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Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney

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SUMMARY

Development of the mammalian kidney is initiated by ingrowth of the ureteric bud into the metanephric blastema. In response to signal(s) from the ureter, mesenchymal cells condense, aggregate into pretubular clusters, and undergo epithelialisation to form simple epithelial tubules. Subsequent morphogenesis and differentiation of the tubular epithelium lead to the establishment of a functional nephron.

Here we demonstrate that Wnt-4, a secreted glycoprotein which is required for tubule formation, is sufficient to trigger tubulogenesis in isolated metanephric mesenchyme, whereas Wnt-11 which is expressed in the tip of the growing ureter is not. Wnt-4 signaling depends on cell contact and sulphated glycosaminoglycans and is only required for triggering tubulogenesis but not for later events. The Wnt-4 signal can be replaced by other members of the *Wnt* gene family including Wnt-1, Wnt-3a, Wnt-7a and Wnt-7b. Further, dorsal spinal cord, which has been thought to mimic ureteric signaling in tubule induction induces *Wnt-4* mutant as well as wild-type mesenchyme suggesting that spinal cord derived signal(s) most likely act by mimicking the normal mesenchymal action of Wnt-4. These results lend additional support to the notion that Wnt-4 is a key auto-regulator of the mesenchymal to epithelial transformation that underpins nephrogenesis adding another level of complexity in the hierarchy of molecular events mediating tubulogenesis.

Key words: Wnt, Wnt-4, Induction, Tubulogenesis, Kidney development, Metanephros

INTRODUCTION

The development of vertebrate organs requires intricate cell and tissue interactions to assure the concerted program of cell growth, differentiation and morphogenesis. The mammalian kidney, mainly of mouse and rat, has long been studied as a model system to reveal both the embryological principles and more recently the molecular control of vertebrate organ formation (for reviews see Saxen, 1987; Lechner and Dressler, 1997; Vainio and Muller, 1997).

Mouse renal development is characterised by the continuous interaction of epithelial and mesenchymal compartments both of which derive from the intermediate mesenchyme. These are the nephric duct and its derivative the ureter, and the nephrogenic mesenchyme which lies adjacent to these ducts. As a consequence of these interactions three embryonic kidneys are laid down from anterior to posterior in time and space. While the initial organ, the pronephros is only a very transient structure established at 8-8.5 days post coitum (d p.c.), the mesonephros extends by posterior elongation of the nephric duct and subsequent tubule induction in the adjacent mesonephrogenic mesenchyme between 9 and 11 d p.c. Although forming elaborate tubules, the mesonephros of the male never becomes a functional organ but contributes to the ductal network of the rete testis.

Metanephric development is initiated when a bud emerges from the nephric duct at the level of the hind limbs around 10.5 d p.c. The ureteric duct subsequently invades the metanephric blastema which lies at the posterior end of the intermediate mesoderm.

In a process repeated many times, mesenchymal cells condense around the tip of the ureter, i.e. bud, aggregate, epithelialise and undergo morphogenetic movements and cellular differentiation programs to form a major part of the nephron the functional unit of the vertebrate kidney. The ureter continues to grow and to branch forming the collecting duct system of the mature organ. 7-10 days post partum nephron formation ceases most likely as the mesenchymal stem cells in the periphery of the kidney are exhausted.

In order to achieve the complex architecture of the mature kidney, the morphogenetic and cellular differentiation programs of both the nephric duct and the mesenchymal derivatives have to be highly integrated making it very likely that multiple signaling systems between and within the two compartments are operative.

Classical kidney organ culture experiments have primarily

focused on the signals exchanged between mesenchyme and ureter upon their initial contact. Separation and recombination experiments have shown that isolated metanephric mesenchyme undergoes apoptosis unless provided with a permissive stimulus which leads to epithelial tubule formation (Grobstein, 1953; Saxen, 1987). Similarly, ureter tissue degenerates upon separation from metanephric mesenchyme, and undergoes limited or altered branching morphogenesis when recombined with heterologous mesenchymal tissues suggesting that metanephric mesenchyme secretes signals essential for ureter survival and correct branching morphogenesis (Saxen, 1987; Kispert et al., 1996; Sainio et al., 1997).

A search for heterologous tissues that may be a more convenient source of a factor capable of replicating ureteric signaling has identified the dorsal spinal cord as a potent inducer of tubulogenesis. Indeed, most of our understanding of cell interactions in kidney development comes from the application of spinal cord derived signals to isolated metanephric mesenchyme in culture. These studies have demonstrated that induction appears to be cell contact dependent, requires approximately 24 hours of contact between spinal cord and mesenchyme, can be blocked by metabolic inhibitors, and cannot be transferred from induced to uninduced mesenchyme (homeogenetic induction) (Grobstein, 1953; Saxen, 1987, and references therein).

It is important to note that while this assay has generally been thought to provide information about ureteric-like inductive signals there is no evidence that this is the case. For example, induction of tubules may require the action of several signals, some from the ureter others from the mesenchyme. Supplying any of these may be sufficient to trigger tubule formation.

While recently progress has been made in identifying mesenchymally derived signaling molecules required for ureter proliferation and branching morphogenesis (see Sariola and Sainio, 1997, for review) the nature of ureteric signals has remained elusive. Several growth factors have been discussed as potential inducers (Hammerman, 1995) but none of them has conclusively been shown to be required and sufficient for tubule induction. A combination of FGF2 and a pituitary extract can induce tubulogenesis, suggesting that tubule induction is a multi-step process mediated by soluble and possibly insoluble factors (Perantoni, 1991; Perantoni et al., 1995).

Several findings have implicated members of the Wnt family of secreted glycoproteins in signaling processes operating during metanephric development. *Wnt-11* is expressed in the tips of the growing ureter where tubule inducing activity is thought to arise (Kispert et al., 1996). In contrast, *Wnt-4* is expressed in pretubular mesenchyme cells shortly before they aggregate and transform to simple epithelial tubules. Loss of function studies indicate that *Wnt-4* is required for tubule formation (Stark et al., 1994). Finally, *Wnt-7b* is expressed somewhat later in the collecting duct epithelium which derives from the ureteric duct (Kispert et al., 1996). NIH3T3 cells stably expressing *Wnt-1*, which is not expressed in the kidney but is expressed in the dorsal spinal cord, are able to induce tubulogenesis in isolated rat metanephric mesenchyme (Herzlinger et al., 1994). This suggests that a member of the Wnt family may normally participate in tubule induction. Thus, induction in culture by the spinal cord might mimic ureteric signaling by Wnt-11 *or* mesenchymal signaling by Wnt-4.

Here we describe a number of experiments in which we investigate the role of *Wnt-11*, *Wnt-4* and a number of other family members in tubule induction. From these results we conclude that Wnt-4, but not Wnt-11, is able to induce tubule formation, and that spinal cord mediated tubulogenesis may reflect the normal mesenchymal function of Wnt-4 rather than that of a ureteric bud derived signal.

MATERIALS AND METHODS

Mice

Wnt-4 heterozygotes were derived and genotyped as described previously (Stark et al., 1994). Embryos for kidney dissections were derived from matings of Swiss Webster (SW) wild-type animals or *Wnt-4* heterozygotes. For timed pregnancies plugs were checked in the morning after mating, noon was taken as 0.5 days post coitum (d p.c.).

Cell lines

Cell lines stably expressing various *Wnt* genes or *lacZ* were prepared essentially as described (Pear et al., 1993). Full-length cDNAs encoding *Wnt-1* (van Ooyen and Nusse, 1984), *Wnt-3a* (Roelink and Nusse, 1991), *Wnt-4*, *Wnt-5a*, *Wnt-7a*, *Wnt-7b* (Gavin et al., 1990), *Wnt-11* (Kispert et al., 1996) and *lacZ* were cloned into the retroviral expression vector pLNCX which confers expression of foreign genes under the control of the CMV promotor (Miller and Rosman, 1989). Bosc23 packaging cells were transfected with recombinant DNA constructs. Viral supernatants were collected 48-72 hours later and used to infect NIH3T3 cells. After 10 days of selection in G418, pools of cells were used for recombination experiments. 50,000 cells were plated in 50 μl of medium on polycarbonate filter and grown for 18-24 hours at 37°C in 5% CO_2.

Organ culture techniques

Metanephric kidneys from SW or *Wnt-4* intercrosses were dissected in PBS. Metanephric mesenchyme was dissected manually from the ureter (bud stage, 10.75 d p.c., to early T stage, 11.5 d p.c.), following a 2 minute incubation in 3% pancreatin/trypsin (Gibco-BRL) in Tyrode's solution. In recombination experiments with wild-type mesenchymes samples were pooled before being distributed to individual experiments. In experiments with *Wnt-4* mutant embryos metanephric mesenchyme from each kidney of the embryo was kept separate. The remainder of an embryo was used for genotyping by Southern analysis. In recombination experiments with dorsal spinal cord metanephric mesenchyme from two kidneys was surrounded by two dissected pieces of dorsal spinal cord from the same embryo on a 1 μm polycarbonate filter (Costar). For direct recombination experiments with *Wnt* expressing cells two mesenchymes were placed on top of modified NIH3T3 cells. For transfilter experiments 50,000 cells in 50 μl medium were seeded on a 1 μm filter 18-24 hours prior to the recombination. Cells were then covered with a 1 μm filter and two mesenchymes placed on this filter. Filters (4-6 mm in size) were supported by stainless steel grids on the surface of the culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1× penicillin/streptomycin). Medium was changed every 2 days. For glycosaminoglycan dependence of induction the medium was supplemented with 30 mM $NaClO_3$ after 0 hours, 24 hours and 48 hours, respectively. In experiments concerning pore size dependence of induction the pore size of the upper filter in the transfilter set-up was varied from 0.05 μm, 0.1 μm, 0.4 μm, 0.8 μm to 1 μm. The number of cultures performed are indicated in the text. For marker experiments at least 6 specimens were processed.

For in situ hybridisation 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. For histological analysis filters were fixed in Bouin's solution and stored in 70% ethanol at 4°C.

In situ hybridisation analysis

In situ hybridisation analyses on whole mount cultures were performed as described (Kispert et al., 1996). Full-length cDNAs for *WT-1* (Pritchard-Jones et al., 1990), *Pax-2* (Dressler et al., 1990), *Pax-8* (Plachov et al., 1990), *Wnt-4* (Gavin et al., 1990) and *E-cadherin* (Ringwald et al., 1987) were labeled with digoxigenin for whole mount detection.

Histological analysis

Samples were dehydrated, embedded in wax and sectioned at 5 μm. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.

Documentation

Brightfield images of cultures and marker stainings were taken with a binocular on Kodak 64T slide film. Histological sections were photographed on the same film on a Leitz Axiophot. Slides were scanned and figures composed in Adobe Photoshop 4.0.

RESULTS

Spinal cord mimics a mesenchymal signal for tubule induction

The identification of *Wnt-4* as a mesenchymal signal essential for tubule formation provides an excellent new tool for readdressing the role of spinal cord explants as heterologous inducers of kidney tubulogenesis. Clearly, if the spinal cord mimics a ureteric signal upstream of *Wnt-4*, this signal would not rescue the mesenchymal requirement for *Wnt-4* in tubulogenesis. To test this possibility, isolated metanephric mesenchyme from individual embryos derived from intercrosses between mice heterozygous for a likely null allele of *Wnt-4* were cultured on a polycarbonate filter in direct contact with dorsal spinal cord from the same embryo. In the absence of spinal cord, all mesenchyme cultures rapidly degenerated as expected. Surprisingly, when cultured in the presence of spinal cord, mesenchyme from *Wnt-4* mutant embryos developed as well as that of wild-type or heterozygous siblings (Table 1). After 48 hours induction was visible as bright round zones of condensing mesenchyme. After 96 hours the zones of condensing mesenchyme had undergone epithelialisation to form complex tubules. At 144 hours epithelial tubular structures and glomeruli indicated that full differentiation of induced tubules occurred in all recombinants (Fig. 1). Thus, the induction of tubulogenesis in *Wnt-4* mutant mesenchyme indicates that spinal cord signaling acts by either mimicking the action of Wnt-4 itself, or a factor downstream of *Wnt-4*. Further, although *Wnt-4* is expressed in the spinal cord (Parr et al., 1993), the observation that spinal cord from *Wnt-4* mutants is capable of induction indicates that *Wnt-4* expression in the spinal cord is not essential for this process. This leaves open the involvement of other *Wnts e*xpressed in this tissue.

Table 1. Induction of tubulogenesis in *Wnt-4*/*Wnt-4* mutant metanephric mesenchyme by dorsal spinal cord

		#Induced/#Total		
Exp. #	# Recombinants	+/+	*Wnt-4*/+	*Wnt-4*/*Wnt-4*
1	8	2/2	5/5	1/1
2	7	1/1	3/3	3/3
3	7	3/3	3/3	1/1
4	5	1/1	3/3	1/1
5	9	3/3	4/4	2/2
6	11	7/7	4/4	–
7	11	3/3	4/4	4/4
Total	58	20/20	26/26	12/12

Isolated metanephric mesenchyme was recombined with dorsal spinal cord from the same embryo on a nucleopore filter. Induction was monitored by bright field microscopy. Embryos of a total of seven litters were analysed in this way.

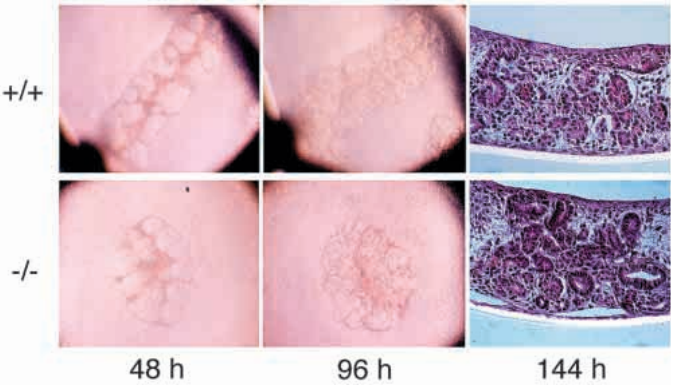

Fig. 1. Induction of tubulogenesis in wild-type and *Wnt-4* mutant metanephric mesenchyme by dorsal spinal cord. Isolated metanephric mesenchyme and dorsal spinal cord from the same 11.5 day embryo were recombined on a nucleopore filter. After 48 hours and 96 hours cultures were monitored as whole mounts using bright field microscopy, after 144 hours as histological sections. Induction of tubulogenesis in wild-type and *Wnt-4*/*Wnt-4* mutant metanephric mesenchyme were indistinguishable. After 48 hours induction was visible as bright round zones of condensing mesenchyme. After 96 hours zones of condensing mesenchyme had undergone epithelialisation to form complex tubules. At 144 hours epithelial tubular structures and glomeruli indicate a full differentiation of induced tubules in either case.

Various Wnts are sufficient to trigger tubulogenesis

In order to investigate whether *Wnt-4* is sufficient for tubulogenesis, and if this property is shared by other *Wnts* normally expressed in the spinal cord (Parr et al., 1993), we established NIH3T3 cell lines stably expressing various *Wnt* genes and performed direct recombinations between *Wnt* expressing cells and isolated wild-type metanephric mesenchyme. Co-cultures with *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-7a* and *Wnt-7b* expressing cells developed on schedule with those induced by spinal cord, forming complex epithelial tubules with differentiated glomeruli at 144 hours (Fig. 2; Table 2). In contrast, cells expressing *Wnt-5a*, *Wnt-11* or a *lacZ* control did not support survival and differentiation of metanephric mesenchyme (Fig. 2; Table 2). Although we cannot exclude the possibility that the *Wnt-5a* and *Wnt-11* cell lines did not produce the respective Wnt protein, *Wnt* mRNA expression was comparable amongst the various lines.

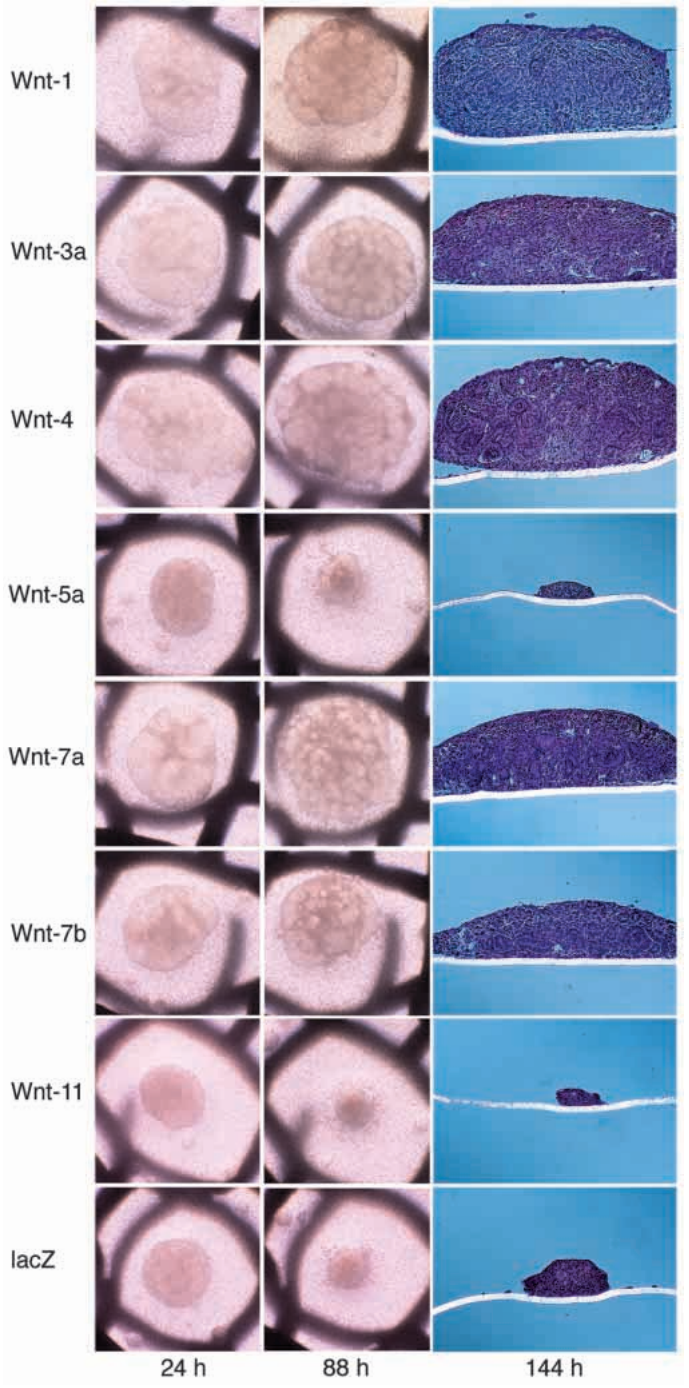

Fig. 2. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing various *Wnt* genes. Brightfield microscopy (24 hours, 88 hours) and histological analysis (144 hours) of direct recombinations between NIH3T3 cells expressing *Wnt* genes and isolated metanephric mesenchyme. After 24 hours bright zones indicating induction are visible in recombinants between wild-type mesenchyme and *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-7a* and *Wnt-7b* expressing cells, respectively. These condensing mesenchymal cells have epithelialised and formed tubular structures after 88 hours. After 144 hours highly elaborate tubular structures are apparent. In contrast, cells expressing *Wnt-5a*, *Wnt-11*, or as a control *lacZ*, respectively, did not support survival and differentiation of metanephric mesenchyme.

Table 2. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing various *Wnt* genes

Cell line	#Induced/#Total
Wnt-1	16/16
Wnt-3a	14/14
Wnt-4	14/14
Wnt-5a	0/12
Wnt-7a	12/12
Wnt-7b	11/12
Wnt-11	0/12
lacZ	1/14
Mesenchyme	1/12

Isolated metanephric mesenchyme from 2-3 11.5 day kidneys was placed on top of NIH3T3 cells expressing various *Wnt* genes. As control mesenchymes were placed on NIH3T3 cells expressing *lacZ* and were placed onto filter without underlying cell layer, respectively. Induction was scored after 6 days using the morphological appearance of the culture (as documented by brightfield microscopy), and histological analysis of selected samples. For each cell type 2-3 independent experiments were performed.

These experiments suggest that a subset of *Wnt g*enes, which includes *Wnt-4* and not *Wnt-11*, are able to induce tubule formation. As all of these are expressed in the spinal cord at the time of assay (Parr et al., 1993), it is likely that these signals account for the robust inducing activity of the spinal cord. However, of these *Wnt-4* is the only member which is actually expressed in and which is also required for mesenchymal aggregation (Stark et al., 1994).

Wnt-4 triggers the complete program of tubular differentiation

In order to investigate whether Wnt-4 is sufficient to induce fully developed tubules in isolated metanephric mesenchyme we analysed the induction properties of NIH3T3 cells expressing *Wnt-4* more carefully by assessing the differentiation state of the mesenchyme by histological and molecular criteria. Tubule induction by spinal cord was classically shown to work through polycarbonate filters of a certain pore size (Grobstein, 1956). We seeded *Wnt-4* expressing cells on one filter and separated these cells from isolated mesenchyme by another filter of 1 μm pore size. Induction took place transfilter (Fig. 3), though with a delay when compared with direct recombinants. Further, transfilter cultures where also less compact and flatter. Zones of condensed mesenchyme formed after 24 hours, aggregating mesenchyme and simple epithelial bodies after 48 hours, epithelial tubules after 96 hours and glomeruli by 8 days.

To verify that these morphological features reflected an underlying differentiation of the mesenchyme in response to Wnt-4 we examined the temporal and spatial expression of a number of molecular markers (Fig. 4). *WT-1* was broadly expressed after 1 day refining to small intensely labeled foci by 8 days of culture. This expression profile parallels the expression of this gene during metanephric development (Pritchard-Jones et al., 1990) which is first expressed in condensing mesenchyme, then in simple epithelial bodies before it is restricted to podocytes in the glomeruli. In the recombinants *WT-1* expression seems to mark glomeruli after 8 days in agreement with the histological analysis. Like *WT-1*, *Pax-2* is also broadly expressed after 1 day, but becomes restricted to epithelial bodies and is lost after 4 days reflecting

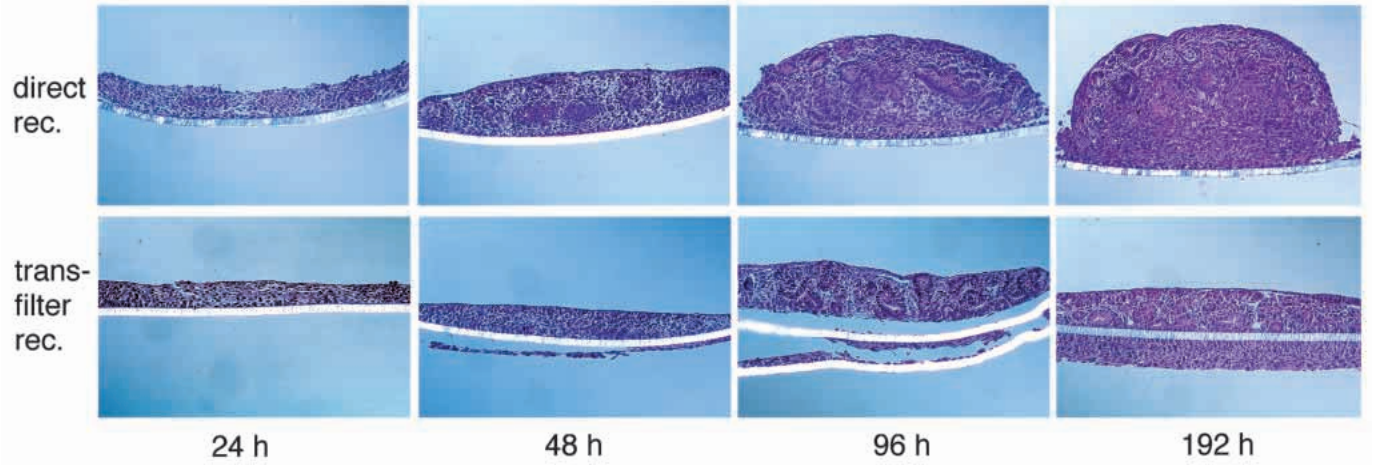

Fig. 3. Histological analysis of tubule induction in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4*. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme directly (direct rec.) and in a transfilter set-up (transfilter rec.), respectively, and analysed by sectioning and histological staining after 24 hours, 48 hours, 96 hours and 192 hours of culture, respectively. Tubule induction in transfilter assays appeared slightly delayed compared to direct recombinations. After 48 hours zones of condensed and aggregated mesenchyme, after 96 hours epithelial tubules were apparent. After 8 days in culture fully differentiated tubular structures including glomeruli were noticed.

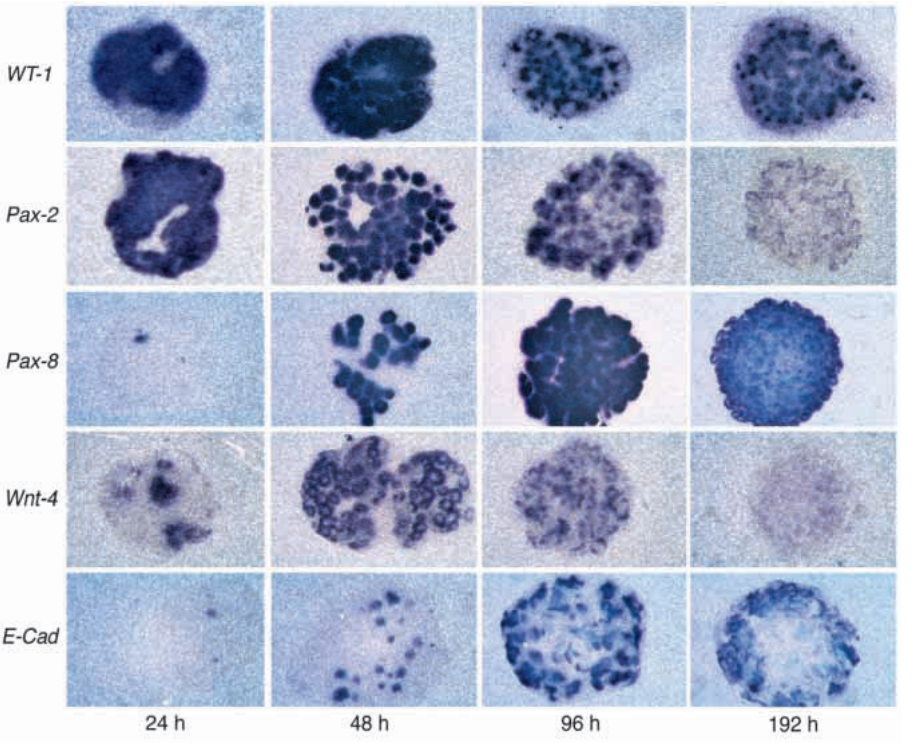

Fig. 4. Marker analysis of tubule induction in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4*. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up and scored for marker expression by in situ analysis after 24 hours, 48 hours, 96 hours and 192 hours of culture, respectively. Expression of *WT-1*, *Pax-2*, *Pax-8*, *Wnt-4* and *E-cadherin*, respectively, were in accordance with expression data known from in vivo and in vitro studies of tubular differentiation. See text for details.

initial expression in condensing metanephric mesenchyme, continuing expression in simple epithelial bodies and subsequent down-regulation as glomeruli start to differentiate (Dressler et al., 1990). *Wnt-4* is expressed in aggregating mesenchyme, in the epithelial bodies which they generate and is subsequently down-regulated as these mature into S-shaped bodies (Stark et al., 1994). *Pax-8*, a paired-box transcription factor, has a similar early expression to *Wnt-4* which has been shown to depend on Wnt-4 activity (Plachov et al., 1990; Stark et al., 1994). In cultures, *Wnt-4* is transiently expressed between 24 hours and 96 hours, peaking at 48 hours. *Pax-8* expression extends longer in S-shaped bodies. *E-cadherin* which is expressed in the proximal tubules in vivo (Vestweber et al., 1985) is present after 24 hours and is maintained consistent with the differentiation of epithelial tubules along the proximal distal axis. Thus, both the molecular and morphological analysis indicate that tubulogenesis in isolated metanephric mesenchyme induced by Wnt-4 follows a similar progression to that observed in the metanephric kidney in vivo. At the stage at which we isolate the metanephric mesenchyme

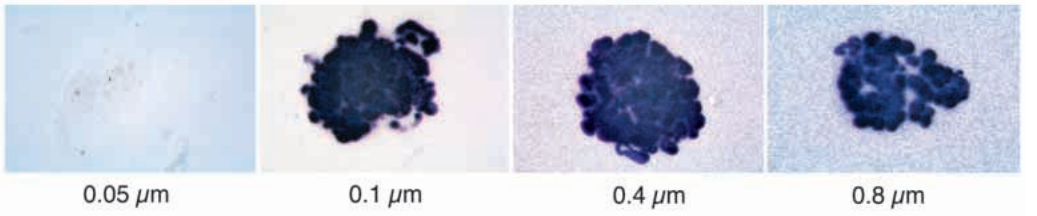

Fig. 5. Pore size dependence of tubule induction by *Wnt-4* expressing cells. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up with various pore sizes of the nucleopore filter. Induction was scored after 4 days by *Pax-8* expression in whole mount in situ analysis. Pore sizes of 0.1 μm and bigger supported full induction of metanephric mesenchyme whereas 0.05 μm pore size dramatically reduced or in most cases abolished induction.

(T-stage of the ureter) initial ureteric signaling has occurred as evidenced by the condensation of mesenchyme around the tip of the ureteric bud. However, this alone is insufficient to support mesenchymal survival and tubulogenesis. In contrast, *Wnt-4* expressing cells are sufficient to support these processes. In order to exclude the possibility that Wnt-4 only maintains *Wnt-4* expression in the isolated mesenchyme we also used mesenchyme derived from 10.75 d p.c. embryos when the ureter bud had just emerged and the metanephric mesenchyme can first be identified. *Wnt-4* expressing cells triggered the complete differentiation program as judged by brightfield observation (12 out of 12 cases) and by molecular criteria (*Pax-8* induction in 8 out of 8 cases after 4 days of culture).

Wnt-4 signaling requires cell contact

We explored tubule induction in isolated metanephric mesenchyme with respect to filter pore size. Experiments using the spinal cord as a heterologous inducer suggest a requirement for cell-cell contact as pore sizes below 0.1 μm, which prevent the extension of cytoplasmic processes, block induction (Saxen, 1987). Transfilter cultures with *Wnt-4* expressing NIH3T3 cells were performed with separating filters ranging from 0.05 μm to 1 μm in pore size, scoring for *Pax-8* induction after 4 days of culture. Pore sizes of 0.1 μm and above supported induction whereas pores of 0.05 μm almost completely abolished induction leading to degeneration of the mesenchyme (Fig. 5; Table 3). Further, we were not able to induce tubulogenesis with supernatants from *Wnt-4* expressing cells (data not shown). Thus, Wnt-4 may act as an insoluble cell bound factor. Such a mode of action agrees well with the known tight association of Wnt proteins with ECM (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Burrus and McMahon, 1995). It also makes it unlikely that Wnt-4 mediated induction occurs through a secondary, soluble factor.

Table 3. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* in transfilter assays with increasing pore size

Pore size (μm)	#Induced/#Total
0.05	3*/13
0.1	14/16
0.4	14/14
0.8	6/6
1	3/3

Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* in a transfilter set-up. Induction was scored after 4 days with in situ hybridisation analysis using *Pax-8* as a probe.

*In each of the specimens scored as induced only 1-4 spots of *Pax-8* expression were seen in contrast to 15-30 with all the other pore sizes.

Wnt-4 signaling requires sulphated glycosaminoglycans

Wnt signaling has been reported to depend on sulfated glycosaminoglycans (GAG)s which might act as cofactors for binding the Wnt protein on the responsive cell (Kispert et al., 1996; Hacker et al., 1997). We were therefore interested to see whether the presence of 30 mM $NaClO_3$, which is known to be a competitive inhibitor of sulfation of GAGs (Kjellen and Lindahl, 1991), may influence induction in our assay. We added this compound at the start of transfilter culture, or 24 and 48 hours later. As a control chlorate was omitted completely. *Pax-8* expression was again scored as a marker for tubule induction after 4 days of culture (Table 4; Fig. 6). When chlorate was added at 0 hours mesenchyme degenerated and *Pax-8* expression was consequently negative. However, addition of chlorate after 24 hours did not influence *Pax-8* expression. Hence, GAGs do not seem to be involved in tubule maturation and differentiation, in agreement with other reports (Davies et al., 1995). Tubule induction does, however, depend on sulfated GAGs in the first 24 hours, the period essential for complete induction by the spinal cord. Although our results point to an important role for GAGs in Wnt-4's action, their precise role remains unclear.

Wnt-4 signaling is only required to trigger tubulogenesis

In order to test whether *Wnt-4* expressing cells can rescue a

Table 4. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* in the presence of 30 mM $NaClO_3$

30 mM $NaClO_3$ added after time in culture (hours)	#Induced/#Total
0	0/19
24	12/19
48	14/17
–	12/15

Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* in a transfilter set-up. 30 mM $NaClO_3$ was added to the medium after the indicated times of setting-up the culture. Induction was scored after 4 days with in situ hybridisation analysis using *Pax-8* as a probe.

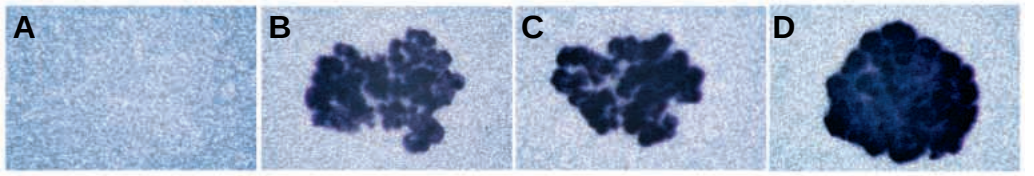

Fig. 6. Glycosaminoglycan dependence of tubule induction by *Wnt-4* expressing cells. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up with addition of 30 mM $NaClO_3$ in the medium. Induction was scored after 4 days by *Pax-8* expression using whole mount in situ hybridisation analysis. Addition of 30 mM $NaClO_3$ after 24 hours (B) or 48 hours (C) of culture did not affect tubule induction compared to untreated controls (D) whereas administration of 30 mM $NaClO_3$ at the beginning of the culture (A) abrogated tubule induction completely.

Wnt-4 mutant mesenchyme we performed direct recombination experiments in culture. Interestingly, *Wnt-4* expressing cells were equally efficient at inducing tubule formation in wild type or *Wnt-4* mutant metanephric mesenchyme (Table 5). Brightfield microscopy and histological analysis of specimen after 6 days in culture revealed the full spectrum of tubular differentiation including glomerulus formation (Fig. 7). Thus, as with spinal cord mediated induction, *Wnt-4* expression in the mesenchyme itself is not required for tubule formation, but supplying *Wnt-4* in adjacent cells is sufficient to trigger the inductive process. These results suggest that whereas *Wnt-4* plays an essential role in initial tubulogenesis, it may not be required for later morphogenesis of the tubule. In agreement with the fact that various *Wnt* genes can trigger tubulogenesis in wild-type mesenchyme *Wnt-1* expressing cells were also sufficient to trigger tubulogenesis in mesenchyme mutant for *Wnt-4* (Table 5).

Table 5. Induction of tubulogenesis in *Wnt-4*/*Wnt-4* mutant metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* or *Wnt-1*

		#Induced/#Total		
Exp. #	# Recombinants	+/+	*Wnt-4*/+	*Wnt-4*/*Wnt-4*
With NIH3T3 cells expressing *Wnt-4*:				
4	42	7/7	18/18	17/17
With NIH3T3 cells expressing *Wnt-1*:				
2	20	5/5	11/12	3/3

Isolated metanephric mesenchyme from embryos of *Wnt-4*/+ intercrosses was placed on top of NIH3T3 cells expressing *Wnt-4* or *Wnt-1*. Induction was scored after 6 days using the morphological appearance of the culture (as documented by brightfield microscopy).

DISCUSSION

Mammalian metanephric development is a highly coordinated process characterised by a continuous interaction of the epithelial ureter and the surrounding metanephric mesenchyme. Classical organ culture experiments have pointed to the fact that these two compartments achieve coordinated development by use of reciprocal signaling systems. First, the metanephric blastema induces a bud from the adjacent nephric duct which invades and branches into the mesenchyme. This process appears to be mediated by GDNF which is secreted by the metanephric mesenchyme and sensed by the c-ret/GDNFRa receptor complex on the ureter (see Sariola and Sainio, 1997, for review). Next, the metanephric mesenchyme undergoes tubulogenesis upon a permissive stimulus from the ureter.

Since ureter itself is a weak inducer, indeed, ureter has never been proven to induce in transfilter assays of tubule induction, heterologous tissues, most notably dorsal spinal cord, have been used for a long time to mimic ureteric signaling. More recently, several studies have implicated *Wnt* genes in ureteric signaling and in dorsal spinal cord activity. The four key observations are:

(1) *Wnt-11* is expressed in the ureter tips (Kispert et al., 1996).

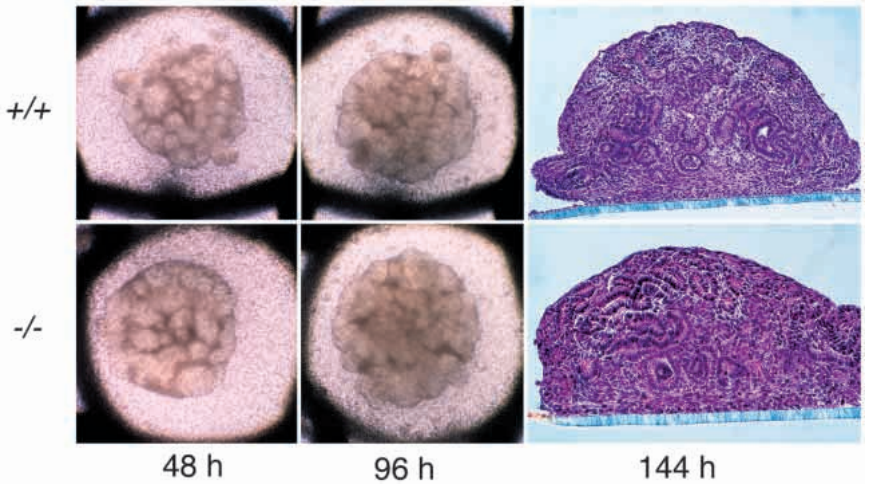

Fig. 7. Induction of tubulogenesis in wild-type and *Wnt-4* mutant metanephric mesenchyme by NIH3T3 cells stably expressing *Wnt-4*. Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* which were supported by a nucleopore filter. After 48 hours and 96 hours cultures were monitored as whole mounts using bright field microscopy, after 144 hours as histological sections. Induction of tubulogenesis in wild-type and *Wnt-4*/*Wnt-4* mutant metanephric mesenchyme by *Wnt-4* expressing cells were indistinguishable.

(2) Cells expressing *Wnt-1* can induce tubulogenesis (Herzlinger et al., 1994).

(3) *Wnt-4* is a critically required for epithelialisation of condensed mesenchyme (Stark et al., 1994).

(4) Various *Wnt* genes are expressed in the spinal cord (Parr et al., 1993).

With respect to these findings our experimental data are of dual importance. First, they point to the nature of signals emitted by the heterologous inducer spinal cord and clarify the action of these signals with respect to ureter signaling. Second, they argue for a decisive role for Wnt-4 signaling in the mesenchyme, adding another signaling system to tubulogenesis, a process which has not been fully appreciated up to now.

Spinal cord signaling and Wnt-4

Classical kidney organ culture experiments have identified numerous tissues of diverse embryonic origins, which can elicit tubulogenesis in isolated metanephric mesenchyme (Unsworth and Grobstein, 1970; Saxen, 1987). As these tissues are robust inducers, they, rather than the ureter itself, have been widely used to define the process of kidney tubule induction. In our recombination experiments we have demonstrated that these inducers, exemplified by dorsal spinal cord tissue, can trigger tubulogenesis in *Wnt-4* mutant mesenchyme. Therefore, it seems unlikely that the signals emitted from the spinal cord, and possibly other heterologous inducing tissues, mimic an endogenous ureteric signal(s) which would still require *Wnt-4* for tubule formation. We suggest they act on the level of *Wnt-4* in the mesenchyme. Since dorsal spinal cord used in the assays was also mutant for *Wnt-4* other *Wnts* may replace Wnt-4 activity in the mesenchyme. By using cell lines expressing various *Wnt* genes we show that *Wnt-1*, *Wnt-3a*, *Wnt-7a*, *Wnt-7b*, all of which are expressed in spinal cord, can evoke tubulogenesis in isolated metanephric mesenchyme. The additive action of several Wnt proteins may also explain why dorsal spinal cord represents such a strong source of inducer. Further, *Wnts* are widely expressed during embryogenesis (Parr et al., 1993; Lee et al., 1995) and could explain why so many tissues can trigger tubulogenesis in isolated metanephric mesenchyme.

In summary, our results suggest a different interpretation of the use of kidney cultures to elucidate the nature of the ureteric signal involved in inducing the mesenchyme. Experiments which have used heterologous sources of tubule inducers, most notably the spinal cord, may not have been investigating the nature of ureteric signaling, but rather the mesenchymal action of signals such as Wnt-4. At present, the exact nature of ureteric signaling remains obscure. It seems clear that a primary signal from the ureter leads to survival and initial condensation of metanephric mesenchyme. This primary signal might be required for a sufficient length of time to allow auto-induction of the mesenchyme by Wnt-4. Alternatively, a secondary signal from the ureter tip might be necessary to induce *Wnt-4* expression in aggregating mesenchyme.

Our experiments argue against a role for Wnt-11 as a ureteric signal for mesenchymal aggregation. As a secreted glycoprotein expressed at the ureter tip Wnt-11 was a prime candidate for such an activity. However, we were not able to get tubulogenesis with cells expressing *Wnt-11*. Recent loss-of-function experiments also support this notion. Mice homozygous for a likely null mutation of *Wnt-11* do not exhibit an overt kidney phenotype (S. Vainio, A. Kispert and A. P. McMahon, unpublished observation). At this time, the function of *Wnt-11* is unclear.

Wnt-4 is a mesenchymal signal for tubulogenesis

Analysis of *Wnt-4* mutants has demonstrated a critical role for *Wnt-4* in kidney development. Homozygous pups die 24 hours after birth due to small agenic kidneys consisting of undifferentiated mesenchyme intermingled with collecting duct tissue. Histological and marker analysis revealed that primary condensation of mesenchymal cells around the ureter tips as well as ureteric branching occurs normally. However, mutant kidneys quickly become growth retarded and the mesenchyme remains undifferentiated lacking pretubular cell aggregates and epithelial tubules. Since kidney size as well as cell death initially remain unaffected, proliferation is unlikely to be controlled by *Wnt-4*. Rather, the lack of *Wnt-4* expression itself and of epithelial structures in the mutant mesenchyme argues that Wnt-4 may autoinduce the epithelialisation of condensed mesenchyme. In this study we have shown that mesenchymally derived *Wnt-4* is not only required but also sufficient for induction of tubulogenesis in the mammalian kidney. Judging by histological and molecular markers Wnt-4 can elicit the complete program of tubular differentiation in isolated metanephric mesenchyme. The activity of Wnt-4 contrasts with other factors thought to regulate mesenchymal development. For example, FGF (Perantoni et al., 1995) and EGF (Weller et al., 1991; Koseki et al., 1992) can both support mesenchymal survival but are not sufficient for tubulogenesis. Like Wnt-4, BMP-7 has been suggested to induce tubules (Vukicevic et al., 1996) but loss-of-function studies indicate it is not essential for tubule formation in vivo as some glomeruli form in *BMP7* mutants (Dudley et al., 1995; Luo et al., 1995). In contrast, loss of *Wnt-4* leads to a complete absence of glomeruli.

Wnt-4 activity shows all the characteristics which have previously been ascribed to induction by dorsal spinal cord tissue. Signaling is cell-contact dependent. Below a certain pore size in the transfilter assay the formation of cellular processes which penetrate the filter pores is inhibited and isolated mesenchyme degenerates. Cell contact dependence agrees well with the fact that Wnt proteins interact with extracellular matrix (ECM) components (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Burrus, 1994; Burrus and McMahon, 1995). The chlorate inhibition experiments defines a critical period of 24 hours for induction. This is in agreement with classical studies which showed that the inducer tissue can be removed after this time with tubulogenesis proceeding undisturbed (Saxen, 1987). Possibly, every cell has to get in contact with the inducer to initiate tubulogenesis. We suggest that further differentiation, i.e. aggregation and epithelialisation of mesenchymal cells is only initiated when a certain number of cells (a small community) has received the Wnt-4 signal. At this time mesenchymal development is independent of ureteric signaling.

Chlorate acts as a competitive inhibitor of sulphotransferases and inhibits the sulphation of glycosaminoglycans (Kjellen and Lindahl, 1991). Our inhibition studies point to a critical role of these ECM compounds in tubulogenesis. Numerous studies have shown that branching morphogenesis of the ureter as well

as branching of other epithelia requires an intact ECM (Platt et al., 1987; Davies et al., 1995; Roskelly et al., 1995; Kispert et al., 1996). Since presence of chlorate after 24 hours does not influence tubulogenesis GAGs do not seem to be involved in tubule maturation and differentiation, in agreement with other reports (Davies et al., 1995). Tubule induction does, however, depend on sulfated GAGs in the first 24 hours, the period essential for complete induction by the spinal cord. Although our results point to an important role for GAGs in Wnt-4's action, their precise role remains unclear. It is possible that chlorate acts on the Wnt secreting cell. Recent experiments in the fly which indicate that GAG synthesis is also required for *wingless* (*Drosophila Wnt-1*) signaling (Reichsman et al., 1996; Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) suggest that GAGs are required in the responsive cell for signal transduction. Most likely as is the case for FGFs, GAGs may act as co-receptors, facilitating presentation or increasing the local concentration of the ligand.

Wnt-4 expression in the metanephric mesenchyme is initiated in the aggregating mesenchyme and maintained in the comma shaped bodies before it is downregulated in S-shaped bodies. Therefore, *Wnt-4* might have a later function in tubulogenesis which is masked in the earlier requirement to form a tubule. However, cells expressing *Wnt-4* are sufficient to induce tubulogenesis in *Wnt-4* mutant mesenchyme arguing that *Wnt-4* expression may not have a later role. It seems that Wnt-4 probably acts as a trigger to start an intrinsic program in the mesenchymal cells which then proceed to form complex nephron like structures. From our experiments it is not clear whether in vivo Wnt-4 acts in an autocrine or a paracrine fashion. However, it seems clear that the signal acts very locally. Interestingly, although these experiments demonstrate that *Wnt-4* is itself a target of its own signaling activity, induced metanephric mesenchyme is not able to induce tubule formation when recombined with uninduced mesenchyme. This lack of homeogenetic induction could be taken as evidence against Wnt-4 acting as a mesenchymally derived tubule inducer. However, there are many other explanations for this result. For example lateral inhibition mechanism as is commonly used in fine grained patterning may limit Wnt-4 action. Alternatively, levels of induced Wnt-4 may not be high enough to induce more mesenchymal cells. More directly, a number of potential Wnt antagonists have been described that could potentially modulate Wnt-4 signaling (Moon et al., 1997; Rattner et al., 1997; Glinka et al., 1998).

Our view is that kidney tubulogenesis is a multi-step process with a hierarchy of signaling systems. A permissive signal from the ureter to the mesenchyme triggers survival and tubulogenesis in the mesenchyme, signals from the mesenchyme to the ureter are required for proliferation and branching morphogenesis of the ureter. Most likely, other signaling systems within the ureter are required for local adhesion and proliferation changes which may mediate branching morphogenesis, and within the mesenchyme for tubulogenesis as evidenced by the role of *Wnt-4*. Taking the complexity of developmental events in the mesenchyme into account, it is conceivable that additional signaling systems control the ratio between interstitial and metanephrogenic cells, between condensing and non-condensing cells, and the maintenance of the mesenchymal stem cells in the periphery. Undoubtedly, the functional analysis of other secreted factors will shed light on these diverse events in kidney development.

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