanephric mesenchyme has allowed the partial characterization of the requirements for ureter morphogenesis and metanephric induction. Organ culture experiments have demonstrated that the extracellular matrix is required for branching morphogenesis in the kidney (Ekblom, 1981; Roskelly et al., 1995). Chemicals known to interfere with proteoglycan metabolism partially or completely abolish ureter morphogenesis (Davies et al., 1995; Klein et al., 1989; Lelongt et al., 1988; Platt et al., 1987). A similar result was reported when polyanionic compounds were added to kidney cultures (Ekblom et al., 1978). Further, neonatal mouse kidney epithelial cells undergo morphogenesis in matrigel supplemented with FGF (Traub et al., 1990), and Madin-Darby canine kidney epithelial cells can form tubules in collagen gel in the presence of hepatocyte growth factor (Montesano et al., 1991).

The induction by the ureter and other heterologous inducers,

most notably spinal cord, depends on cell contacts. The induction process itself is relatively slow and cannot be transferred by induced mesenchymal cells to uninduced cells (Grobstein, 1953, 1955, 1956; Saxen and Lehtonen, 1978; Saxen et al., 1976; Unsworth and Grobstein, 1970; Wartiovara et al., 1974). Although many growth factors have been identified in the kidney (Hammerman, 1995), none of these has been shown to individually induce tubulogenesis. A combination of FGF2 and a pituitary extract can induce tubulogenesis, suggesting that tubule induction is a multistep process mediated by soluble and insoluble factors (Perantoni, 1991; Perantoni et al., 1995).

Recently, it was shown that members of the *Wnt* family are both sufficient and necessary for tubule development. *Wnt-4* is expressed in the condensing mesenchyme and the comma- and S-shaped bodies in the metanephric kidney. Gene targeting in ES cells has demonstrated that Wnt-4 acts as an autoinducer of mesenchymal aggregation (Stark et al., 1994). Cells expressing *Wnt-1* can induce tubule formation in isolated metanephric mesenchyme (Herzlinger et al., 1994). *Wnt-1* is not expressed in the kidney, but is expressed in the dorsal spinal cord, the strongest known inducer of tubulogenesis (Wilkinson et al., 1987). It is conceivable that *Wnt-1* mimics the activity of a *Wnt* family member specifically expressed in the kidney, either *Wnt-4* in the mesenchyme or an unknown *Wnt* in the ureter.

In order to gain insight in the complexity of *Wnt* gene function in the developing kidney, we have systematically analyzed expression of all available members of the mouse *Wnt* gene family (14 in total) (Lee et al., 1995) in the metanephric kidney. Here we report that, in addition to *Wnt-4* (Stark et al., 1994), *Wnt-7b* and *Wnt-11* show specific sites of non-overlapping expression. *Wnt-7b* is expressed in the maturing collecting ducts, while *Wnt-11* expression is restricted to the ureter tips. We provide evidence that activation of *Wnt-11* expression in the nephric duct depends on signals from the metanephric blastema. Maintenance of expression in the ureter tips, however, is independent of specific metanephric mesenchymal signals as *Wnt-11* expression persists as the ureter undergoes morphogenesis in heterotypic mesenchyme. Proteoglycans appear to play a major role in the cell or matrix interactions that regulate *Wnt-11* expression.

MATERIALS AND METHODS

Mouse stocks

Mice heterozygous for the *Danforth's short-tail* (*Sd*) mutation were purchased from the Jackson Laboratory. The mutation was maintained on the background RSV/Le *Re*/*Re Sd*/+ *Va*/+, (*re-rex, Va-varitint waddler*). Heterozygotes were identified by an absent or rudimentary tail. *Wnt-4* heterozygotes were derived and genotyped as described previously (Stark et al., 1994). Embryos for the analysis of the normal pattern of *Wnt-11* expression and for kidney dissections were derived from matings of Swiss Webster wild-type animals (purchased from the Jackson Laboratory). For timed pregnancies, plugs were checked in the morning after mating, noon was taken as 0.5 days of gestation.

Isolation and sequencing of *Wnt-11* cDNAs

A *Wnt-11* cDNA fragment corresponding to the highly conserved exon 4 was cloned using a PCR amplification strategy on 9.5 days p.c. embryonic cDNA (Gavin et al., 1990). The *Wnt-11* PCR-fragment was labeled with [^{32}P]CTP by random priming (GibcoBRL Random Prime Labeling Kit) and used to screen a newborn mouse kidney cDNA library (Wada et al., 1993). Hybridization was performed under stringent conditions in Church buffer at 68°C (Church and Gilbert, 1984). Approximately 1×10^6 plaques were screened and 19 positive plaques purified. 11 independent cDNAs were subcloned into pBluescript SKII using the ExAssist System (Stratagene). cDNAs were sequenced (Sanger et al., 1977) with a Sequenase Kit (USB).

In situ hybridization

Whole-mount in situ hybridization was performed as described by Parr et al. (1993) and modified according to Knecht et al. (1995). Digoxigenin probes were synthesized using the Digoxigenin RNA Labeling Kit (Boehringer Mannheim). A probe to *Wnt-11* was generated from the full-length cDNA pnk3 by linearization with *Xho*I and transcription with T3 RNA polymerase. A probe corresponding to approximately 1.2 kb of the 3′-part of the coding region of *c-ret* was generated from the plasmid pmc-ret (a kind gift of F. Constantini) by linearization with *Bam*HI and transcription with T3 RNA polymerase. A probe corresponding to nucleotides 3612-4951 of the *c-ros* cDNA was obtained by linearizing the plasmid pmc4 (a kind gift of C. Birchmaier) with *Xba*I and transcribing with T7 RNA polymerase. Riboprobes were used at approximately 1 μg/ml.

Stained embryos, kidneys and cultures were transferred into 80% glycerol and photographed on Ektachrome Tungsten 64 color slide film (Kodak) using a Leica Wild M10 photomicroscope. Composites were generated using Adobe Photoshop v3.0 and Canvas v3.5 on a Power Macintosh.

In situ hybridization analysis on sections was performed according to published procedures (Wilkinson et al., 1987). Generation of PCR fragments used as in situ probes was described by Gavin et al. (1990). The in situ probes for *Wnt-4* and *Wnt-7b* were described before (Parr et al., 1993), the *Wnt-11* riboprobe was prepared from the PCR-fragment.

Organ culture techniques

Metanephric kidneys and lungs from 11.5 days p.c. wild-type Swiss Webster embryos were dissected in PBS. In some cases, parts of the mesonephric duct derivatives were left attached to increase the size of the specimen, which facilitated later in situ hybridization analysis. For recombination experiments, ureters were manually separated from metanephric mesenchyme, and lung mesenchyme from lung epithelium, following a 2 minute incubation in 3% pancreatin/trypsin (GibcoBRL) in Tyrode's solution. Metanephric kidneys were placed on 4-6 mm diameter 0.1 μm Nucleopore filters (Costar) supported by stainless steel grids on the surface of the culture medium (Dulbecco's modified Eagle's medium (Sigma #D5796) supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine (GibcoBRL #1273), 1× penicillin/streptomycine (GibcoBRL #0511). Medium was modified with one or more of the following chemicals: 30 mM $NaClO_3$ (Sigma #S3171), 10-100 μg/ml pentosan polysulphate (Sigma #P8275), 50 μM suramin (CB Chemicals, Woodbury, CT), 100 μg/ml chondroitin-6-sulphate from shark cartilage (ICN), 100 μg/ml low molecular weight heparin (CalBiochem), chondroitinase ABC (Sigma #C2905), heparitinase III (Sigma #H8891). In recombination experiments, lung mesenchyme was placed adjacent to one or two ureters or ureter tips on one filter. Samples were incubated in 5% CO_2 in air at 37°C. For all kidney culture experiments, a minimum of 6, in most cases 12, specimens were processed.

For in situ hybridization analysis, filters were submerged in cold methanol for 10 seconds and then fixed in 4% paraformaldehyde in PBS overnight prior to stepwise transfer into methanol and storage at −20°C.

Whole-mount β-galactosidase staining

Metanephric kidney cultures from transgenic experiments were processed for β-galactosidase activity according to published procedures (Whiting et al., 1991). The cultures were stained for periods

ranging from 30 minutes to 12 hours according to the strength of expression. The reaction was stopped by washing in PBS and post-fixation in 4% paraformaldehyde/PBS overnight. Specimen were then processed through a graded series into 80% glycerol and photographed as described.

RESULTS

Identification of *Wnt* genes with specific expression patterns in the metanephric kidney

In order to identify *Wnt* genes specifically expressed in the metanephric kidney, PCR fragments from all available mouse *Wnt* genes (14 so far) were used to generate antisense riboprobes for in situ hybridizations on sections of 14.5 days p.c. metanephric kidneys. Three *Wnt* genes with specific and non-overlapping expression domains were identified: *Wnt-4*, *Wnt-7b* and *Wnt-11* (Christiansen et al., 1995).

In the 14.5 days p.c. metanephric kidney, *Wnt-4* is expressed in the mesenchymal condensates, their simple epithelial derivatives, the comma- and S-shaped bodies, and in the centrally located stroma. *Wnt-7b* transcripts are restricted to the collecting ducts and *Wnt-11* expression to the tips of the ureter (Fig. 1). The expression and requirement for *Wnt-4* in kidney development has been addressed earlier (Stark et al., 1994), the expression of *Wnt-7b* in the collecting ducts makes it unlikely that it is involved in inductive events between the ureter and the mesenchyme. In contrast, *Wnt-11* expression correlates with sites of epithelial-mesenchymal interactions, and growth and branching morphogenesis of the ureter. *Wnt-11* is the first secreted protein with such an expression pattern making it a candidate mediator of these phenomena. We therefore focused our work on the characterization of *Wnt-11*.

In situ hybridization analysis of *Wnt-11* expression during murine development

Prior to urogenital development, we detect *Wnt-11* expression first in a punctate pattern in the posterior half of the embryo at 6.75 days p.c., where the primitive streak has formed (Fig. 2A). At 7 days p.c., two expression domains can clearly be distinguished. The node and, at the posterior end of the embryo, the extraembryonic mesoderm and base of the allantois (Fig. 2B,C). Expression in the node disappears at around 8 days p.c. whereas the expression in the posterior trunk persists in the tailbud until at least 11.5 days p.c. (Fig. 2D-H). At 7.75 days p.c. (before somite formation), we detect *Wnt-11* transcripts in the anlage of the heart, which is located rostral-lateral to the invaginating foregut anlage (Fig. 2D). Expression in the forming heart tube continues until at least 9.5 days p.c. and is confined to the myocardial layer (not shown). At around 8.25 days p.c., *Wnt-11* expression is detected in the most rostral somites at the dorsalmost aspect, probably marking the future dermatome (Fig. 2E). At 9.5 days p.c., expression is weak in the newly forming and stronger in more mature somites (Fig. 2F,G). During tail formation (11.5 days p.c.), expression is very strong in the newly forming somites at the tail tip. Rostrally, expression becomes progressively restricted to the caudal half of the somite (Fig. 2H). At 10.5 days p.c., we detect *Wnt-11* in the branchial arches (Fig. 2H and not shown). *Wnt-11* shows a particular dynamic expression pattern in the limb bud. *Wnt-11* expression accompanies the emerging forelimb bud at 9.25 days p.c. (Fig. 2G). An initially broad distal domain of staining is later confined to a distal ectodermal strip of cells identical to the apical ectodermal ridge. Finally, from 10.5 to 11 days p.c., expression is observed in the condensing skeletal rudiments (Fig. 2H) and later in the perichondrium (data not shown).

Expression in the urogenital system is first detected in the mesonephric duct at around 9 days p.c. at a very low level (Fig. 2F). Expression follows the posterior elongation of the duct (Fig. 2G). At 10.5 days p.c., the mesonephric duct has reached the position of the metanephric mesenchyme which is located at approximately the level of the hindlimb buds. At this time, we see a strong *Wnt-11* signal in the epithelium of the region of the nephric duct that is facing the metanephric mesenchyme (Fig. 3A). Thus, expression is asymmetric in the nephric duct at this time. This expression domain demarcates the region of the nephric duct that will form the ureter bud a few hours later. Expression of *Wnt-11* is confined to the rounded tip of the invading ureter whereas the stalk region is devoid of signal (Fig. 3B). At 11 days p.c., the ureter bud branches for the first time and the *Wnt-11* expression domain is split in two (Fig. 3C). Consequently, when the first branching event is

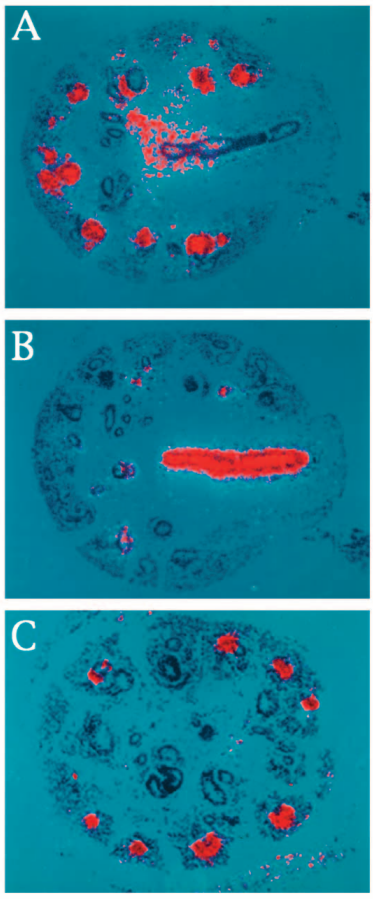

Fig. 1. Expression of *Wnt* genes in the metanephric kidney. In situ hybridization with [^{35}S] ribopobes was used to detect specific expression of *Wnt-4* (A), *Wnt-7b* (B) and *Wnt-11* (C) in sections of a 14.5 days p.c. metanephric kidney. *Wnt-4* is expressed in the mesenchymal condensates and the comma- and S-shaped bodies, and in the centrally located stroma, *Wnt-7b* in the collecting ducts and *Wnt-11* in the ureter tips.

completed at 11.5 days p.c., *Wnt-11* expression is again confined to the tip of both branches of the ureter (Fig. 3D). At more advanced stages of metanephric development, the inductive interactions take place in a narrow zone in the periphery of the kidney. Whole-mount in situ hybridization of 12.5 to 18.5 days p.c. kidneys shows a spotted pattern of *Wnt-11* expression in a surface view of the kidney (Fig. 3E). Sections reveal that *Wnt-11* expression is precisely confined to the growing tips of the ureter in this peripheral region where nephrogenesis is continuing (Fig. 3F).

Regulation of *Wnt-11* expression in the metanephric kidney

Activation of *Wnt-11* expression

The upregulation of *Wnt-11* expression in the epithelium contacting the metanephric blastema suggests that signals from the metanephric mesenchyme are involved in *Wnt-11* activation. In order to address this issue, we studied *Wnt-11* expression in embryos homozygous for the *Danforth's short-tail* (*Sd*) mutation. Heterozygotes are characterized by a short or absent tail and the occasional absence of one kidney. Homozygous mutant embryos show a severe truncation of the axis and gross urogenital defects. Kidneys are hypoplastic or agenic, a phenotype that was attributed to a failure of metanephric mesenchyme induction due to a delay or absence of ureter outgrowth (Glucksohn-Schoenheimer, 1943, 1945). Rather than influencing the ureter directly, the *Sd* mutation most likely interferes with the necessary proximity of the nephric duct and the metanephric blastema, which is required for outgrowth of the ureteric bud (Glucksohn-Waelsch and Rota, 1963).

We harvested embryos at 11-11.5 days p.c. when homozygous *Sd* embryos are readily distinguishable due to the occurrence of edemas in the tail tip and spina bifida in the posterior trunk. Heterozygotes show only a slightly thinned tail at this time (Glucksohn-Schoenheimer, 1945). The frequency of these phenotypes was as expected for a Mendelian trait. From 46 embryos isolated in total, 16 were classified as wild-type, 26 as *Sd*/+ and 13 as *Sd*/*Sd*. All wild-type embryos showed *Wnt-11* expression in the tips of the bifurcated ureter. From the 26 embryos scored as *Sd*/+, 12 had a wild-type staining pattern, 5 had bilateral but unequal staining, 5 showed unilateral staining only and 4 had very weak or no detectable staining. In the homozygous mutants we observed three different phenotypes. In 8 cases we did not detect any staining but the ureter was absent; in three cases, we noticed a unilateral staining at the tip of a long unbranched ureter stalk and, in 2 cases, staining was weak but bilateral (Fig. 4). These results reveal that *Wnt-11* expression is not strictly dependent on *Sd*. They are compatible with the notion that *Sd* does not affect kidney development directly but rather disturbs (secondarily) the proximity between the ductal elements and the metanephric mesenchyme. The missing contact between metanephric blastema and the nephric duct prevents ureter outgrowth and *Wnt-11* activation.

Maintenance of *Wnt-11* expression in the ureter tips

After the initial activation in the nephric duct, *Wnt-11* expression is strictly confined to the tips of the ureter as long as branching morphogenesis and mesenchymal induction occur. As reciprocal signals from the induced mesenchyme undergoing tubulogenesis are thought to regulate ureter devel-

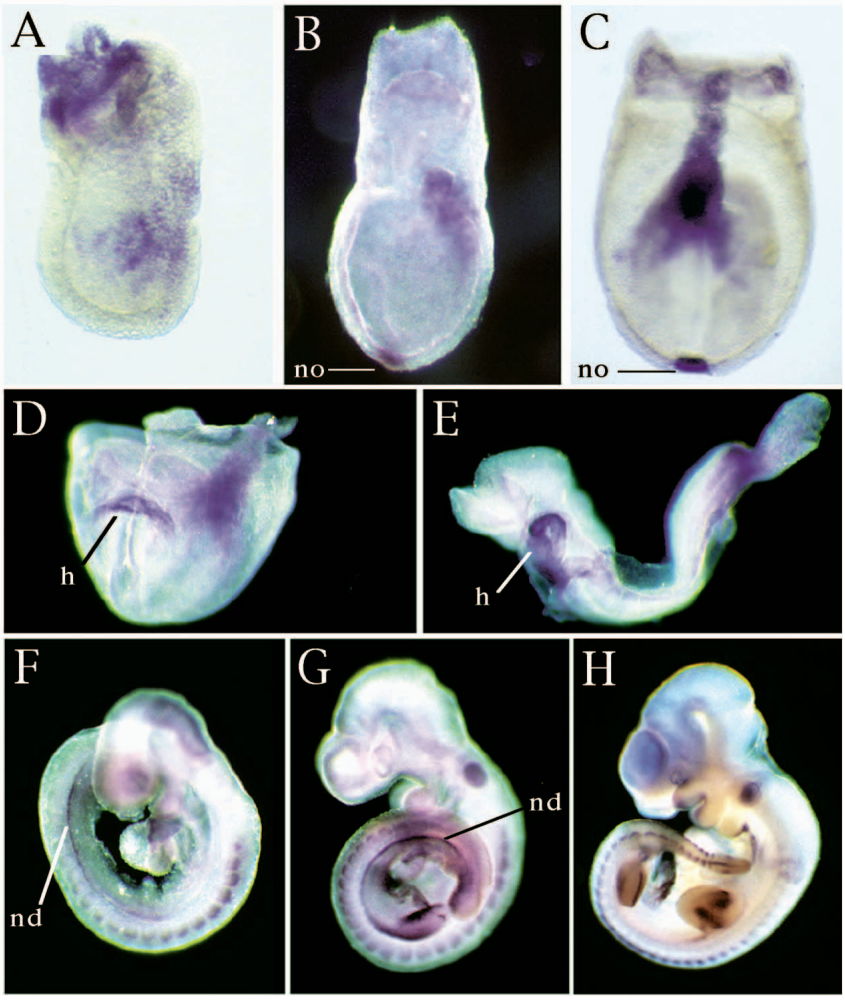

Fig. 2. Spatial expression of *Wnt-11* between 6.75 days and 11.5 days p.c. of mouse development as detected by whole-mount in situ hybridization. (A) 6.75 days p.c., weak *Wnt-11* expression in scattered cells in the posterior half of the embryo. (B) 7.0 days p.c., onset of expression in the node and in the extraembryonic mesoderm including the allantois. (C) 7.5 days p.c., enhanced expression in these two regions. (D) *Wnt-11* expression comes on in the primitive heart anlage, and is maintained in the posterior region. (E) 8.5 days p.c., *Wnt-11* is strongly expressed in the heart tube and the posterior trunk, weak expression in the newly forming somites. (F) 9 days p.c., *Wnt-11* is expressed along the length of the mesonephric duct. (G) *Wnt-11* expression accompanies the outgrowth of the forelimb buds, (H) 10.5 days p.c., expression is maintained in the somites, in the apical ectodermal ridge and the proximal region of the limb and is initiated in the first and second branchial arch. no, node; h, primitive heart anlage; nd, mesonephric duct.

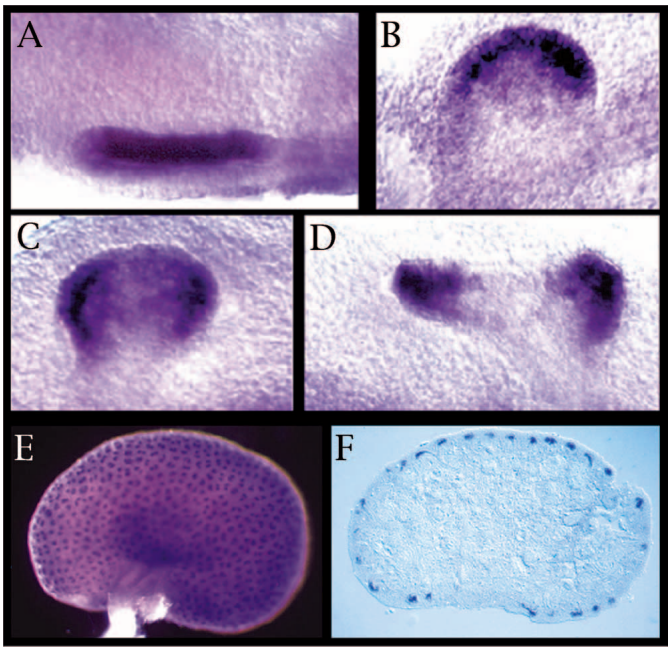

Fig. 3. *Wnt-11* expression in the metanephric kidney. Posterior trunk regions of embryos and isolated metanephric kidneys were stained as whole mounts for *Wnt-11* expression. (A) *Wnt-11* expression is first seen at 10.5 days p.c. in the epithelium of the ureter adjacent the metanephric mesenchyme. (B,C) With the ingrowth of the ureteric bud (10.75 days p.c., 11 days p.c.), the signal is only detected at the rounded tip. (D) The expression domain splits in two after the bifurcation of the ureter has been completed at 11.5 days p.c. (E) *Wnt-11* expression as seen in 16.5 days p.c. metanephric kidney stained as a whole mount. (F) Sectioning reveals that expression is confined to the tips of the growing ureter in the nephrogenic zone.

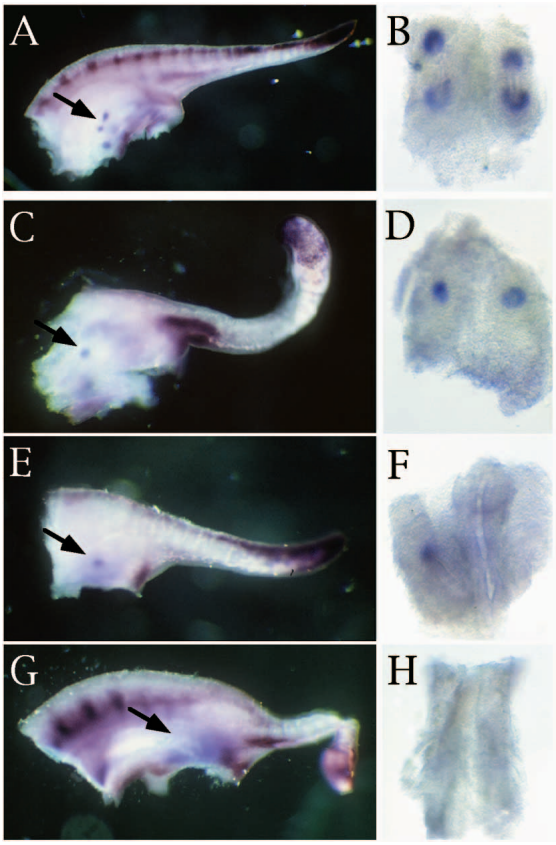

Fig. 4. *Wnt-11* expression in *Danforth's short-tail* mice. (A,B) *Wnt-11* expression in the posterior trunk and kidneys of *Sd*/+ embryos. *Wnt-11* expression is lost in the most caudal somites, but still present in the ureter tips (see arrow). (C-H) *Wnt-11* expression in the posterior trunks and kidneys of *Sd/Sd* embryos. All embryos are characterized by large edema in the tail. *Wnt-11* expression in the somite is partially or completely lost, but persists in the tail bud. *Wnt-11* expression is found in the tip of the ureter on both sides (D), on one side (F) or is absent (H) (see arrow).

opment (for review see Saxen, 1987), it is conceivable that mesenchymal factors are required to maintain and restrict *Wnt-11* expression to this region of the ureter.

Mice homozygous for the *Wnt-4* mutation fail to form pretubular cell aggregates and, consequently, do not develop more mature mesenchymal structures (Stark et al., 1994). We analyzed *Wnt-11* expression in the *Wnt-4* mutant background in whole kidneys and in sections by in situ hybridization at 12.5 and 14.5 days p.c. (Fig. 5). *Wnt-11* expression persists at the tips of the ureter until at least 14.5 days p.c. though, at 14.5 days p.c., the tips are very much reduced in number and they do not show the half moon form indicative of tip branching observed in wild-type kidneys (Fig. 5D). Thus, *Wnt-11* expression is independent of Wnt-4 signaling and also of signals provided by pretubular cell aggregates or epithelial bodies. However, the clear reduction of ureter tips as noted previously (Stark et al., 1994) suggests that the fine tuning of ureteric growth and branching critically depends on the intact architecture of the mesenchymal and epithelial elements.

Earlier reports indicated that metanephric mesenchyme is unique in its ability to support growth and branching of ureteric epithelium (Bishop-Calame, 1965a,b; Saxen, 1987). We recombined ureters isolated at 11.5 days p.c. after the first branching ('T-stage') with lung mesenchyme from the same embryo. Unexpectedly, we found that lung mesenchyme is able to support branching morphogenesis of the ureter though with a pattern more reminiscent of lung epithelium (Fig. 6A). We believe this effect was not due to contaminating metanephric mesenchyme since isolated ureters degenerated rapidly when cultured on nucleopore filters and tubule induction never occurred in our cultures. We obtained identical results when we recombined ureter tips with lung mesenchyme (data not shown) suggesting that the growth and branching potential of the ureter resides in this morphologically distinct structure.

To determine whether the ureteric epithelium preserved its molecular identity under these conditions, we examined *Pax-2* expression in the explants. *Pax-2* encodes a transcription factor that is both expressed in the ureter and required for urogenital development (Dressler et al., 1990; Torres et al., 1995). We used a transgenic line that expresses β-galactosidase exclusively in the nephric duct, its derivatives and the ureter under the control of *Pax-2* genomic sequences (A. Kispert, D. H. Rowitch and A. P. McMahon, unpublished observations). We detected β-galactosidase activity in the epithelium until at least day 6 of culture suggesting that the epithelium retains is ureteric identity in the recombinants (Fig. 6B). Moreover, *Wnt-11* expression is also maintained and localized to the tips for at least 4 days (Fig. 6C). Intriguingly, *c-ret*, a receptor tyrosine

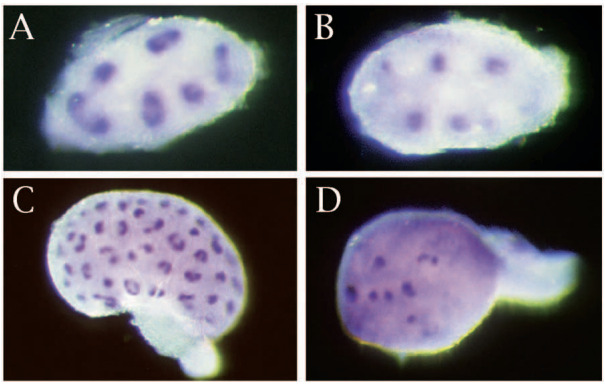

Fig. 5. *Wnt-11* expression in kidneys mutant for *Wnt-4*. *Wnt-11* is expressed in the ureter tips in the mutants, although the number of branches is clearly decreased in comparison to the wild-type. (A) 12.5 days p.c. +/+, (B) 12.5 days p.c. –/–, (C) 14.5 days p.c. +/+, (D) 14.5 days p.c. –/–. Embryos were derived from matings of *Wnt-4* heterozygotes and genotyped individually, kidneys were stained as whole-mounts for *Wnt-11* expression.

kinase which is normally coexpressed with *Wnt-11* at the ureteric tips remains coexpressed with *Wnt-11* in the recombinants suggesting a common mechanism of regulation (Fig. 6D). Thus, maintenance of *Wnt-11* and *c-ret* expression in the ureter tips is not kidney mesenchyme specific. Only a general permissive environment may be required to maintain ureter viability and *Wnt-11* transcription.

Requirement of proteoglycans for *Wnt-11* expression

We were interested to investigate whether the extracellular matrix (ECM), and in particular proteoglycans (macromolecules consisting of a protein moiety and a covalently attached often highly sulphated glycosaminoglycan (GAG) (Kjellen and Lindahl, 1991)), could be an essential factor in the maintenance of *Wnt-11* expression. Proteoglycans have been shown to be expressed both in the ureter and the induced mesenchyme (Vainio et al., 1989) and are required for ureter branching and tubule induction, but not for nephron maturation (Davies et al., 1995; Platt et al., 1987). We addressed this problem by administering compounds known to interfere with the integrity and function of proteoglycans, to kidney rudiment cultures, taken at 11.5 days p.c. when only one branch of the ureter had formed (T-stage). After 24 hours in control medium, the ureter had branched several times and tubule induction was evident by the appearance of mesenchymal condensates (Fig. 7A,B). Expression of *Pax-2* (Fig. 7B), *Wnt-11* (Fig. 7C) and the receptor tyrosine kinases *c-ret* and *c-ros* (not shown) confirmed that growth and branching of the ureter in vitro approximated the in vivo condition. For all experimental treatments, at least 6, in most cases 12, kidney rudiments were cultured and processed for any given concentration or time point.

Chlorate competes with sulphate in the synthesis of phospho-adenosine-5′-phosphosulphate, the sulphate donor used by sulphotransferases in the sulphation of polysaccharides (Farley et al., 1978). The addition of chlorate to the culture medium therefore selectively probes for the requirement of the glycosaminoglycan moiety of proteoglycans. Recently, Davies et al. (1995) reported that ureteric bud growth and branching in kidney rudiments is reversibly inhibited by chlorate in the culture medium. Surprisingly, nephron development is apparently unaffected under these conditions. Inhibition is concentration dependent and correlates quantitatively with the deprivation of sulphated GAGs. Chlorate concentrations between 15 mM and 30 mM were found to block growth and branching of the ureter completely. Ureters exhibited a T-shape even after 96 hours in culture. Nephron maturation in these cultures continued seemingly unaffected. In 30 mM chlorate, *Wnt-11* expression was completely abolished after 24 hours, whereas the ureteric marker *Pax-2* was expressed until at least 96 hours of incubation (Fig. 7D,E). Davies et al. (1995) noted that the effect on the ureter is reversible upon removal of the chlorate after 30 hours or competition with a low concentration of sulphate. However, in our hands, we observed major defects in ureter growth and branching after only 18 hours incubation in chlorate, but we did notice a massive wave of new tubule induction after transfer into fresh medium. Branching was highly ‘compressed’ and ureter shape disturbed as demonstrated by *Pax-2* expression (Fig. 7G). *Wnt-11* was expressed in few distinct spots and some smeary background (Fig. 7F). Kidney rudiments cultured for 48 hours in medium containing 30 mM chlorate retained their T-shape after another 48 hours incubation in standard medium but never showed *Wnt-11* expression (not shown).

Chondroitin sulphate and heparin/heparan sulphate are major constituents of the proteoglycan complex. Both sulphated proteoglycans can be selectively degraded with the specific glycanases chondroitinase ABC and heparitinase III, respectively (Davies et al., 1995). 0.5 U/ml chondroitinase ABC and heparitinase III, respectively, had no effect on ureter shape and *Wnt-11* expression, higher concentrations of heparitinase III (2.5 U/ml) and a combination of both enzymes (0.5 U/ml chondroitinase, 0.5-2.5 U/ml heparitinase III) resulted in

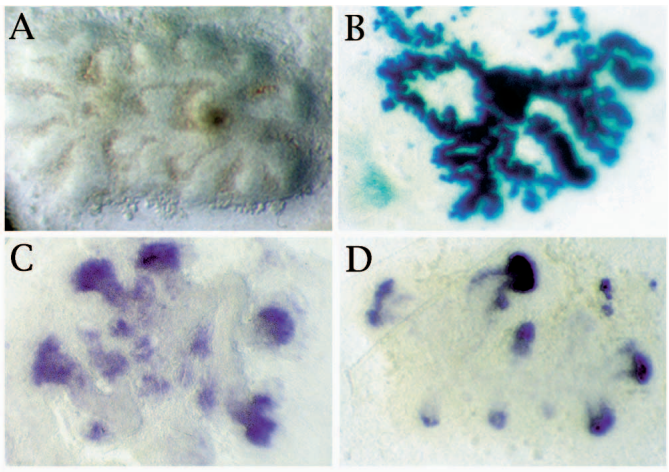

Fig. 6. Growth and branching of the ureter occurs in a recombinant with lung mesenchyme. (A) Isolated ureters grow and branch excessively in lung mesenchyme as seen in a bright-field image. (C) *Wnt-11* expression and (D) *c-ret* expression are confined to the rounded tips of the ureter; (B) the ureter still expresses *Pax-2*. 11.5 days p.c. ureters (bifurcated once) and lung mesenchyme from the same embryo were recombined on a nucleopore filter. Specimen were grown for 2 days (C,D) and 4 days (A,B), respectively, before subjecting them to β-gal staining (A) or whole-mount in situ hybridization (C,D).

branching inhibition and a partial loss of *Wnt-11* expression (not shown).

Thus, sulphated proteoglycans are important for the maintenance of *Wnt-11* expression and chondroitin sulphate, heparan sulphate and heparin might constitute a major fraction of the glycosaminoglycans required for *Wnt-11* regulation.

The effect of chlorate on ureter growth and branching in kidney cultures might result from the loss of bound factors from the matrix. Indeed, Wnts, fibroblast growth factors and a variety of other secreted factors are thought to bind to matrix components, including proteoglycans. We investigated the effect of three sulphated glycosaminoglycans and of suramin, all of which release Wnt proteins into the medium in cell culture systems (Burrus and McMahon, 1995). 100 μg/ml chondroitin-6-sulphate had no effect on ureter morphology and *Wnt-11* expression (Fig. 7H,I). However, kidney rudiments cultured for 3 days in 100 μg/ml heparin, exhibited very limited ureter growth and branching, although tubule induction was normal. *Wnt-11* expression was found to be severely reduced after 24 hours of culture (Fig. 7J,K). Kidneys grown in medium containing 10-100 μg/ml pentosan polysulphate for 24 hours showed no *Wnt-11* expression in the ureter tips, tubule formation was severely reduced, and growth and branching of the ureter were completely inhibited (Fig. 7L,M). Finally, we tested the effect of suramin on kidney cultures. In 50 μM suramin, the ureter epithelium survived and expressed the ureter marker *Pax-2* even after 4 days of culture. However, ureter growth was completely arrested, tubule induction

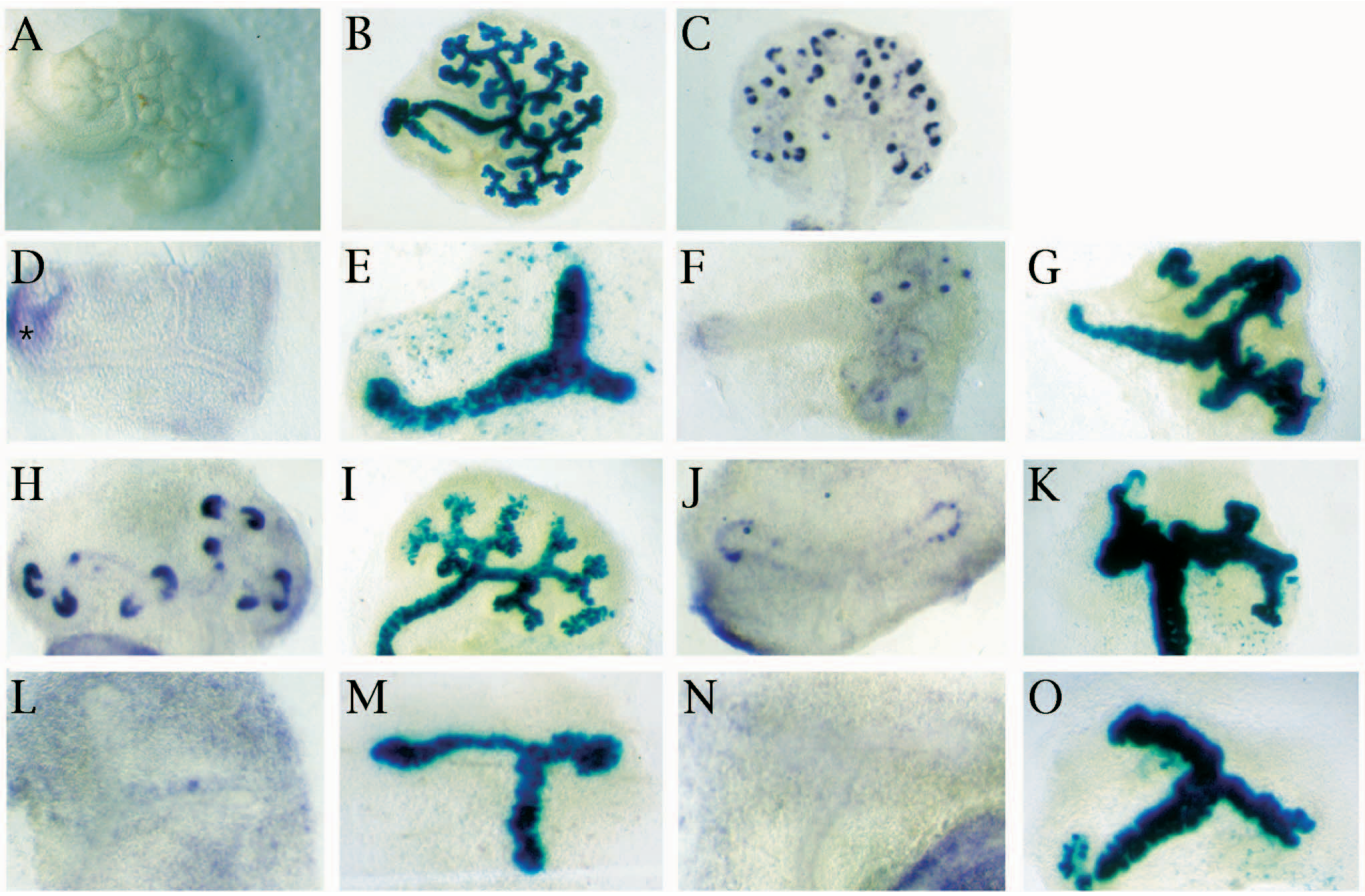

Fig. 7. Sulphated proteoglycans are required for *Wnt-11* expression. All kidney rudiments were isolated at 11.5 days p.c. and grown in culture for various times with supplementation of the culture medium as indicated. Cultures were fixed and stained as whole mounts for *Wnt-11* expression using in situ hybridization or probed for *Pax-2* expression using a β-galactosidase assay. (A) Bright-field image of a 48 hour metanephric kidney culture. Ureter branching and induced tubules are clearly visible. (B) *Pax-2* staining outlines the regular pattern of ureter branching in a kidney rudiment cultured for 48 hours. The ampulla-like ureter tips are evident. (C) *Wnt-11* expression marks the numerous ureter tips after 48 h of growth in culture. (D,E) Kidney rudiments grown in medium supplemented with 30 mM $NaClO_3$ for 24 hours do not show ureter growth. *Wnt-11* expression is lost in the ureter tips (*, but not in the mesonephric duct to the left, D), whereas *Pax-2* expression can be detected even after 96 h in culture (E). (F,G) Kidney rudiments were grown for 24 hours in medium containing 30 mM $NaClO_3$ and were then transferred into standard medium for 48 hours. Numerous induced tubules and clear condensates were scored. *Wnt-11* expression is very spotty and irregular (F), the ureter branching pattern is very disturbed as evidenced by *Pax-2* expression (G). (H,I) Chondroitin-6-sulphate at 100 μg/ml had no effect on *Wnt-11* expression (H) and ureter growth and branching as seen with the *Pax-2* marker (I) after 24 hours culture. (J,K) Supplementation of the medium with 100 μg/ml heparin leads to a drastic downregulation of *Wnt-11* expression after 24 hours, and almost a halt in ureter growth (J). After 72 hours, *Pax-2* expression uncovers a severely growth-retarded ureter with very limited branching (K). Kidney rudiments grown in medium containing 50 μM suramin (L,M) and 100 μg/ml pentosan polysulphate (N,O), respectively, have completely lost *Wnt-11* expression at the ureter tips after 24 hours (L,N). *Pax-2* expression persists after 72 hours in the completely growth-arrested ureter.

extremely rare and *Wnt-11* expression lost after 24 hours (Fig. 7N,O).

To address whether the loss of *Wnt-11* expression in these cultures was solely the result of the loss of the characteristic ampulla-like structure at the ureter tips, which is not evident in chlorate, pentosan polysulphate and suramin cultures after 24 hours, we compared a time course of *Wnt-11* and *c-ret* and *c-ros* (not shown) expression in 30 mM chlorate (Fig. 8). Already after 5 hours, we detected a dramatic reduction of *Wnt-11* expression (Fig. 8C) and after 8 hours expression was almost completely abolished (Fig. 8E). In contrast, expression of *c-ret* and *c-ros*, which exhibit a similar pattern, were only slightly reduced after 8 hours (Fig. 8D,F). After 24 hours, both markers were completely lost from the tips but a weak expression was detected throughout the ureter (Fig. 8J). Thus, the loss of *Wnt-11* precedes gross morphological changes in the ureter (Fig. 8D,F).

Since chlorate exerts its effects intracellularly and acts only on newly synthesized proteoglycans, its effects should be retarded compared to those substances that directly affect the matrix. Not surprisingly, we found that the kinetics of *Wnt-11* downregulation accelerated in cultures that had been supplemented with 100 μg/ml pentosan polysulphate. Even after only 2-3 hours of incubation, *Wnt-11* expression was almost completely abolished, well before *c-ret* transcription was lost in the ureter tips and preceding visible morphological changes (Fig. 8K-N).

DISCUSSION

The development of the mammalian kidney can schematically be divided into three processes. First, the metanephric blastema forms at the caudal end of the intermediate mesoderm. Second, the ureter bud emerges from the nephric duct, enters the metanephric blastema, grows and branches. Third, mesenchymal cells condense, epithelialize and differentiate to form the mature nephron.

We demonstrate here that *Wnt-11* activation in the nephric duct is dependent on the metanephric blastema and its subsequent expression correlates with branching morphogenesis of the ureter. These results are consistent with the mesenchymal blastema providing a signal or signals that activate *Wnt-11* and lead to ingrowth of the ureteric bud. Support for the existence

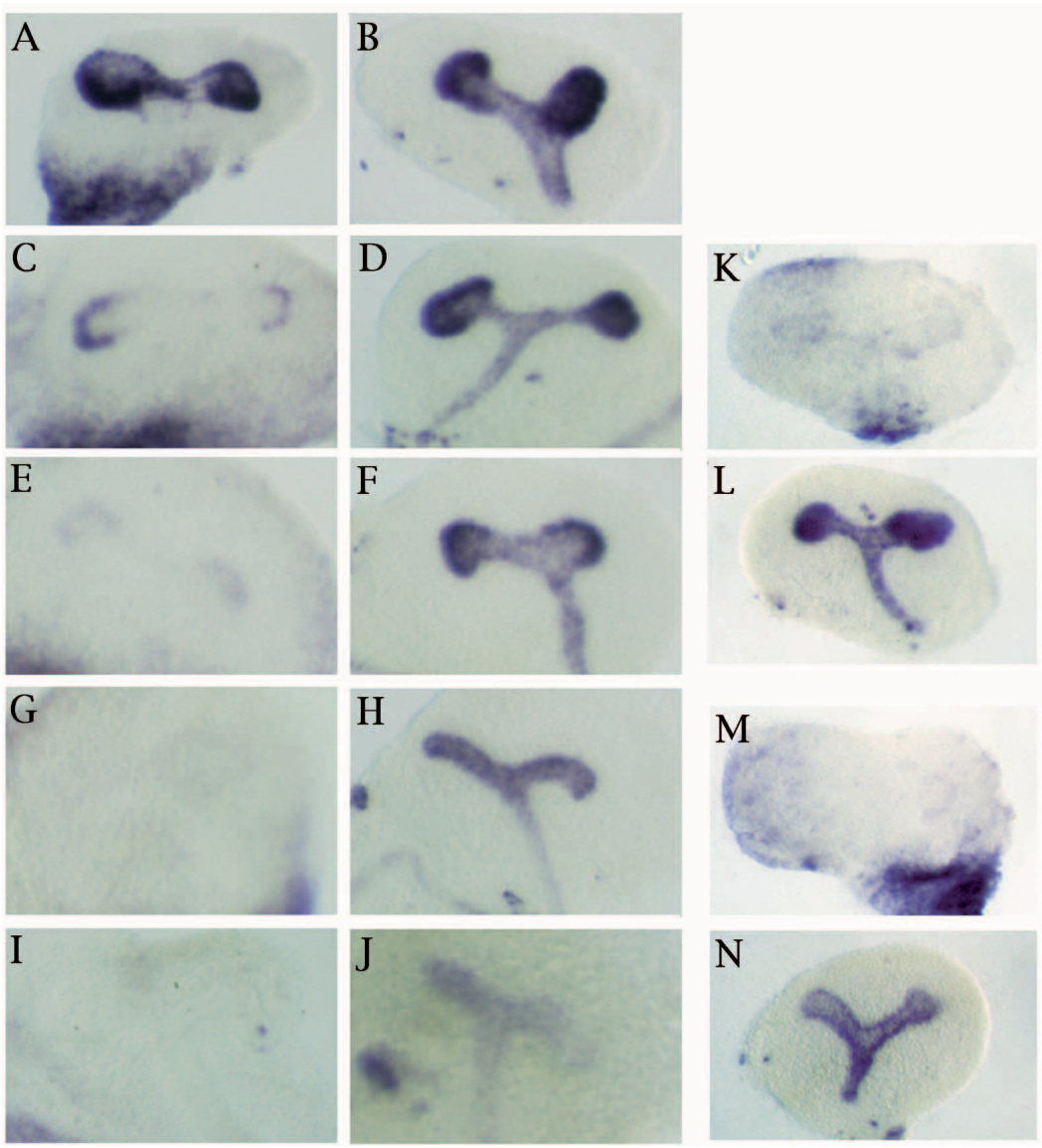

Fig. 8. Kinetics of *Wnt-11* downregulation in cultures of metanephric kidneys treated with $NaClO_3$ and pentosan polysulphate, respectively. Kidney rudiments were isolated at 11.5 days p.c. (bifurcated once) and cultured in standard medium (A,B) and in medium supplemented with 30 mM $NaClO_3$ (C-J) and 100 μg/ml pentosan polysulphate (K-N), respectively. Cultures were fixed after 2 hours, in the case of the standard medium, after 5 hours (C,D), 8 hours (E,F), 16 hours (G,H), and 24 hours (I,J) when grown in medium supplemented with $NaClO_3$ and after 3 hours (K,L) and 6 hours (M,N), respectively, in the pentosan polysulphate experiments. Cultures were subsequently subjected to whole-mount in situ hybridization to detect *Wnt-11* expression (A,C,E,G,I,K,M) and *c-ret* expression (B,D,F,H,J,L,N), respectively. Loss of *Wnt-11* expression is a rapid consequence of chlorate treatment and is even accelerated in the pentosan-polysulphate-treated cultures. *Wnt-11* downregulation precedes loss of *c-ret* transcription and morphological changes at the ureter tip.

of a mesenchymal signal promoting ureter outgrowth comes from genetic studies. For example, in the *Wilms tumor-1* (*WT-1*) mutant, the metanephric blastema undergoes apoptosis and subsequently, in the absence of the mesenchyme, the nephric duct fails to bud (Kreidberg et al., 1993). Similar studies on *Sd* kidneys in vivo and in culture indicate that abnormalities in formation, growth and differentiation of the ureteric bud that result in the partial or complete failure of the metanephric mesenchyme differentiation have their origins in altering the interactions between the nephric duct and the blastema as intact kidney rudiments of *Sd* homozygous embryos are able to undergo differentiation in culture (Gluecksohn-Schoenheimer, 1943;, 1945; Gluecksohn-Waelsch and Rota, 1963).

Interestingly, we noticed a frequent unilateral loss and unequal bilateral expression of *Wnt-11* in *Sd* heterozygotes, which most likely reflects variation in the proximity of the metanephric mesenchyme and the nephric duct tissues rather than direct genetic interactions. A second model, that positional cues intrinsic to the nephric duct autonomously regulate the activation of *Wnt-11*, is clearly not supported by our data.

Our experiments specifically addressed the regulation of *Wnt-11* activation, which correlates with outgrowth of the ureteric bud. Additional experiments will be necessary to address whether *Wnt-11* is required for bud outgrowth. The observation that *Wnt-11* activation precedes budding supports such a model.

Recombination experiments in culture have suggested that following invasion of the kidney mesenchyme, survival, growth and branching of the ureter are also controlled by factors specific to the metanephric mesenchyme (Bishop-Calame, 1965a,b; Saxen, 1987). Our findings contradict this view. In our experiments, the ureter epithelium grows and branches in lung mesenchyme though the branching pattern resembled that of lung rather than ureteric bud epithelium. Further, *Wnt-11* expression is retained and remains restricted to the tips of the ureter. Thus, it is unlikey that metanephric-specific factors are required for branching morphogenesis of the ureter. While we cannot exclude that our recombinants are contaminated with metanephric mesenchyme, it is unlikely because isolated ureters without lung mesenchyme degenerated and we never observed tubule induction in the recombinants. Unfortunately, there is no suitable marker to assay for small numbers of contaminating cells. Interestingly, it was thought for some time, that mouse submandibular epithelium critically depends on specific submandibular mesenchymal factors for branching. However, it now appears that lung mesenchyme is also able to support budding and cytodifferentiation of salivary epithelium (Lawson, 1972, 1974).

One interpretation of our results is that the ureteric epithelium contains an intrinsic morphogenetic program, and that the metanephric mesenchyme provides nonspecific survival/proliferation factors. Interestingly, Nogawa and Ito (1995) have reported that branching morphogenesis of mouse lung epithelium occurs in mesenchyme-free conditions. All that is required is a basement membrane matrix and the soluble growth factor aFGF. Further, neonatal mouse kidney epithelial cells (Traub et al., 1990) and Madin-Darby canine kidney epithelial cells can form tubules on manipulations of the matrix and addition of some soluble growth factors (Montesano et al., 1991). Our data suggest that the metanephric mesenchyme plays two roles in ureter branching. First, it provides general proliferative factors, a role that can be mimicked by heterologous mesenchymes. Second, it controls the 'kidney'-specific rate and mode of ureter morphogenesis probably by the concerted interaction of more mature mesenchymal elements with the ureter. Our data also show that the localization of *Wnt-11* expression to the tips of the ureter is independent of any specific signals provided by the kidney mesenchyme though the precise cellular dynamics of expression may be modulated specifically.

Numerous studies have pointed to the importance of the extracellular matrix (ECM) in epithelial-mesenchymal interactions in general and in kidney development in particular (Roskelly et al., 1995). The ureter epithelium is surrounded by a basal lamina consisting of type IV collagen, glycoproteins and proteoglycans (Ekblom, 1981). Induction of mesenchymal cells results in the remodeling of the interstitial type of ECM into an epithelial type (Aufderheide et al., 1987; Ekblom et al., 1980). Components known to interfere with proteoglycan metabolism and structure have profound effects on kidney development. In kidney cultures grown in medium supplemented with β-D-xyloside, a competitor of xylosylated core proteins at the level of galactosyltransferase, synthesis of macromolecular proteoglycans is decreased. Kidney development is perturbed and, in particular, branching morphogenesis is abnormal (Klein et al., 1989; Lelongt et al., 1988; Platt et al., 1987). Recently Davies et al. (1995) showed that chlorate, a competitive inhibitor of sulphate in the sulphation of proteoglycans, has an effect similar to that of β-D-xyloside in inhibiting branching and growth of ureters and tubule induction in cultures of kidney rudiments. In both cases, nephron maturation occurred normally suggesting that sulphated proteoglycans are not required for nephron development.

We find that *Wnt-11* expression is rapidly lost on chlorate treatment. This is followed by a downregulation in expression of the receptor tyrosine kinases *c-ret* and *c-ros* and loss of the ampulla-like morphology at the termini of the ureteric buds. These morphological and molecular changes most likely explain the absence of tubule induction under these conditions. Our findings suggest that proteoglycans are an important component in a signaling process that maintains *Wnt-11* expression and the integrity of the tips of the ureter bud. As our recombination experiments demonstrate that only the tips are necessary for branching morphogenesis, loss of tips most likely accounts for the cessation of ductal development. At present, it is not clear whether proteoglycans themselves and/or some associated factors modulate the development of the ureteric bud. For example, recent studies have pointed out that proteoglycans act as low affinity receptors for many growth factors including fibroblast growth factors and transforming growth factors (for review see Schlessinger et al., 1995; Rapraeger, 1995). Moreover, Wnts are thought to interact with ECM components (Bradley and Brown, 1990; Burrus and McMahon, 1995; Papkoff and Schryver, 1990). All of these factors may be displaced from matrix association by polysulphated compounds, which, in our experiments, block ureteric bud development and tubule induction. The effect of the polysulphated compounds on ureter branching correlates directly with their effect on *Wnt-11* expression. *Wnt-11* expression in the ureter tip was unaffected by chondroitin sulphate, reduced by heparin and completely abolished by pentosan polysulphate and suramin in the culture medium. Loss of *Wnt-11* expression

is a rapid response to addition of pentosan polysulphate arguing for a direct interference with a signaling pathway regulating *Wnt-11* expression, possibly Wnt-11 itself.

Our data argue that *Wnt-11* is likely to play an important role in regulating development of the metanephric kidney possibly in the branching morphogenesis of the ureter epithelium. In *Drosophila melanogaster*, *wingless* is expressed in the Malpighian tubules and is required for proliferation in the morphogenesis of this organ (Skaer and Martinez Arias, 1992) pointing to a possible evolutionary conservation of *Wnt* gene function in the ductal epithelium during development of excretory structures. *Wnt-11* is coexpressed with the receptor tyrosine kinase *c-ret* in the nephric duct and in the ureter tips during normal kidney development, in *Wnt-4* mutant kidneys and in heterologous recombination experiments. Moreover, both genes are downregulated under conditions that interfere with the structure and function of proteoglycans. Taking the requirement of *c-ret* for ureter growth and branching into account (Schuchardt et al., 1994), it is tempting to speculate that *Wnt-11* and *c-ret* both act in the ureter tip as part of a cascade of genes controlling growth and branching.

Finally, we cannot exclude additional roles for *Wnt-11* in kidney development. Intriguingly, Herzlinger et al. (1994) have shown that cells expressing *Wnt-1* are able to induce tubulogenesis in isolated metanephric mesenchyme. *Wnt-1* is expressed in dorsal spinal cord (Wilkinson et al., 1987), the strongest heterologous inducer of tubulogenesis (Grobstein, 1956). However, *Wnt-1* is not expressed in the kidney, suggesting that Wnt-1 mimics a real Wnt-like inducer. The expression pattern of *Wnt-11* in the metanephric kidney is fully compatible with the notion that Wnt-11 is this endogenous inducer. We cannot rigorously exclude this possibility at present, but thus far we have been unable to induce tubule formation using conditions in which Wnt-1 acts as a strong inducer (A. K. and A. P. M., unpublished observation). Consequently, we favor a model in which *Wnt-11* is required within the ureter as a survival/proliferation factor.

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