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Wnts as kidney tubule inducing factors

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ABSTRACT Since the discovery that inductive tissue interactions regulate nephrogenesis, one of the aims has been to identify the molecules that mediate this induction. The small size of embryonic tissue has limited the possibilities to identify the inducers biochemically, even though such efforts were directed to study, e.g. neural induction (for a comprehensive review, Saxén and Toivonen, *Primary embryonic induction,* Academic Press, London, 1962). The rapid progress in molecular biology made it possible to identify genes from minute amounts of tissue and provided techniques to generate recombinant proteins to assay their action in classic experimental systems. This led to the identification of some signals that are involved in primary and secondary inductive interactions during embryogenesis. Here, we will review evidence suggesting that secreted signaling molecules from the Wnt gene family mediate kidney tubule induction..

KEY WORDS: *Wnt, frizzled, frzb-2, tubule induction, Wnt-4 knock out, epithelial-mesenchymal interactions*

Classic and modern assay systems to screen for tubule inducing signals

The work of Glifford Grobstein (1953) demonstrated that kidney development depends on interactions between the ureter bud and metanephric mesenchyme (Fig. 1A). If the ureter was removed no tubules appeared in the metanephric mesenchyme *in vitro*. These findings indicated that during ingrowth of ureter bud into kidney mesenchyme, the epithelial cells secrete substances that trigger epithelial transformation of mesenchymal cells. By assaying activities of heterologous inductors, Grobstein and later Saxén and his associates concluded that tissues other than ureter bud also induced tubules (for a comprehensive review see; Saxén, 1987). Ureter turned out to be a relatively weak inducer whereas a dorsal part of the embryonic spinal cord was a very potent one (Fig. 2A). Such studies lead to the conclusion that a permissive inducer was expressed by several other embryonic tissues as well. By using varying pore sizes in a transfilter assay developed by Grobstein and later applied by Saxén and his colleagues, the conclusion was reached that cell contacts, or close proximity is necessary for transmission of tubule induction and that induction does not occur if only cellular processes are exposed to mesenchyme (Saxén, 1987). These conclusions are supported by more recent genetic studies (for review see Vainio and Müller, 1997).

Even though many embryonic tissues induce tubules, these tissues are still small and biochemical purification of inductive fractions was not an attractive approach. Furthermore, it was not known if the inductive substances were present in minute amounts, if they were unstable for purification, or fold into an active conformation only locally and in a highly regulated manner.

A step onwards was to screen inductive activities of cell lines. Such an approach was successful e.g. in attempts to characterize mesoderm inducers (Sokol *et al.,* 1990). Auerbach (1977) had preliminary data of conditioned media from neural teratoma cells that induced tubules. As a more direct approach, Barasch and his colleagues (1996) immortalized cells from the ureter bud. Conditioned media from these cells rescued apoptosis of kidney mesenchymal cells but did not induce tubules. However, when a trypsinized pellet of the same cells was used, tubules were induced. The authors concluded that two distinct ureter signals are involved, a secreted one to rescue apoptosis and a diffusion-limited basolateral molecule to induce the epithelial transformation program.

A more direct way to assay for tubule inducing signals was to test activity of recombinant proteins. When evidence became available that growth factors may be involved in control of proliferation and cell fate choices, their activity in the kidney tubule induction assay was also tested. The Perantoni laboratory screened extracts of pituitary gland and found that these, in conjunction with FGF-2, induced tubules. This was in line with the two signal models for induction (Perantoni *et al.*, 1995). Screening for activities of a panel of other growth factors only revealed a role for EGF (Weller *et al.,* 1991) and FGF-2 (Barasch *et al.,* 1997) in control of apoptosis during kidney development, but neither of them induced tubules (for secreted factors present in the kidney see http://www.ana.ed.ac.uk/anatomy/database/ kidbase/ kidhome.html).

Abbreviations used in this paper: PG, proteoglycans; GAG, glycosaminoglycans.

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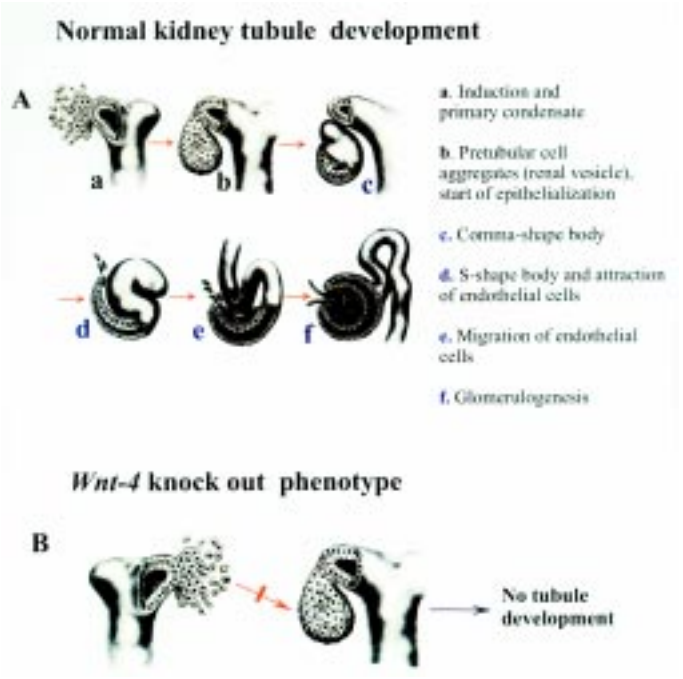

Fig. 1. **Schematic drawing of normal stages in kidney tubule development (A). (B)** *Step at which the process is disrupted in Wnt-4 knock mice. (Figures adapted from Saxén, 1987).*

Wnts: a family of secreted signals regulates key developmental steps

The Wnt gene family was among the last of the so far identified growth and differentiation factors to be tested in the kidney model system. Wnt signals consist of a large secreted glycoprotein family of growth and differentiation factors that regulate key developmental steps; e.g. Wnt-3a is necessary for gastrulation, Wnt-1 for midbrain development, and Wnt-7a for limb D-V polarity (for review see Lee *et al.,* 1995). Wnts consist of 350-400 amino acids with 50-60% identity. The first one of the Wnts to be discovered, Wnt-1, encodes a protein of 41-44 KDa and is the ortholog of *Drosophila wingless*. Wnt-1 contains 23 cysteines, and 22 of these are conserved in other mouse Wnt proteins as well. Secreted Wnt binds to the cell surface and extracellular matrix, but purification of biologically active Wnt members have failed. Reception of the Wnt signal involves frizzled, which makes it a candidate Wnt receptor. Several components in the signal transduction pathway have been identified and a model of how Wnt signaling regulates expression of downstream genes has been determined (for review; Cadigan and Nusse, 1997; for updates see; http://www.stanford.edu/~rnusse/wntwindow.html;).

Sequential activation and non-overlapping expression of *Wnt* genes during kidney development

The finding that Wnt signaling plays important roles in different developmental systems raised the possibility that these signals may also be involved in kidney development. Screening of expression of the family members revealed sequential and non-overlapping expression (Fig. 3). *Wnt-11* gene expression is upregulated in the ureter bud at the initiation of kidney development, around 10.5 dpc. Thereafter *Wnt-11* is expressed in the newly formed ureter tips during kidney morphogenesis. The *Wnt-7b* gene is activated a few days later (around 13.5 dpc) also in the ureter bud. *Wnt-7b* expression is confined to the collecting duct and is excluded from the tip area where *Wnt-11* is expressed. *Wnt-4* expression appears in the kidney mesenchyme at around 11 dpc and thereafter it is confined to the pretubular cell aggregates (renal vesicles) that will form the nephrons (Kispert *et al.,* 1996). Hence, expression of these *Wnt* genes is sequential and non overlapping during kidney development. This suggests unique developmental role/s for them.

Wnt-4 is a necessary signal for development of the nephron

Initial hints that Wnts are involved in inductive interactions in the kidney came from genetic studies. When Wnt-4 function was inactivated *in vivo* by gene targeting, homozygous newborn mice carrying a likely null allele had small kidneys. Closer studies of kidneys of a newborn mouse demonstrated lack of glomeruli and that the mesenchyme remains morphologically undifferentiated.

In situ hybridization analysis revealed that *Wnt-4* gene expression is upregulated in each renal vesicle that is induced by ureter tip to generate the nephron. This expression was consistent with the knock-out phenotype. Closer studies demonstrated that kidneys

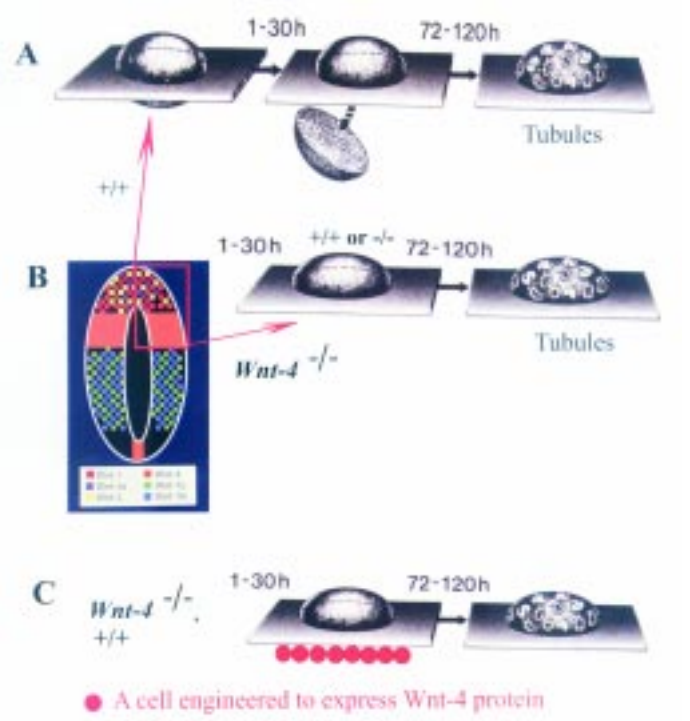

Fig. 2. Outline of the transfilter model system used to assay tubule induction *in vitro* (A). (B) *Spinal cord expresses a panel of Wnt genes. The dorsal piece of spinal cord of Wnt-4 knock out (-/-) embryos rescues tubule development of mutant mesenchyme (-/-). It also induces tubules in wild type (+/+) mesenchyme.* **(C)** *Cells that express Wnt-4 induce tubules in the same manner as spinal cord in (B) (Kispert et al., 1998). (Figures adapted from Saxén, 1987 and Parr et al., Development. 119: 247, 1993).*

defective for *Wnt-4* signaling do not undergo mesenchymal-epithelial transformation, which is necessary for nephrongenesis. Screening of expression of other genes important for kidney development demonstrated that *WT-I, N-Myc* and *Pax 2* were still expressed in the mutant. As expression of these genes is induced as an early response to tubule induction, the data suggest that Wnt-4 signaling may not control the earliest steps of kidney development but that Wnt-4 comes into action to control formation of the pretubular cell aggregates (Fig. 1B).

Wnt-4 may regulate tubule development via Pax 8, a homeobox containing a transcription factor from the *Pax* gene family. *Wnt-4* expression precedes that of *Pax 8* and their expression overlaps in the pretubular cell aggregates. Further support that Wnt-4 may regulate tubule morphogenesis via Pax proteins is that *Pax 8* gene expression is perturbed in *Wnt-4* mutant kidneys (Fig. 4; Stark *et al.,* 1994). Pax gene expression correlates with *Wnt* gene expression also in the developing midbrain (Lee *et al.,* 1995). It remains to be directly shown whether *Pax* genes regulate Wnt expression in the kidney.

One of the downstream consequences of induction in the kidney is increased adhesion of mesenchymal cells. Upregulation of *Wnt-4* expression correlates to the formation of cellular aggregates that also express E-cadherin (uvomorulin). As β-catenin is in the Wnt signal transduction pathway and also regulates cadherin mediated cell adhesion, one likely function for Wnt-4 is to regulate cell adhesion. β-catenin, which is present in kidney mesenchyme (Vainio *et al*., unpublished data), may be in the nucleus in complex with known targets of Wnt signals, such as Lef/TCF, to regulate gene expression. After induced Wnt-4 expression, β-catenin may be released from the Lef/TCF complex, shuffle back into the cytoplasm and bind to cytoplasmic domains of cadherins, such as E-cadherin, to mediate adhesion. Even though the precise biochemical mode of action of Wnt-4 remains to be shown, it is clearly a key signal required to assemble the nephron.

Evidence that Wnt-4 acts as a classic tubule inducing signal

A traditional view of tubule induction is as follows: each ureter tip secretes an inductive signal that triggers the tubulogenic program in mesenchymal cells in contact with the ureter. Being exposed to the signal, the induced cells start to migrate, proliferate and adhere to each other by homotypic cell-cell interactions necessary for subsequent tubule morphogenesis to take place. The finding that Wnt-4 was a necessary mesenchymal signal for tubule development leads to the conclusion that, in addition to this possible initial epithelial signal, “a second signal” in the form of Wnt-4 is involved (Fig. 4). BMP-7 is a candidate signal to cooperate with Wnt-4 (Godin *et al.,* 1998; for a review see Vainio and Müller, 1997).

Besides kidney, *Wnt-4* gene is expressed in the dorsal spinal cord used in the classic transfilter assay as an inducer tissue (Stark *et al.,* 1994). Several other *Wnt* genes are expressed in the spinal cord as well (Fig. 2B). To study possible role of Wnts in induction of kidney tubules, Herzlinger *et al.* (1994) generated cells engineered to express another Wnt family member, *Wnt-1.* Coculture of these cells with kidney mesenchyme induced tubules, suggesting that Wnts may be sufficient also for tubule induction. However, *Wnt-1* is not expressed in the kidney excluding its possible role *in vivo.* Wnt-1 may mimic the action of another Wnt secreted by the ureter tip. Alternatively, it may stimulate the same mesenchymal signaling

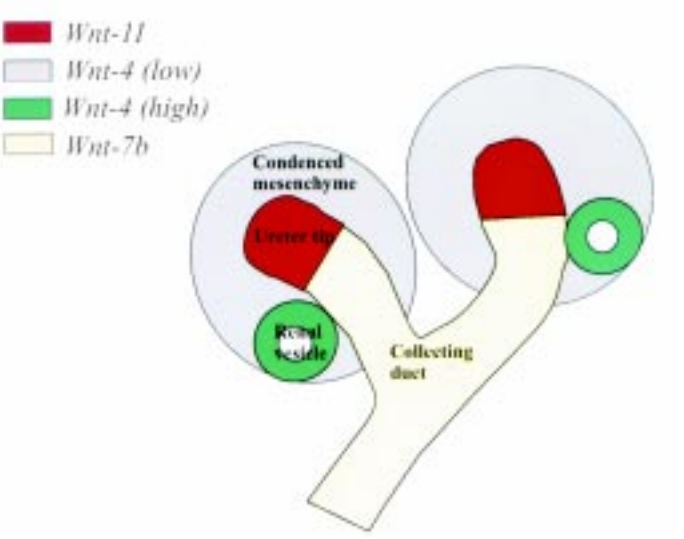

Fig. 3. A summary of Wnt gene expression in the kidney. *See text and Stark et al., 1994; Kispert et al., 1998, for details.*

pathway that is regulated by Wnt-4. Because it is not currently possible to generate functional recombinant Wnt proteins, Wnt functions were tested by using cell lines engineered to express various Wnts by retroviral vectors. Such studies indicated that, in addition to Wnt-1, Wnt3a, Wnt-7a, Wnt-7b induce tubules whereas Wnt-5a and Wnt-11 do not. As our studies demonstrated expression of only *Wnt-11*, *Wnt-4* and *Wnt-7b* genes from the currently known Wnts in the embryonic kidney, we may conclude that Wnt signaling in the kidney is redundant. This suggestion is also supported by the fact that spinal cord from *Wnt-4* knock out embryos rescued *Wnt-4* mutant mesenchyme, but it was also sufficient to induce tubules in wild type mesenchymes (Fig. 2B) (Kispert *et al.,* 1998). Hence, other Wnts present in the spinal cord and apparently synthesized in *Wnt-4* knock out tissue are likely to be able to rescue the lack of Wnt-4 (Fig. 2B). Based on these findings, it also seems clear that Wnt receptors can respond to more than one Wnt. Our recent data show that the mesenchyme expresses only a few candidate Wnt receptors of the currently known Frizzleds and include Frizzled 7 (Fig. 5 and Uusitalo *et al*., unpublished data).

As the Wnt-4 mutant mesenchyme does not express functional Wnt-4 protein due to the engineered mutation, the rescue experiment indicates that Wnt-4 signaling acts only to trigger tubule development. Hence, even though Wnt-4 is expressed in the pretubular cell aggregates during their development, Wnt-4 may not have morphogenic activity post-induction. An alternative option is that the Wnt-4 receptor is specifically downregulated from the renal vesicle or that a Wnt-4 antagonist is concurrently activated. We find evidence for both of these hypotheses. Frizzled receptor expression is lost from the renal vesicle that still expresses *Wnt-4* gene and a candidate Wnt-4 antagonist, a secreted Frizzled related protein (sFRP). *SFRP-2* expression in kidney mesenchyme correlates to that of *Wnt-4* (Fig. 3; Uusitalo *et al*. unpublished data). Even though the mechanism of *Wnt-4* action in the mesenchymal cells remains to be elucidated, the rescue experiment reveals a role for Wnt-4 in the tubule induction process.

The inductive role of Wnt-4 was directly tested by using cells engineered to express Wnt-4. Wnt-4 cells not only rescue the mutant mesenchyme but are also able to induce tubules in wild type

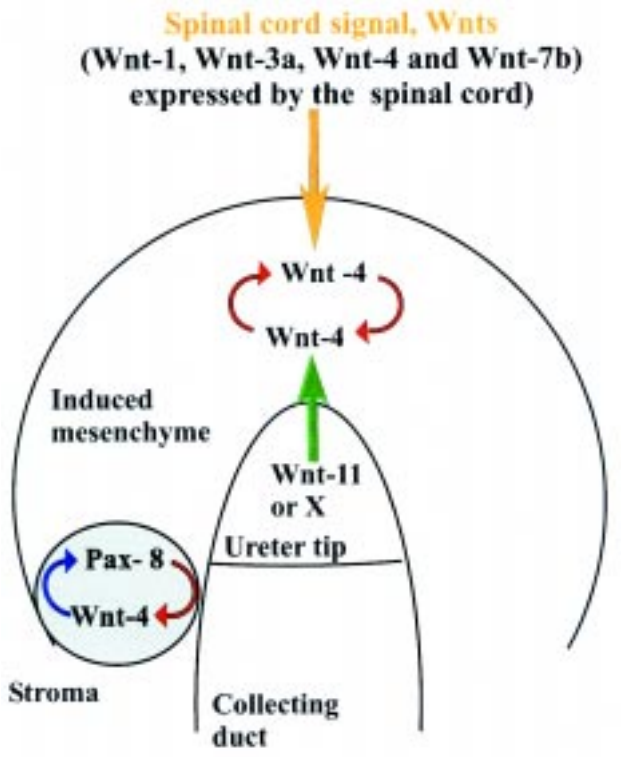

Fig. 4. A model showing how Wnt-4 operates as "a second" tubule inductive signal. *A ureter derived signal, in the form of Wnt-11 or X leads to induction of expression of Wnt-4, which autoregulates itself and triggers tubule morphogenesis. Wnt-4 signaling involves Pax 8. The spinal cord acts as an inducer tissue as it expresses a panel of Wnts including Wnt-4 which triggers the autoregulated Wnt-4 gene expression to induce tubules.*

mesenchyme (Figs. 2C and 5). How can these results be explained? The simplest conclusion is that by expressing Wnt-4 the spinal cord induces tubules because of the property of Wnt-4 to autoregulate its own expression. This is likely to be the situation as coculture of Wnt-4 producing cells with kidney mesenchyme induce also its own gene expression in the mesenchyme undergoing differentiation. What the data brings up is that the spinal cord assay may not faithfully model the ureter mediated inductive steps occurring *in vivo,* but models a downstream signaling event. The spinal cord may act as an inducer tissue as it expresses itself this downstream "second" signal (Fig. 4).

How do we explain the fact that *Wnt-4* knock out kidneys show activation of "kidney induction markers" such as *WT-I, N-Myc* and *Pax 2* in conjunction with the data that Wnt-4 cells induced tubules? This fact is well in line with the hypothesis that Wnt-4 acts as a downstream signal from the ureter one. This yet to be identified "upstream ureter signal" is still expressed in the *Wnt-4* knock out and *induces WT-I, N-MYC* and *Pax-2* gene expression. Furthermore, our data indicates unchanged expression of epithelial genes in *Wnt-4* knock out such as *Wnt-11* and *c-ret* (Stark *et al*., 1994).

Does a Wnt member also mediate inductive signaling from ureter to mesenchyme?

As *Wnt-7b* and *Wnt-11* are expressed only in the ureter, could these mediate also the "upstream" signaling event in the kidney to initially trigger tubule development via Wnt-4? *Wnt-7b,* which induces tubules, is not expressed in a pattern that is expected from an endogenous inducer, as it is not expressed at the very tip of the ureter, which is the source of the initial inducer (Fig. 3 and our unpublished data). This may exclude Wnt-7b as an endogenous inducer but calls for genetic experiments to test its role. Being expressed at the ureter tips, Wnt-11 is another candidate. However, as already mentioned, Wnt-11 did not induce tubules in the same assays where several other Wnts did (Kispert *et al.,* 1998). It remains to be seen if Wnt-11 plays a role in kidney development or if signals other than Wnts mediate early inductive tissue interactions in the kidney.

Kinetics and cell contacts in Wnt mediated tubule induction

By using the transfilter assay, Saxén and his colleagues defined a minimal induction time for tubules by removing the inducer tissue (embryonic spinal cord) at different time points from contact with mesenchyme. Such studies suggested that a 24 h induction pulse is sufficient for commitment to tubular cell lineage (Saxén, 1987). The kinetics of Wnt-4 cell mediated induction follows rather well that observed with the spinal cord. Furthermore, the cut off level for spinal cord mediated tubule induction is 0.05 μm. This pore size prevented also Wnt-4 mediated induction (Kispert *et al.,* 1998) and is consistent with the model that cell-cell contacts or proximity are necessary also for Wnt mediated induction.

Proteoglycans are required for Wnt signal transmission

Wnt signal transmission may depend on close cellular proximity as they bind to heparan sulfate decorated molecules. In the kidney, *Wnt-11* gene expression depends on glycosaminoglycans (GAG) as gene expression is rapidly lost if GAG synthesis is perturbed (Kispert *et al*., 1996). By using the transfilter assay and Wnt-4 expressive cells, it was shown that proteoglycans (PGs) are necessary for Wnt signal transmission during the 24 h tubule induction period but not for subsequent tubule morphogenesis.

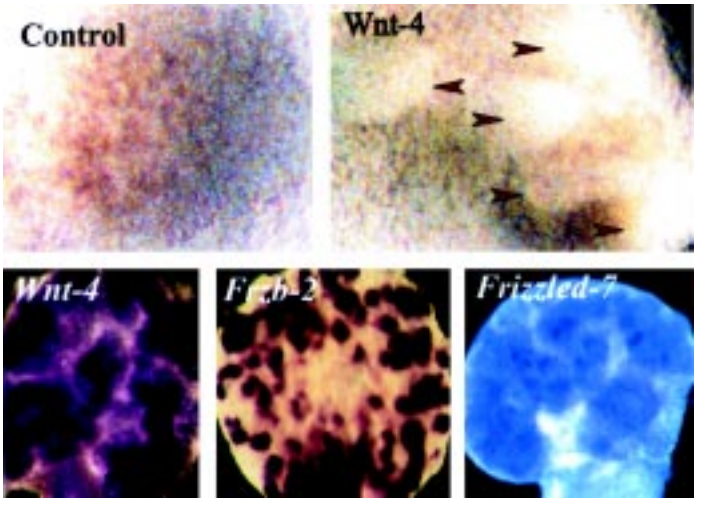

Fig. 5. Wnt-4 pathway in kidney tubule induction and development. *3T3 cells infected with a retrovirus carrying a Wnt-4 cDNA devoid of the initiation cordon, do not induce tubules (control) whereas those infected with full length Wnt-4 do (red arrowheads) 48 h culture. Wnt-4 expression overlaps with that of SFRP-2 (Frzb-2) and Frizzled-7 which are candidate genes in the Wnt-4 signaling pathway.*

Administration of $NaClO_3$ during the first 24 h culture period blocked induction but not tubule development if $NaClO_3$ was added post induction (Kispert *et al.,* 1998). This finding suggests that in the kidney, Wnt-4 signaling depends on PGs. Genetic experiments in *Drosophila melanogaster* provided evidence that enzymes in the GAG biosynthesis are in the wingless signal transmission pathway (Hacker *et al.,* 1997). One candidate PG involved in the Wnt induction is Syndecan-1. Syndecan is expressed in the ureter and its expression is induced during the 24 h inductive period (Vainio *et al.,* 1992). Syndecan binds at least FGFs, but its affinity to Wnts is currently unknown (Bernfield *et al.,* 1993). It will be interesting to see if cell surface PGs, such as Syndecans or Glypicans have a function to concentrate different growth factors and contribute to selection of cell fates as a result of their interactions. The function of PGs may be to localize and concentrate Wnts. Alternatively, they may have a more active role and modify conformation. Wnts, PGs may also operate in the presentation of the Wnt ligand to its candidate frizzled receptor. The findings that Wnt signaling plays an essential role in kidney tubule induction has opened the way for detailed molecular analysis.

Future directions

Even though the genetic data strongly suggest that Wnts mediate kidney tubule induction, induction with recombinant Wnt protein would be a final proof for this. The genetic approach has proven to be useful to study kidney development (for a review see Vainio and Müller, 1997) and allows new techniques to be developed to study e.g. Wnt signal transduction. Finally, current Wnt data still leaves the ureter derived upstream signal/s uncharacterized. Fortunately, we are now equipped with efficient methods to address the ureter inducers as well. It will be interesting to learn how transmission of induction *in vivo* is concurrently coordinated with the molecular control of ureter branching and pattern formation within the metanephric mesenchyme and how Wnt-4 cells trigger epithelial development at the molecular level.

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