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GDNF and its receptors in the regulation of the ureteric branching

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ABSTRACT Recent transgenic and organ culture experiments have inevitably shown that glial cell line-derived neurotrophic factor (GDNF) is a mesenchyme-derived signal for ureteric budding and branching. The signalling receptor complex for GDNF includes a dimer of Ret receptor tyrosine kinase and two molecules of GDNF family receptor α1. α-receptors are not only needed for the ligand binding and Ret activation, but they might mediate signals without Ret. While GDNF is clearly required for ureteric branching, tissue recombination studies have shown that it is not sufficient for the completion of ureteric morphogenesis, and other signalling molecules are needed. Different experimental models have resulted in somewhat contradictory results on their molecular identity, but transforming growth factor-β1, - β2, fibroblast growth factor-7 and hepatocyte growth factor form, obviously among others, a redundant set of growth factors in ureteric differentiation. Three other members of the GDNF family, neurturin, artemin and persephin, are also expressed in the developing kidney, and at least neurturin and persephin promote ureteric branching *in vitro*, but their true *in vivo* roles are still unclear.

KEY WORDS: *GDNF, neurturin, artemin, persephin, Ret receptor tyrosine kinase, ureteric branching, kidney morphogenesis*

Wolffian duct elongation and budding

The nephric duct or Wolffian duct (WD) commences its development in the region of the pronephros and its early development is dependent on BMP-4 signals from the surface ectoderm (Obara-Ishihara *et al.*, 1999). WD elongates thereafter caudally, and becomes the common denominator of the different types of renal organs: pro-, meso- and metanephros. In mouse and rat, WD develops buds only in the cranial area of the mesonephros and just before the cloaca, where a ureteric bud is formed and triggers metanephrogenesis (Fig. 1). It is uncertain at the moment whether the caudal mesonephric tubules open in WD or not. This is obviously not the case in mouse and rat (Sainio *et al.*, 1997a), but in other species, such as pig, the mesonephros is a well-developed and probably functional embryonic organ.

The molecular regulation of pro- and mesonephrogenesis is poorly known. However, developmental and morphological similarities between the vestige embryonic and the permanent kidneys led Vise *et al.* (1997) to propose that all nephric organs might use similar molecular pathways during their development. This is clearly true for transcription factor *Pax-2*, expressed throughout nephrogenesis from pro- to metanephros, as Pax-2-deficient mice show severe defects already in pronephric differentiation, and they lack meso- and metanephros (Rothenpieler and Dressler, 1993). Some other genetic experiments have not verified such a molecular homogeneity throughout nephrogenesis. For instance, glial cell line-derived neurotrophic factor (GDNF) -deficient mice lack metanephros, but pro- and mesonephros seem to develop normally (Pichel *et al.*, 1996; Sainio and Sariola, unpublished data). Transcription factor WT-1 -deficient mice also lack metanephric kidneys and the caudal set of mesonephric tubules, while the cranial set of tubules develops (Sainio *et al.*, 1997a). Based on the present molecular data, one could tentatively conclude that the morphological similarity between different types of renal organs might reflect some molecular similarity in the fundamental transcriptional regulation of different nephric organs, in particular by Pax-2, but the signalling networks promoting budding, and possibly tubule induction, seem to be more diverse and distinct for each renal organ.

Abbreviations used in this paper: ART, artemin; CaM, caudal mesonephros; CrM, cranial mesonephros; FGF-7, fibroblast growth factor-7; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF family receptor α; GPI, glycosylphosphaditylinositol; HGF, hepatocyte growth factor; NTN, neurturin; PLCγ, phopholypase-C γ; PSP, persephin; TGF-β, transforming growth factor β; WD, Wolffian duct.

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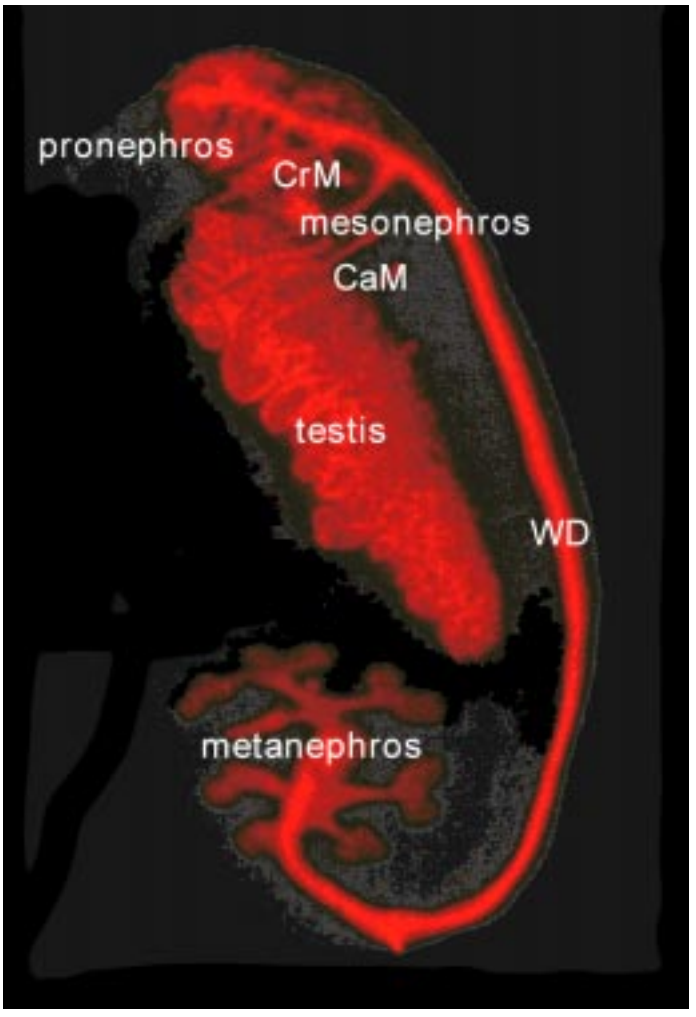

Fig. 1. Whole-mount of a 15-day old embryonic rat urogenital ridge with pro-, meso- and metanephros. *Developing testis is seen above mesonephros. Indirect immunofluorescence for cytokeratin. WD, Wolffian duct; CrM, cranial mesonephros; CaM, caudal mesonephros.*

Ureteric branching and differentiation

During the last decades, research on the molecular regulation of kidney differentiation has focused in the search for inductive substances in tubule induction. However, a signalling molecule for ureteric budding and branching was first found when the function of a novel neurotrophic factor, GDNF, was approached by targeted disruption of the gene and kidney culture experiments. The molecule had been known for its potent action in the maintenance of midbrain dopaminergic and cholinergic neurones of the central nervous system and spinal motoneurones (Lin *et al.*, 1993; Henderson *et al.*, 1994; Buj-Bello *et al.*, 1995; Trupp *et al.*, 1995). It is approximately hundred times more potent in maintaining these sets of neurones than any other neurotrophic factor.

In situ hybridisation showed that *GDNF* mRNA is not only expressed in the expected neuronal regions, but also in the pretubular mesenchymal condensates of the embryonic metanephric kidney (Hellmich *et al.*, 1996; Suvanto *et al.*, 1996). Moreover, *GDNF* mRNA is abundantly expressed in the developing gastrointestinal tract. The distribution of *GDNF* mRNA suggested that GDNF might act as a signalling molecule in kidney morphogenesis and it became a candidate molecule for the maturation and survival of the enteric neurones. While this expression pattern was naturally interesting as such, it was even more challenging that the receptor for GDNF was still unknown.

Because the GDNF expression in the pretubular condensates is developmentally strictly regulated, it was logical to assume that the unknown receptor should be searched for from the tips of the ureteric epithelium. Our binding assays with ^{125}I-labelled GDNF verified that it binds to the tips of the ureteric buds (Sainio *et al.*, 1997b). Only few orphan receptors were known from this site, Ros and Ret receptor tyrosine kinases (Pachnis *et al.*, 1993). Ros-deficient mice do not show any renal abnormalities (Sonnenberg-Riethmacher *et al.*, 1996), but Ret-deficient mice either lack kidneys or develop renal hypodysplasia, and they also lack enteric neurones below stomach (Schuchardt *et al.*, 1994). We therefore favoured Ret as a receptor for GDNF. This hypothesis was strengthened by the overlapping expression patterns of GDNF and Ret in several organs, in particular in gastrointestinal tract and kidney. By various biochemical and biological approaches Ret was soon identified as the signalling receptor for GDNF (Durbec *et al.*, 1996; Trupp *et al.*, 1996; Vega *et al.*, 1996; Treanor *et al.*, 1996), but the molecular landscape became more complicated.

The signalling receptor complex for GDNF contains not only Ret but a novel glycosylphophaditylinositol (GPI)-linked protein that was initially termed GDNF receptor α (Jing *et al.*, 1996; Treanor *et al.*, 1996), later named as GDNF family receptor α1 (GFRα1) by the GFRα Nomenclature Committee (Davies *et al.*, 1997). The GFRα1 receptor had been actually characterised a year earlier and the GDNF binding was clear, but the publication had been hampered by the lack of any detectable response upon GDNF binding. The missing piece of evidence came from the phenotype of the GDNF-deficient mice. Like Ret-deficient mice, the mice lacking GDNF do not develop kidneys and enteric innervation (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). Once the high similarity between Ret- and GDNF-null phenotypes became public, the nature of the signalling receptor complex for GDNF was easy to discover. A GDNF dimer binds first to two GFRα1 molecules probably leading to their dimerisation. The complex then binds to and induces Ret dimerisation and autophosphorylation (Fig. 2). This sequence of events has been verified by several groups and experimental models, but also alternative models for the construction of the GDNF receptor complex have been presented (review: Sariola and Sainio, 1997).

Four members in the GFRα family have now been characterised, one of which is only known from chicken. Simultaneously, the GDNF ligand family has expanded with three novel members, neurturin (NTN) (Kotzbauer *et al.*, 1996), persefin (PSP) (Milbrandt *et al.*, 1998) and artemin (ART) (Baloh *et al.*, 1998). Like GDNF, also NTN, PSP and ART have seven conserved cysteine residues in the same spacing and therefore GDNF family ligands belong to transforming growth factor–β (TGF-β) superfamily. It is rather surprising that they all activate Ret in a complex with GFRα-receptors. The primary α-receptor for GDNF is GFRα1 (Jing *et al.*, 1996; Treanor *et al.*, 1996; Cacalano *et al.*, 1998; Enomoto *et al.*, 1998), for NTN GFRα2 (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Jing *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997; Rossi *et al.*, 1999), for ART GFRα3 (Baloh *et al.*, 1998;

Worby *et al.*, 1998), and for PSP GFRα4 (in chicken) (Enokido *et al.*, 1998) (Fig. 2). The identification of Ret as the signalling receptor for GDNF, NTN, ART, and PSP was conceptually important, since TGF-βs signal via transmembrane serine-threonine kinases, whereas Ret is a typical receptor tyrosine kinase. It is also quite intriguing that a "dedicated" neurotrophic factor, GDNF, has a completely non-neurotrophic function outside the nervous system functioning as a signalling molecule in the developing kidney.

GDNF as a regulator of ureteric branching

GDNF, expressed by the nephrogenic mesenchyme, activates Ret–GFRα receptor complex on the tips of the ureteric bud. Thus, this ligand-receptor pair seemed to provide a straightforward molecular explanation for the classic model of heterotypic inductive interactions. This turned out to be an oversimplification. NTN was soon found from the tips of the collecting ducts, the same cells that express Ret (Widenfalk *et al.*, 1997). NTN also activates Ret and shows the same biological responses as GDNF in explant culture (Davies *et al.*, 1998). However, there is an important difference between these two ligands in the initiation and timing of their expression. GDNF is expressed earlier than NTN, already in the kidney rudiment, while the latter appears only two days later. This sequential expression pattern would suggest that ureteric branching is first regulated by an epithelio-mesenchymal tissue interaction between the ureteric bud (with Ret/GFRα1) and the nephrogenic mesenchyme (with GDNF), but later on a cell autonomous mechanism cannot be ruled out. However, more information is needed about the biology of the GDNF ligand family and their signal transduction, because NTN-deficient mice do not show any renal disturbances (Milbrandt *et al.*, 1998).

GDNF signal transduction

GDNF triggers Ret autophosphorylation and activates several intracellular signalling cascades (Durbec *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996; for review, see Unsicker, 1998). It is noteworthy that recent *in vivo* and *in vitro* studies have shown that the biological response to GDNF requires TGF–β1 (Krieglstein *et al.*, 1998). It remains however unknown how these different ligands co-operate.

Ret is a typical transmembrane tyrosine kinase receptor with one exception. It has a unique structure in the extracellular domain with the Ca^{2+} binding cadherin-like motif. Recent experiments have shown that Ca^{2+} ions are required for Ret activation and therefore they are components of the signalling complex formed by GDNF, Ret, and GFRαs (Nozaki *et al.*, 1998).

The role of the second receptor component, the GFRα proteins, in GDNF signalling is intriguing. Current experimental evidence suggests that GFRα1 (and possibly other members of the GFRα family) just bind GDNF and present the GFRα1-GDNF complex to Ret. Several new lines of evidence, however, suggest that GFRα proteins may mediate the GDNF signalling on their own. Firstly, Ret and GFRα1 expression patterns, although having a lot of similarities, do not overlap in some tissues, where GFRα1 receptor is expressed alone without Ret (Luukko *et al.*, 1997, 1998; Trupp *et al.*, 1997; Widenfalk *et al.*, 1997). Secondly, GPI-linked proteins, associated with so called lipid rafts (Simons and Ikonen, 1997), mediate intracellular signalling events *in vitro* and *in vivo*. Thirdly,

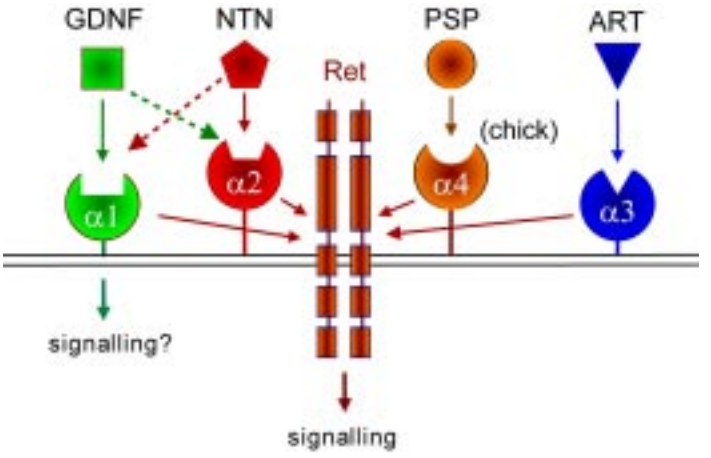

Fig. 2. GDNF family proteins bind to GPI-linked GFRα receptors and the complex is then delivered to Ret. *Ret dimerisation and autophosphorylation triggers intracellular signalling events leading to different cellular responses. GFRα1 is the primary co-receptor for GDNF and NTN signals are mediated by GFRα2. ART binds to GFRa3 and PSP can bind to chicken GFRα4 to activate Ret. The mammalian GFRα4 has not yet been characterised. There is recent experimental evidence that GDNF can signal Ret-independently via GFRα1.*

we have recently observed that postnatal rat cochlear ganglion neurones expressing GFRα1, but not Ret, are survival-dependent on GDNF (Ylikoski *et al.*, 1998). The open question is whether GFRα proteins could mediate GDNF signals via a non-Ret pathway either directly or in association with other proteins.

Our recent experiments with rat dorsal root ganglion neurones from wild type as well as Ret-deficient mice have demonstrated that GDNF can signal Ret-independently, leading to a long lasting elevation in the intracellular concentration of Ca^{2+} (Titievsky *et al.*, 1999). Thus, GPI-linked GFRα1 receptors can signal without Ret upon GDNF binding via activation of src-type kinases and subsequent recruitment of phospholipase-Cγ signalling pathway (Titievsky *et al.*, 1999). Whether GFRα1 is sufficient to activate src kinases due to the GFRα1 clustering in the rafts and subsequent direct phosphorylation of PLCγ or due to additional accessory proteins is still an open issue. The question about the physiological significance of the GFRα1 mediated long-lasting elevation of intracellular Ca^{2+} for the cells and the question whether NTN, ART and PSP can directly signal via their cognate GFRα receptors also wait for an answer. It will be also interesting to know whether the non-Ret GFRα1-mediated signalling is executed at any stage of the kidney development.

The regulation of ureteric branching

It is clear that GDNF is a signal for the promotion of ureteric budding from WD and for ureteric branching. We can also speculate that NTN expression in the tips of the growing ureteric tree makes the growth and branching less dependent on the mesenchyme-derived signals during late embryogenesis. These conclusions do not, however, exclude an involvement of other signals in the ureteric development (Fig. 3).

Hepatocyte growth factor (HGF) is expressed by the nephrogenic mesenchyme and antibodies to HGF inhibit ureteric branch-

ing in organ culture (Woolf *et al.*, 1995). HGF was shown to be less significant *in vivo* by targeted disruption of the gene, because these mice develop normal kidneys (Schmidt *et al.*, 1995). The same dilemma is true with TGF–β1. Antibodies to TGF–β1 inhibit ureteric branching in organ culture, but targeted disruption of the gene did not confirm the importance of the gene for nephrogenesis *in vivo* (Shull *et al.*, 1992). Several TGF-β family members are, however, expressed by the kidney and redundancy would be an explanation for these discrepancies between organ culture and knockout experiments (Sariola 1996). Accordingly, TGF-β2-null mice show regularly defects in kidney morphogenesis (Sanford *et al.*, 1997).

We approached the roles of HGF and TGF–β1 in ureteric branching by a heterologous tissue recombination assay. When an early ureteric bud is recombined with any other mesenchyme of the embryo, it does not branch. However, when it is microsurgically recombined with lung mesenchyme and GDNF is added to the culture medium, the bud branches (Sainio *et al.*, 1997). It is noteworthy that the ureteric bud does not branch when recombined with gut that is a rich source for GDNF, underlying the importance of the type of the mesenchyme in the regulation of ureteric branching. However, if a late stage, T-shaped bud, is recombined with lung mesenchyme, it branches without GDNF supplementation (Kispert *et al.*, 1996). This tissue recombination system shows that both the type of the mesenchyme and the stage of the bud are elementary in the branching response.

Not only exogenous GDNF, but also TGF–β1 and HGF, promote ureteric branching in lung mesenchyme-ureteric bud recombination cultures (Sainio *et al.*, 1997b). These data obviously recall for further information about the receptor identities of the ureteric cells and the intracellular signalling cascade activated by them. We do not know at present how Ret and other receptors on the ureteric bud mediate their signals to nuclei, and which of these signalling cascades are important for the branching response. A plausible explanation for the discrepancies between the *in vivo* and *in vitro* experiments is that the decisive factor might be that the critical signalling molecules are present in the right place in the kidney rudiment at the right time, but biochemistry inside the cell is redundant. However, it might be that GDNF and other signalling molecules do not activate branching directly, but via a change in the extracellular matrix. For instance, kidney morphogenesis is critically dependent on integrin α8β1 and the mutant mice lacking it do not develop kidneys (Muller *et al.*, 1997). There is no information about the possible interplay of the GDNF and integrin systems.

Open questions

The same question arises often when a novel molecule has been either mapped in the embryonic kidney or it has received a function in nephrogenesis: why are molecules acting in neuronal differentiation also important in kidney morphogenesis? Is this only economics of evolution or do we misunderstand the biology of nephrogenesis? The classic view on the regulation of nephrogenesis implies that it is an interplay between the nephrogenic mesenchyme and the ureteric bud. However, the embryonic kidney has many other cell lineages such as the stromal cells, neuronal cells and endothelial cells.

The long tradition of cultivating embryonic kidneys in organ culture excludes any active participation of blood vessels in the nephron induction and differentiation, because nephron formation is efficient in organ culture where angiogenesis does not take place properly. Angiogenic growth factors have been identified from the pretubular condensates, suggesting that angiogenesis is regulated by the induced renal cells (Risau and Ekblom, 1986; Esser *et al.*, 1998). However, we cannot exclude the participation of the stromal, and maybe neuronal cells, in tubule induction and ureteric branching. These cell types are always present in the explant culture and their differentiation coincides with the induction of tubules and branching of the ureteric bud. Both early and recent experimental data suggest that the simplified view on the tubule induction should be re-evaluated. Disialylated ganglioside D3, GD3, is exclusively expressed on the renal stromal cells and antibodies to GD3 inhibit kidney morphogenesis in organ culture (Sariola *et al.*, 1988) Similarly, targeted disruption of the BF2 winged helix transcription factor, expressed specifically by the stromal cells, results in hypoplastic kidneys (Hatini *et al.*, 1996). Thus, the stromal cells clearly affect tubule formation, but the mechanisms remain unknown.

The normal fate of the stromal cells is to die. Although they represent the majority of renal cells at midgestation, there are only few of them left in the adult kidney. While their function in nephrogenesis remains unknown, neural crest chimeras between quail and chicken have shown that they may be derived from the neural crest (LeDouarin and Teillet, 1973). If this would be the case in mouse as well, the key question is why these cells are so abundant in the embryonic kidney and how do the contribute to the regulation of nephron formation?

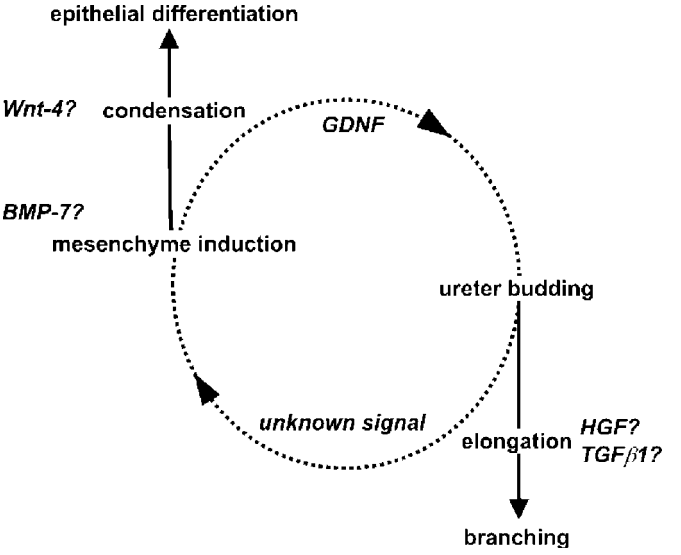

Fig. 3. Schematic representation of the inductive signalling between the ureteric bud and the nephrogenic mesenchyme. *GDNF promotes ureteric budding but HGF, TGFβ1 and FGF-7 among others, influence the growth and elongation of the branches. Once in contact with uninduced nephrogenic mesenchyme, the tip of the bud induces it. One of the first consequences of induction is transient upregulation of GDNF. It promotes further budding etc.*

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