

Dominant effects of RET receptor misexpression and ligand-independent RET signaling on ureteric bud development

Shankar Srinivas¹, Zaiqi Wu¹, Chiann-Mun Chen¹, Vivette D'Agati² and Frank Costantini^{1,*}

¹Department of Genetics and Development and ²Department of Pathology, College of Physicians and Surgeons, Columbia University, 701 W 168th Street, New York, NY 10032, USA

*Author for correspondence (e-mail: fdc3@columbia.edu)

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SUMMARY

During kidney development, factors from the metanephric mesenchyme induce the growth and repeated branching of the ureteric bud, which gives rise to the collecting duct system and also induces nephrogenesis. One signaling pathway known to be required for this process includes the receptor tyrosine kinase RET and co-receptor GFR α -1, which are expressed in the ureteric bud, and the secreted ligand GDNF produced in the mesenchyme. To examine the role of RET signaling in ureteric bud morphogenesis, we produced transgenic mice in which the pattern of RET expression was altered, or in which a ligand-independent form of RET kinase was expressed. The *Hoxb7* promoter was used to express RET throughout the ureteric bud branches, in contrast to its normal expression only at the bud tips. This caused a variable inhibition of ureteric bud growth and branching reminiscent of, but less severe than, the *RET* knockout phenotype. Manipulation of the level of GDNF, *in vitro* or *in vivo*, suggested that this defect was due to insufficient rather than excessive RET signaling. We propose that RET receptors expressed ectopically on

ureteric bud trunk cells sequester GDNF, reducing its availability to the normal target cells at the bud tips. When crossed to *RET* knockout mice, the *Hoxb7/RET* transgene, which encoded the RET9 isoform, supported normal kidney development in some *RET*^{-/-} animals, indicating that the other major isoform, RET51, is not required in this organ. Expression of a *Hoxb7/RET-PTC2* transgene, encoding a ligand-independent form of RET kinase, caused the development of abnormal nodules, outside the kidney or at its periphery, containing branched epithelial tubules apparently formed by deregulated growth of the ureteric bud. This suggests that RET signaling is not only *necessary* but is *sufficient* to induce ureteric bud growth, and that the orderly, centripetal growth of the bud tips is controlled by the spatially and temporally regulated expression of GDNF and RET.

Key words: RET signaling, Ureteric bud, GDNF, Kidney development, Mouse

INTRODUCTION

Development of the metanephric kidney has long been known to depend on reciprocal inductive interactions between the ureteric bud (UB) and the metanephric mesenchyme (Saxen, 1987). While the mesenchyme induces the growth and branching of the UB, which gives rise to the renal collecting system (Erickson, 1968; Grobstein, 1953, 1955), the UB branches induce the surrounding mesenchymal cells to condense into epithelial vesicles, which differentiate into the various segments of the nephron (Grobstein, 1953, 1955; Saxen, 1970). While nephrogenesis has been intensively studied for many years, until recently less attention has been paid to the developmental control of UB growth and branching. However, this process is equally critical for normal renal development: as the kidney develops, each new branch of the UB induces new nephrons, so that the histoarchitecture of the mature kidney is determined largely by the pattern of growth and branching of the UB (Ekblom, 1992).

Gene expression studies (reviewed by Bard et al., 1994) have suggested a number of growth factors and receptors that may play a role in UB development, and the importance of several such gene products has been demonstrated through gene knock-out studies or through inhibition of gene expression in organ culture systems (reviewed by Lechner and Dressler, 1997). The first gene with a demonstrated role in UB development was the proto-oncogene *RET*, which encodes a receptor tyrosine kinase (Takahashi and Cooper, 1987). *RET* is initially expressed throughout the Wolffian duct, and when the ureteric bud evaginates from the Wolffian duct and branches within the metanephric mesenchyme, RET expression continues throughout the entire UB epithelium. By the time the UB has branched several times, expression of *RET* is restricted to the distal tips of the UB in the peripheral nephrogenic zone (Pachnis et al., 1993; Tsuzuki et al., 1995). In *RET*^{-/-} mouse embryos, the UB either fails to form, resulting in renal agenesis, or its growth and branching are severely retarded leading to hypodysplasia (Schuchardt et al., 1994, 1996). This

led to the proposal that RET serves as the receptor for a ligand, produced by the metanephric mesenchyme, which stimulates the outgrowth of the UB from the Wolffian duct as well as its subsequent growth and repeated branching (Schuchardt et al., 1996).

This model was substantiated by the discovery of a RET ligand, glial cell line-derived neurotrophic factor (GDNF) (Durbec et al., 1996; Lin et al., 1993; Robertson and Mason, 1997; Trupp et al., 1996; Vega et al., 1996; Worby et al., 1996), which is expressed in the metanephric mesenchyme (Hellmich et al., 1996; Miyamoto et al., 1997; Suvanto et al., 1996). GDNF binds to RET in conjunction with GFR α -1, a glycosylphosphatidylinositol- (GPI) linked cell surface co-receptor (Jing et al., 1996; Treanor et al., 1996). In the developing kidney, GFR α -1 mRNA is expressed at the highest levels in the peripheral portion of the UB, although in a broader domain than RET (Baloh et al., 1997; Sainio et al., 1997), and at lower levels in condensed mesenchyme and early epithelial tubules (Sainio et al., 1997; Suvanto et al., 1997). Soluble GFR α -1, produced by cleaving the GPI linkage, can also mediate the binding of GDNF to RET (Jing et al., 1996; Treanor et al., 1996). Targeted mutagenesis of either *GDNF* (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) or *GFR α -1* (Enomoto et al., 1998) results in bilateral renal agenesis in homozygotes. These observations have established that GDNF is an inductive factor produced by the metanephric mesenchyme, which signals through GFR α -1 and RET, thus stimulating the evagination of the UB from the Wolffian duct, and its subsequent growth and branching within the metanephros.

While these studies have demonstrated that GDNF/RET signaling is *required* for normal UB development, the specific consequences of this signal for the UB remain to be elucidated. Does the GDNF/RET signal promote survival, proliferation, or differentiation of the cells at the tips of the developing UB? Does it stimulate branching, or influence the specific pattern of growth and branching? While several recent studies have attempted to address these questions through the addition of GDNF to kidney rudiments growing in organ culture (Pepicelli et al., 1997; Sainio et al., 1997; Vega et al., 1996), they remain to be fully resolved. In this paper, we address the related question of the significance of the restricted sites of expression of RET and GDNF. After the first few branches of the UB, expression of RET is restricted to the distal tips of the UB branches, the sites at which new UB branches are formed. GDNF is expressed in the undifferentiated metanephric mesenchyme, which is also localized in the peripheral nephrogenic zone. It has been observed that the early UB will grow toward localized sources of GDNF (Durbec et al., 1996; Pepicelli et al., 1997; Sainio et al., 1997), and that addition of soluble GDNF to organ cultures results in increased numbers of UB tips (Pepicelli et al., 1997; Vega et al., 1996). Together, these observations suggested that the restricted expression of RET and GDNF might be important to regulate the extent, and possibly the pattern, of UB growth and branching.

To examine this question, we have produced transgenic mice in which a wild-type RET cDNA is expressed under the control of the *Hoxb7* promoter. Unlike *RET*, *Hoxb7* is expressed in a constitutive pattern throughout the developing UB, and its expression persists in the collecting ducts, pelvis and ureter in the adult kidney. *Hoxb7* promoter sequences have been shown

to direct expression of a *lacZ* reporter gene in the same pattern as the endogenous *Hoxb7* gene (Kress et al., 1990; Vogels et al., 1993). Our hypothesis was that constitutive expression of wild-type RET would cause excessive or ectopic branching of the ureteric bud, and thus disrupt or transform the normal histoarchitecture of the kidney. However, we were concerned that RET receptors ectopically expressed in the UB trunk regions might be ineffective if GDNF were restricted to the periphery of the kidney. Therefore, we also produced transgenic mice in which a ligand-independent, constitutively active form of RET was expressed under the *Hoxb7* promoter.

MATERIALS AND METHODS

Mouse strains

All constructs were injected into (B6 \times CBA) F_2 zygotes, and transgenic mice were bred to the same F_1 hybrid strain, or to *RET* $^{+/-}$ mice (on a mixed 129/SvEv and MF1 background). *GDNF* $^{+/-}$ mice (Sanchez et al., 1996) were obtained from Dr M. Barbacid (Bristol-Myers Squibb).

Transgene constructs

The *Hoxb7* promoter fragment (sequences from -1316 to +81 of the *Hoxb7* gene), obtained from Dr Jacqueline Deschamps (Kress et al., 1990), was excised from the pGEM-Blue vector with *KpnI* and *AvaI*, the ends blunted, and recloned into the *EcoRV* site of pBluescript KS $^+$ in both orientations. A cDNA encoding the RET9 isoform, cloned into the *EcoRI* and *SacI* sites of pBluescript KS $^+$, was provided by Dr Vassilis Pachnis. The PTC2 RET cDNA (Bongarzone et al., 1993), cloned into the *XbaI* site of the pMAM-neo vector, was obtained from Dr Marco Pierotti. The human β -globin sequence was a 1.7 kb *BamHI/PstI* fragment that included the last 20 base pairs of exon2, all of intron 2 and exon 3, and the polyadenylation signal. This fragment was cloned into the *BamHI* and *PstI* sites of pBluescript KS $^+$.

To make the *Hoxb7/RET9* construct, the human β -globin sequence was excised with *BamHI* and *Clal*, and the *BamHI* site blunted with Klenow DNA polymerase. This fragment was ligated downstream of the RET cDNA by digesting the RET clone with *EcoRV* and *Clal*. The ligated RET and globin sequences were subsequently excised with *SacII* and *Clal*, and the *SacII* end was blunted. This fragment was ligated downstream of the *Hoxb7* promoter by cutting the vector with *HindIII* and *Clal* (the *HindIII* site was blunted prior to ligation). The entire transgene was excised for microinjection using *XbaI* and *Clal*. The RET cDNA portion of the final RET9 construct was sequenced using multiple overlapping primers generated on the basis of the published sequence.

The PTC2 cDNA was excised from pMAM-neo by partial digestion with *EcoRI*, digested with *DraI* to delete the 3' poly(A) tail, and cloned into pBluescript KS $^+$ cut with *EcoRI* and *SmaI*. To produce the transgene, a *NotI/HindIII Hoxb7* fragment and a *HindIII/SpeI* PTC2 fragment were ligated into a human β -globin-containing pBluescript KS $^+$ vector cut with *SpeI* and *NotI*. The entire transgene was then excised with *KpnI* and *NotI*.

Production of transgenic mice

Transgene DNA for microinjection was prepared as described by Hogan et al. (1994). Briefly, the transgene fragment was gel purified and then subject to CsCl gradient centrifugation. Peak fractions were dialyzed against a vast excess of injection buffer (10 mM Tris-HCl, 0.1 mM EDTA) at 4°C for >48 hours. The transgene solution was then centrifuged through 'Ultrafree-MC' centrifugal filtration units (Millipore) to remove particulate impurities. The DNA was diluted to 3 ng/ μ l and used for pronuclear injection into mouse eggs. Potential transgenic mice were screened by the PCR and confirmed by Southern blotting.

Polymerase chain reaction

The primers used to screen the *RET9* mice spanned the RET/ β -globin junction. The primer in RET (Ab: 5'TTCGGACTCACTGCTGTA-TGAC3') and the primer in globin (K: 5'ACGATCCTGAGACTT-CCACT3') amplified a band of 370 bp. The primers used to screen the PTC2 mice spanned the junction of the *Hoxb7* promoter and PTC2 cDNA. The *Hoxb7* primer (R2: 5'GGGGTCTTTTGGTGTA-AATC3') and the PTC2 primer (Ad: 5'CCGCATCTTCTCCGTG-TAGAC3') amplified a 550 bp band. The primers used to screen for the *RET* knockout allele (Schuchardt et al., 1994) were P1: 5'TGGGAGAAGGCGAGTTGGAAA3' and P2: 5'TTCAGGAAC-ACTGGCTACCATG3' which amplified a wild-type band of 220 bp, and P3: 5'AGAGGCTATTCGGCTATGACTG3' and P4: 5'CCTG-ATCGACAAGACCGGCTTC3' which amplified a mutant band of 420 bp.

The primers used to screen for the *GDNF* knockout allele were BH128: 5'GCTGGCGAAAGGGGATGTG3', which anneals to the 5' end of the wild-type allele, BH122: 5'GGAGGGAGCGG-TTCTTACAG3' which anneals to the 3' end of the wild-type allele, and BH129: 5'CTTGCCTGGTGGGTTCTCT3' which anneals to the 3' end of the targeted allele. They amplified a 260 bp wild-type band and a 170 bp targeted band.

Southern and northern blots

Southern and northern blot hybridization was performed using standard methods. Total RNA was isolated from adult brains and kidneys by homogenizing the organs in GNTC buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M β -mercaptoethanol), lowering the pH with 2 M sodium acetate pH 4.0, extracting with phenol and precipitating with isopropanol. Poly(A)⁺ RNA was selected using the PolyAttract mRNA isolation system (Promega). Probes for Southern and northern blots were random primed using the 'Rediprime DNA labeling system' (Amersham Life Sciences). Probes were labeled using [α -³²P]dCTP (NEN Dupont). Hybridized blots were exposed to a Molecular Dynamics 'Storage Phosphor Screen' and developed using a Molecular Dynamics PhosphorImager.

A 1.1 kb *HindIII/XhoI* fragment of the mouse RET cDNA was used as a template in probe preparation for both Southern and northern blot hybridization on nucleic acids from *Hoxb7/RET9* transgenic mice. This fragment contains a portion of the extracellular domain, the transmembrane domain and a portion of the intracellular domain. Genomic DNA was digested with *ScaI*, to cut once within the transgene, and Southern blots detected a transgene band at 6.5 kb in addition to endogenous bands. The entire PTC2 cDNA was used as a template for the probe used on Southern blots of DNA from *Hoxb7/PTC2* mice. Genomic DNA was digested with *XbaI*, and the probe detected a 5.0 kb target band. We determined that none of the adult *Hoxb7/PTC2* transgenic mice expressed the transgene by performing northern blot hybridization on poly(A)⁺ RNA from the brain and kidneys. A 1.1 kb *EcoRI/XbaI* fragment from the 3' end of the PTC2 cDNA (corresponding to the intracellular domain of RET) was used as template for the probe. As a positive control, we used poly(A)⁺ RNA from the SK-N-SH human neuroblastoma cell line (ATCC).

Analysis of transgenic kidneys

The cross-sectional area of newborn kidneys was estimated by placing them on a 0.25 mm grid and measuring their length (rostrocaudal aspect) and width (mediolateral aspect) under a dissection microscope. For organ culture, kidneys dissected from E11.5 embryos were cultured on 'Transwell Clear' filter units (Costar) in DMEM/F12 medium containing 5% fetal bovine serum (Hyclone), 0.1 unit/ml penicillin/streptomycin (Gibco BRL) and 2 mM glutamine (Gibco BRL). Kidneys were cultured at 37°C in the presence of 5% CO₂. Lyophilized recombinant rat GDNF (R&D Technologies) was resuspended according to the manufacturers recommendations, and

added directly to the culture medium to a final concentration of 100 ng/ml. Culture medium was replaced every 2 days. To visualize the ureteric bud branches, the cultured kidneys were fixed in fresh 2% paraformaldehyde in PBS at 4°C, permeabilized in 0.1% saponin in PBS at room temperature and stained in 50 μ g/ml FITC-conjugated *Dolichos biflorus* lectin (DB) at 37°C. They were then washed, post-fixed in 2% paraformaldehyde, mounted on glass slides and photographed. To quantitate the extent of ureteric bud branching, the numbers of bud tips in each DB-stained kidney was counted, without prior knowledge of genotype or culture conditions. The numbers of bud tips in wild-type or transgenic kidneys cultured with and without GDNF were compared using Student's *t*-test for paired samples.

For histological analysis, kidneys were fixed in 10% formalin or 4% paraformaldehyde, washed in PBS, dehydrated and embedded in paraffin. Six micron sections were cut and stained with hematoxylin and eosin. *Dolichos biflorus* and *Tetragonolobus lotus* lectin staining of kidney sections was performed as described by D'Agati and Trudel (1992).

In situ hybridization

In situ hybridization was performed as described by Wilkinson (1992) with modifications. Briefly, samples were fixed in fresh ice cold 4% paraformaldehyde in PBS, washed in PBS, then saline, and then dehydrated through an ethanol series. They were then embedded in paraffin and sectioned at 8 μ m. Hybridization was performed using digoxigenin-labeled riboprobes, which were detected using an alkaline-phosphatase-conjugated monoclonal antibody against digoxigenin (Boehringer Mannheim). Alkaline phosphatase activity was detected with BCIP and NBT (Boehringer Mannheim).

The entire RET cDNA was used as a template for riboprobe synthesis. For sense probe, the vector was linearized with *EcoRV*, and T7 polymerase was used to transcribe the RNA. For antisense probe, the vector was linearized with *SacII*, and transcribed using T3 polymerase. A GDNF cDNA, obtained from Dr Andreas Zimmer, was linearized with *XbaI* and transcribed using T7 polymerase (sense probe), or linearized with *HindIII* and transcribed with Sp6 polymerase (antisense probe). The template for the GFR α -1 probe was a partial cDNA cloned by RT-PCR from newborn mouse brain RNA, using the primers Gtop: 5'AACTGCCTGAGTCTGCAAG-ACTCCTGCAAG3' and Gbot: 5'GGAGCAGCATTGATTTG-TGGTTATGTGG3'. The amplified cDNA fragment, which corresponds to 645 base pairs of the 3' end of the coding region, was cloned into the pCRII vector in both orientations. To make the sense and antisense probes, the two vectors were linearized using *HindIII*, and transcribed with T7 polymerase.

GDNF expression was examined in E12.5 and E13.5 (as well as later) embryos by another method (data not shown). *Hoxb7/RET9* transgenic mice were crossed with a mouse strain carrying a *lacZ* knock-in at the *GDNF* locus, in which *lacZ* expression serves as a reporter for the transcriptional activity of *GDNF* (Sanchez et al., 1996). Embryos heterozygous for the *GDNF* knock-in allele, and either transgenic or non-transgenic for *Hoxb7/RET9*, were stained for β -galactosidase (Hogan et al., 1994).

RESULTS

A *Hoxb7/RET* transgene causes dominant renal hypodysplasia of varying severity

To ask whether the expression of RET throughout the ureteric bud, instead of only at its tips, would alter the pattern of UB growth and branching, we expressed a wild-type *RET* cDNA under the control of the *Hoxb7* promoter (Fig. 1A). Four transgenic mouse lines were generated, all of which expressed the transgenic RET mRNA in the adult kidney as well as in the

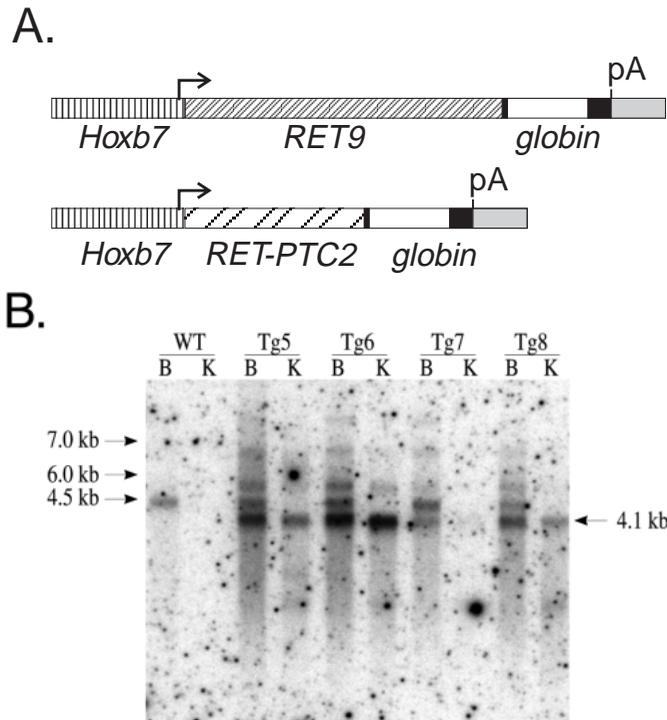


Fig. 1. *Hoxb7* transgene constructs, and expression of RET mRNA in brain and kidney of adult *Hoxb7/RET* transgenic mice.

(A) Schematic diagram of transgene constructs. The *Hoxb7* promoter and 5' flanking sequences were joined to cDNA sequences encoding either the wild-type murine RET9 isoform or the human RET-PTC2 oncogene product. The arrow indicates the start of transcription. Both constructs contained 3' sequences from the human β -globin gene, including an intron (open bar), untranslated exon sequences (black bars) a polyadenylation signal and 3' flanking DNA (grey bars). RET-PTC2 consists of the tyrosine kinase domain of RET fused to a portion of the RI α regulatory subunit of protein kinase A, which allows ligand-independent dimerization and constitutive RET kinase activity (Bongarzone et al., 1993). (B) Northern blot analysis of RNAs from brain (B) and kidney (K) of adult wild-type (WT) mice and individuals from four *Hoxb7/RET* transgenic lines. The arrows on the left indicate the positions of endogenous RET mRNAs in the brain: 4.5 kb (the major species), 6.0 kb and 7.0 kb. The endogenous RET gene is not expressed in the adult kidney, as previously reported (Pachnis et al., 1993). The arrow at right indicates the position of the major 4.1 kb mRNA encoded by the transgene, which is expressed in the kidney as well as the brain.

brain (Fig. 1B). The three lines with the highest expression in the kidney (Tg5, Tg6 and Tg8) were used for further studies. The sustained expression of RET in the kidneys of adult transgenic mice contrasts with the transient fetal expression of endogenous RET in the kidneys of non-transgenic mice (Pachnis et al., 1993), and confirms that the *Hoxb7* promoter is constitutively active in the mature kidney (Kress et al., 1990; Vogels et al., 1993).

While some adult *Hoxb7/RET* transgenic mice appeared normal and healthy, many others became sick or died, and their kidneys were found to be small and cystic (data not shown). Subsequently, to avoid selecting for less severely affected individuals, the kidneys from a random sample of newborn transgenic progeny and their non-transgenic littermates were analyzed histologically. The transgenic kidneys ranged from normal, to moderately small and cystic, to very small and highly dysplastic (Fig. 2). The most severely affected kidneys were comparable in size and dysmorphology to the kidneys observed in some newborn mice homozygous for the *RET* knockout (e.g. Fig. 7E). However, in contrast to *RET* knockout mice, most of

which display unilateral or bilateral renal agenesis, all of the transgenic mice had two kidneys and ureters. Similar results were obtained with all three transgenic lines.

In general, the size of the newborn transgenic kidneys showed an inverse correlation with the degree of dysplasia determined histologically. Therefore, the cross-sectional area of the newborn kidneys was employed as a quantitative measure of the degree of dominant abnormality in *Hoxb7/RET* transgenic mice. As shown in Fig. 3, most kidneys of newborn wild-type mice had a cross-sectional area of 6–10 mm². In contrast, the transgenic kidneys displayed a broad, bimodal size distribution, with about half in the near-normal size range (5–9 mm²) and the other half reduced several-fold in size (2–3 mm²). The variable severity of renal hypodysplasia did not correlate with level of expression of the transgene (data not shown), and is most likely a consequence of the mixed genetic background of the mice.

Developmental basis of dominant renal defects in *Hoxb7/RET* transgenic mice

To examine the developmental basis of the renal abnormalities in *Hoxb7/RET* transgenic mice, fetal kidneys were dissected at various stages and subjected to histological analysis. This revealed that the first signs of developmental retardation could be observed at about E12.5 (data not shown). In situ

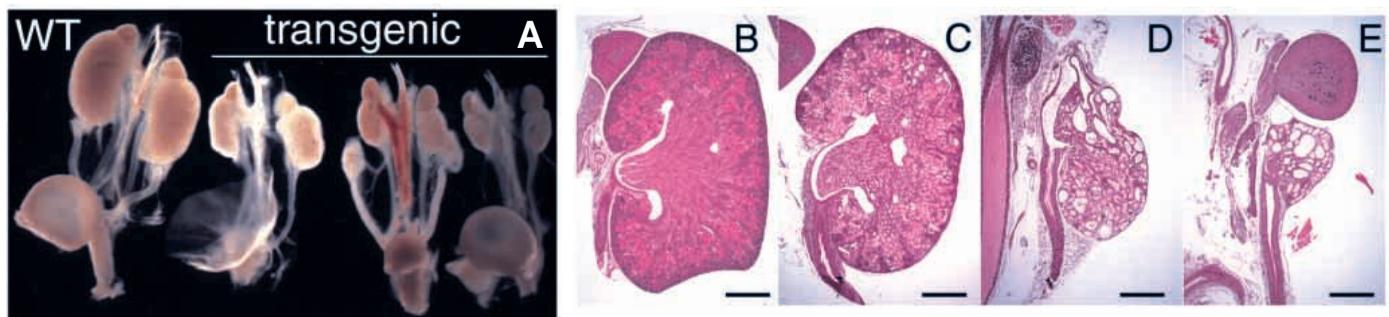


Fig. 2. Dominant renal hypodysplasia in *Hoxb7/RET* transgenic mice. (A) Excretory systems dissected from one wild-type (WT) and three transgenic newborn mice. (B) Sagittal section of a kidney of a wild-type newborn mouse. (C–E) sagittal sections of kidneys of three transgenic newborn mice, showing a range of severity of hypodysplasia. H&E stain. Scale bars, 0.5 mm.

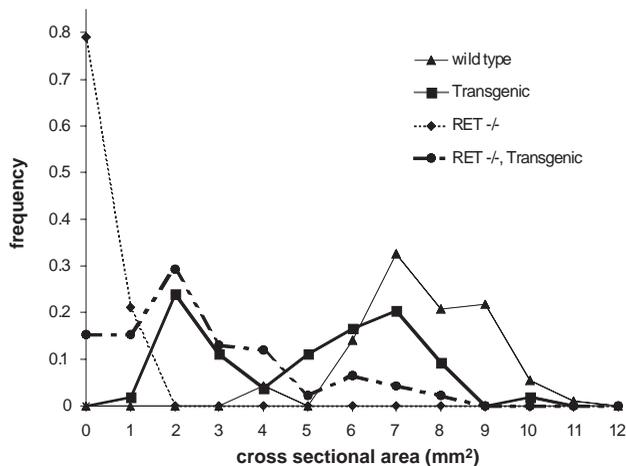


Fig. 3. Kidney sizes in wild-type, *Hoxb7/RET* transgenic, *RET*^{-/-} and transgenic *RET*^{-/-} newborn mice. Kidneys were dissected from newborn mice and the cross-sectional area of each kidney (in the sagittal plane) was measured. The frequency of kidneys in each size category is plotted for each of four genotypic classes: non-transgenic on wild-type background ($n=92$), transgenic on wild-type background ($n=54$), non-transgenic *RET*^{-/-} ($n=38$) and transgenic *RET*^{-/-} ($n=92$) (n indicates the number of individual kidneys measured). Data for three different transgenic lines, Tg5, Tg6 and Tg8 are pooled, as there were no obvious differences between the lines. *RET* $+/+$ and $+/-$ animals were both included in the wild type category as they were indistinguishable in phenotype. A size '0' indicates absence of a kidney.

hybridization studies of the fetal transgenic kidneys showed the expected ectopic expression of RET, as well as abnormalities in the expression of several other genes. RET mRNA was found throughout the ureter and medullary trunks of the ureteric bud (Fig. 4B), in contrast to its normal pattern at the UB tips (Fig. 4A). GFR α -1 mRNA, which is normally expressed in the tip and distal trunk regions of the UB, as well as in nephrogenic vesicles (Fig. 4C), showed greatly reduced expression (Fig. 4D). Similarly, GDNF mRNA, which is normally expressed in the undifferentiated mesenchyme surrounding the UB tips (Fig. 4E), was much reduced (Fig. 4F).

Next, we dissected metanephroi from transgenic E11.5 embryos and their non-transgenic littermates, and monitored their development in organ culture. In normal mouse embryos, the ureteric bud evaginates from the Wolffian duct at E10.5, and by E11.5 it has grown into the metanephric mesenchyme and branched once. All of the transgenic metanephroi contained normal T-shaped ureteric buds at E11.5, and they remained indistinguishable from wild type through the first day of culture (Fig. 5A). Subsequently, the transgenic kidneys began to show delayed development, and by the fifth day of culture many of them were grossly retarded, with relatively few ureteric bud branches and nephron elements (Fig. 5B). To quantitate this effect, we counted the number of UB tips in preparations stained with the UB-specific lectin *Dolichos biflorus* (DB) (Fig. 5C and data not shown). While wild-type kidneys developed an average of 41 (25 UB tips; $n=16$) by the 4th-5th day, the transgenic kidneys had, on average, fewer than half as many UB tips, 15 (14 ($n=18$, $P=0.0003$)).

In summary, rather than causing increased or ectopic growth and branching of the UB, the *Hoxb7/RET* transgene caused a

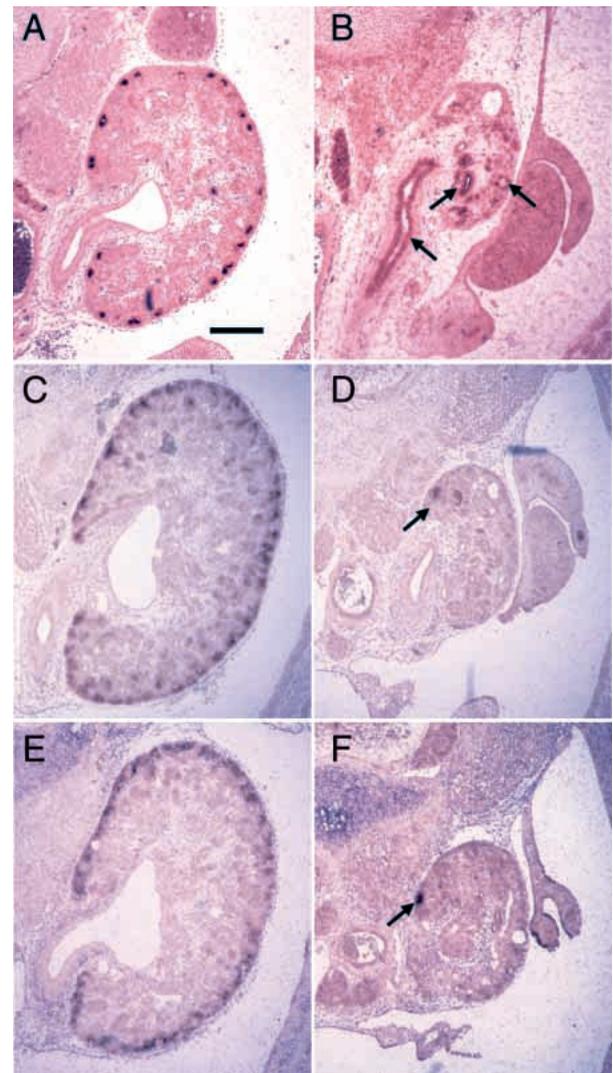


Fig. 4. Expression of RET (A,B), GFR α -1 (C,D) and GDNF (E,F) mRNAs in wild-type (A,C,E) or *Hoxb7/RET* transgenic (B,D,F) kidneys at E15.5, analyzed by in situ hybridization. In wild-type, RET mRNA is expressed only at the tips of the UB (A), while in the transgenic (B) it is expressed throughout the ureter and branches of the UB (arrows). GFR α -1 mRNA, which is normally expressed in the peripheral regions of the UB and in nephrogenic vesicles (C), and GDNF mRNA, which is normally expressed in the mesenchyme surrounding the UB tips (E) are both greatly reduced in the transgenic kidneys (D,F) except in a few areas (arrows). Scale bar, 0.25 mm in all panels.

retardation of kidney development beginning at about E12.5, and reduced expression of two other genes involved in signal transduction through RET.

The role of GDNF levels in the dominant kidney defect in *Hoxb7/RET9* transgenic mice

One potential explanation for the inhibitory effects of the transgene on kidney development was that ectopic RET signaling in the UB trunk cells interfered with their normal growth or differentiation. Alternatively, since the *Hoxb7* promoter is also active in the UB tip cells, which express endogenous RET, overexpression of RET in these cells could

result in an abnormally high level of RET signaling, which might also be deleterious. To test whether either of these explanations might be valid, we examined the effects of varying the level of GDNF to which the kidneys were exposed, either in vitro or in vivo. The rationale was that if RET signaling (either excessive or ectopic) were responsible for the observed developmental defects, then an increase in the level of GDNF should worsen the defects, while a moderate reduction in the level of GDNF should improve kidney development.

One metanephros from each transgenic or wild-type E11.5 embryo was cultured in medium containing 100 ng/ml of recombinant GDNF, and compared to the contralateral metanephros cultured in normal medium. After 4 or 5 days, the kidneys were stained with the DB lectin to examine the branching patterns (Fig. 5C,D). Consistent with previous reports (Pepicelli et al., 1997; Vega et al., 1996), wild-type kidneys cultured with added GDNF ($n=16$) showed a modest increase (average 23%) in the number of UB tips compared to those cultured in normal medium. The transgenic kidneys ($n=18$) showed an even greater improvement in growth and branching of the UB when cultured with 100 ng/ml GDNF, with an average 88% increase in the number of UB tips compared to the contralateral controls ($P=0.05$). Thus, addition of GDNF to the transgenic kidneys partially rescued the defect in UB branching, rather than worsening it.

In a complementary experiment, the *Hoxb7/RET* transgenic mice were crossed with *GDNF* heterozygous (+/-) knockout mice to reduce by half the level of GDNF to which the transgenic UB is exposed in vivo. The kidneys of newborn mice of various genotypes (transgenic or non-transgenic, *GDNF* +/+ or +/-) were dissected and measured. As observed in previous samples of newborn mice, most non-transgenic, wild-type (*GDNF*^{+/+}) kidneys had a cross sectional area in the range 7-10 mm² (Fig. 6) with a mean of 8.2 mm². Transgenic mice on a wild-type background showed a heterogeneous reduction in kidney size, with a mean of 5.45 mm², and 28% of kidneys 3 mm² or smaller. Non-transgenic *GDNF*^{+/-} mice showed a mild reduction in the kidney size distribution (mean 6.3 mm²), consistent with previous reports (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). However, transgenic *GDNF*^{+/-} kidneys were more severely affected than transgenic *GDNF*^{+/+} kidneys, with a mean area of 2.8 mm², and 69% with an area of 3 mm² or less. Therefore, a 2-fold reduction in the gene dosage of GDNF throughout prenatal development exacerbated the developmental defects in *Hoxb7/RET* transgenic kidneys.

In conclusion, the results of both experiments argue against the hypothesis that the inhibition of kidney development in *Hoxb7/RET* transgenic mice is due to excessive or ectopic RET signaling. On the contrary, the ability of added GDNF to rescue partially the defects in vitro suggests that expression of the transgene can somehow interfere with normal GDNF/RET signaling.

The *Hoxb7/RET* transgene can rescue kidney development in *RET* knockout mice

Since the *Hoxb7* promoter is active throughout the UB (including the normal site of RET expression), and since only a fraction of *Hoxb7/RET* transgenic animals show severe dominant defects, we expected that the transgene might be able to substitute for endogenous *RET* during kidney development, in at least some homozygous *RET* knockout mice. When the *Hoxb7/RET* transgene was bred into the *RET*^{-/-} background, it was indeed able to support apparently normal kidney development in some *RET*^{-/-} individuals, although in other cases the rescue was only partial (Fig. 7). This variability in the degree of rescue may be due to a combination of the incompletely penetrant, deleterious effect of the transgene on kidney development, and its ability to substitute for endogenous *RET*. Nevertheless, given that kidneys >1 mm² were never observed in *RET*^{-/-} mice, it is highly significant that >15% of the kidneys in *Hoxb7/RET* transgenic, *RET*^{-/-} mice were within the normal size range and histologically normal, and 70% were ≥ 2 mm² (Fig. 3).

The endogenous *RET* gene encodes two major protein isoforms, RET9 and RET51, which are produced by differential splicing and polyadenylation, and differ only in

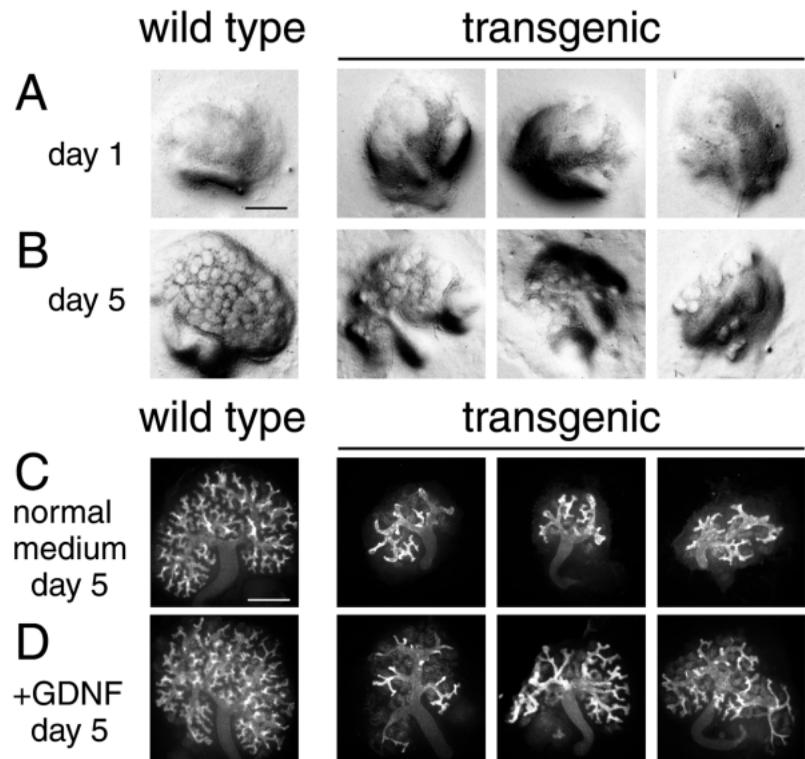


Fig. 5. In vitro culture of wild-type and *Hoxb7/RET* transgenic kidneys and the effects of added GDNF. (A,B) Kidneys were dissected at E11.5, cultured in normal medium and photographed after 1 (A) and 5 (B) days of culture. After 1 day, transgenic kidneys were indistinguishable from wild type, but after 5 days many of the transgenic kidneys showed moderate to severe developmental retardation. (C,D) Improved development of transgenic kidneys with added GDNF. One kidney from each E11.5 embryo was cultured in medium containing 100 ng/ml GDNF (D), while the contralateral kidney from each embryo was cultured in normal medium (C). After 5 days, the kidneys were stained with *Dolichos biflorus* to reveal the branching pattern of the UB. Representative examples of wild-type and transgenic kidneys are shown. Scale bars, 0.5 mm (all images at same magnification).

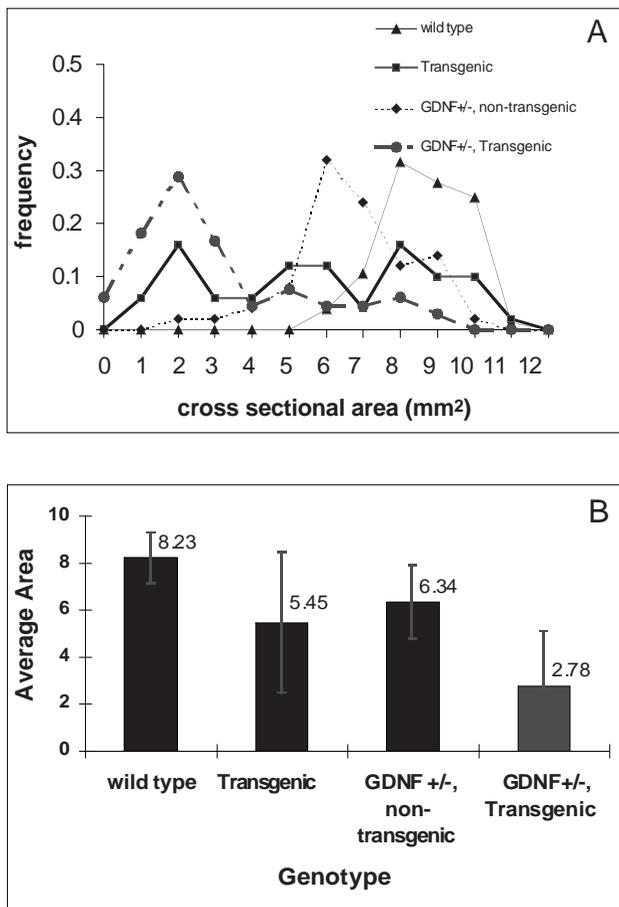


Fig. 6. Effect of heterozygosity for a *GDNF* knockout on development of *Hoxb7/RET* transgenic kidneys. Hemizygous transgenic mice were crossed with heterozygous *GDNF*^{+/-} mice to generate four possible genotypes, and the kidney sizes (sagittal cross sectional area) of all the progeny were measured at birth. The kidney size distributions of the four genotypes are shown in A, and the average size and standard deviation are shown in B. Wild type, *n*=76; Transgenic, *n*=50; *GDNF*^{+/-}, non-transgenic, *n*=50; *GDNF*^{+/-}, Transgenic, *n*=66. For 'Transgenic' vs. '*GDNF*^{+/-}, Transgenic' the *P* value was $<10^{-6}$.

their C-terminal tail (Tahira et al., 1990). While the two isoforms are co-expressed in most if not all tissues where RET is expressed (Pachnis et al., 1993), it is not known whether they serve different functions. As the RET cDNA used to construct the *Hoxb7/RET* transgene encoded only the RET9 isoform, our results indicate that the RET51 isoform is not required for normal kidney development.

A *Hoxb7/RET-PTC2* transgene causes extra-renal growth of the ureteric bud

In addition to the restricted expression of *RET* in the developing kidney, the specific expression patterns of the RET ligand GDNF and the co-receptor GFR α -1 may also be important for the control of ureteric bud development. Because receptor tyrosine kinases are active only when induced to dimerize by ligand binding, RET can only transduce a signal in cells exposed to a soluble ligand, such as GDNF, and a membrane bound (or potentially, soluble) co-receptor such as

GFR α -1. Since GDNF is only synthesized in the peripheral nephrogenic zone, and GFR α -1 is expressed primarily in the peripheral regions of the ureteric bud, RET receptors ectopically expressed in the medullary (trunk) portions of the UB might not be induced to signal. Therefore, the ectopic expression of wild-type RET receptors by the *Hoxb7/RET* transgene may not fully test its ability to alter the pattern of UB growth and branching.

To address this question, we attempted to produce transgenic mice expressing a ligand-independent, constitutively active form of RET, also under the *Hoxb7* promoter (Fig. 1A). RET-PTC2 is a rearranged form of human RET found in certain papillary thyroid carcinomas, in which the RET intracellular domain is fused to a heterologous protein as a result of a chromosomal rearrangement. The RET-PTC2 fusion protein undergoes spontaneous dimerization, resulting in constitutive and ligand-independent tyrosine kinase activity (Bongarzone et al., 1993; Durick et al., 1995). All of the five founder transgenic mice produced with the *Hoxb7/RET-PTC2* construct had very low transgene copy numbers, and when mated to normal mice they transmitted the transgene to only a small fraction of progeny, if any, suggesting that they were highly mosaic for the transgene (data not shown). In addition, we were unable to detect any expression of the transgene by northern blot analysis of adult kidney and brain RNA (data not shown). We therefore suspected that mice with high transgene copy numbers in every cell, expressing this transgene at high levels, had not survived to birth or to weaning age.

We therefore microinjected additional eggs with the *Hoxb7/RET-PTC2* construct, and analyzed the resulting mice before birth. Four of the seven transgenic mice analyzed at E16-E19 displayed renal abnormalities, which were quite distinct from the abnormalities in *Hoxb7/RET* transgenic mice. The *Hoxb7/RET-PTC2* transgenic kidneys were close to normal in size, and none of them had the cystic and dysplastic appearance of *Hoxb7/RET* kidneys. Instead, they contained abnormal nodules, either outside the kidney capsule in one case (Fig. 8A,B) or near the periphery of an otherwise normal kidney in other cases (e.g., Fig. 8C). All of the nodules consisted of branched tubular epithelial structures whose morphology was reminiscent of ureteric bud branches, surrounded by undifferentiated mesenchyme or stroma, with no visible glomeruli or comma- or S-shaped bodies (Fig. 8D-F). Staining with DB lectin confirmed the UB origin of the branched tubular structures (Fig. 9A-C). The nodules failed to stain with the lectin *Tetragonolobus lotus*, a specific marker for proximal tubules, confirming the morphological evidence that the nodules do not contain differentiated nephron elements (Fig. 9D-F).

Thus, expression of a constitutively active form of the RET tyrosine kinase under the *Hoxb7* promoter does not inhibit kidney development in the same way as a similar transgene expressing wild-type RET, but it results in the unrestricted growth of the ureteric bud at the periphery of the kidney, or outside the normal confines of the kidney.

DISCUSSION

It is known that the secreted factor GDNF and its receptor, composed of the RET receptor tyrosine kinase and a co-receptor,

GFR α -1, are essential for normal growth and branching of the ureteric bud during kidney development. However, exactly how this signal transduction pathway regulates the development of the UB is not well understood. RET and GFR α -1 are normally co-expressed only in the UB tips at the periphery of the kidney, while GDNF is expressed in the surrounding metanephric mesenchyme. In this study, we sought to test the developmental significance of these restricted expression patterns, by misexpressing two different forms of the RET tyrosine kinase throughout the UB, under the control of the *Hoxb7* promoter. One transgene encoded a wild-type RET protein, and the second a ligand-independent, constitutively active form (RET-PTC2). Because the UB tips, where RET is normally expressed, are the sites of active growth and branching, we expected that these transgenes might alter the pattern of branching of the UB, and indeed both of them did, but in different ways. The *Hoxb7/RET* transgene, rather than causing extra or ectopic branches, inhibited kidney development in a manner reminiscent of (although less severe than) a *RET* knockout. Studies in which we manipulated the level of GDNF suggested that this defect was caused by a decrease, rather than an increase, in RET signaling. We suggest that ectopically expressed wild-type RET receptors act as a sink, decreasing the available concentration of GDNF around the UB tips. The expression of a ligand-independent form of RET kinase under the same promoter did not inhibit kidney development in the same manner, but instead allowed the UB to grow in abnormal patterns, and in one case to outgrow the normal confines of the kidney. This implies that the growth of the UB is normally restricted by the limited and spatially restricted supply of RET ligand.

A dominant inhibitory effect of ectopic RET expression on kidney development

Many of the *Hoxb7/RET* transgenic mice on a wild-type background had small, cystic kidneys, with a dysplastic histoarchitecture similar to that seen in the rudimentary kidneys that develop in some homozygous *RET* knockout mice. Histological and organ culture studies with transgenic fetal kidneys showed that these defects resulted from a deficiency in UB growth and branching beginning at about E12.5, after the first few branches of the UB formed normally. Although there was no specific defect in nephrogenesis, there was a progressive deterioration in kidney development, as indicated by the reduced expression of GDNF mRNA in the metanephric mesenchyme and GFR α -1 mRNA in the UB and nephrogenic vesicles. As the transgene is expressed specifically in the UB, the effect on GDNF expression is likely to be an indirect one, in which defective growth of the UB tips leads to reduced proliferation of metanephric stem cells (which express GDNF), and in turn to further deterioration of UB development (as evinced by the weak expression of GFR α -1). This conclusion was supported by the observation that GDNF expression was normal at E12.5-E13.5, before the stage when kidney development becomes severely compromised (data not shown). A greatly reduced

level of *GDNF* mRNA in the metanephric mesenchyme has also been seen in mice defective for *Emx2*, a homeobox gene expressed primarily in the UB, whose disruption inhibits UB growth and branching (Miyamoto et al., 1997).

Why did the expression of RET under the *Hoxb7* promoter interfere with normal UB growth and branching? The hypothetical mechanisms can be divided into two classes: (1) those that involve signaling by the exogenous RET molecules, either excessive signaling in the UB tip cells due to increased RET expression, or ectopic signaling in regions of the UB that do not normally express RET; and (2) those that involve inhibition of endogenous RET function. Mechanisms that involve increased or ectopic RET signaling appear to be ruled out by two types of evidence. First, two different experiments, in which we manipulated the level of GDNF available to the transgenic kidneys, indicated that the developmental defects were caused by insufficient rather than excessive RET signaling. Addition of extra GDNF to transgenic kidney primordia cultured in vitro improved the growth and branching of the UB, while halving the GDNF gene dosage in vivo, by breeding the transgene into a GDNF heterozygous knockout background, exacerbated the deleterious effect of the transgene on kidney development. Second, the *Hoxb7/RET-PTC2* transgene, which encodes a

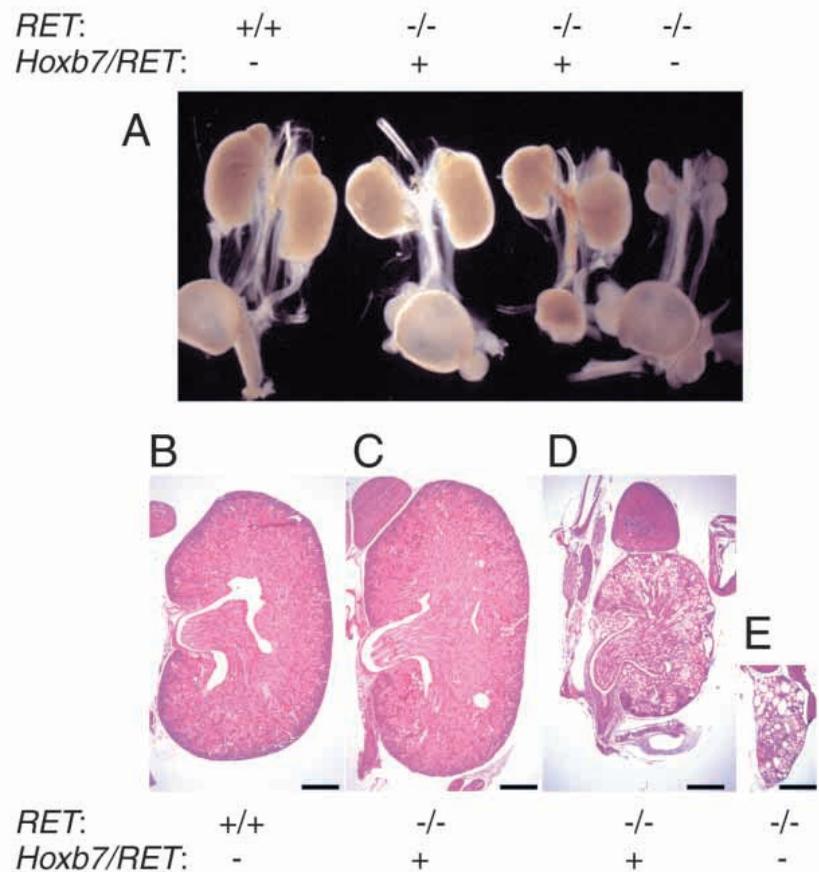


Fig. 7. Ability of the *Hoxb7/RET* transgene to rescue renal development in *RET*^{-/-} knockout mice. (A) Excretory systems dissected from one wild-type (*RET*^{+/+}) newborn mouse (left) and three *RET*^{-/-} newborn mice, two of which inherited the *Hoxb7/RET* transgene. (B-E) Sagittal sections of wild-type newborn kidney (B), two examples of *RET*^{-/-} kidneys rescued by the transgene (C, D) and one kidney from a *RET*^{-/-} non-transgenic newborn mouse (E). C shows an example of a complete rescue, and D shows a partial rescue. H&E stain. Scale bars, 0.5 mm.

ligand-independent form of the RET tyrosine kinase that signals constitutively, did not inhibit kidney development in a similar manner, although it caused a different type of abnormality.

There are several potential mechanisms by which the transgene might have interfered with endogenous RET function. First, it might have carried a mutation so that it encoded a hypomorphic receptor, which could inhibit normal RET signaling through a dominant negative mechanism. This appeared unlikely, since the transgene was able to support normal kidney development in mice homozygous for a knockout of the endogenous *RET* gene. Nevertheless, to rule out this possibility, we sequenced the entire coding sequence of the transgene, and found that it matched the published RET cDNA sequence (Iwamoto et al., 1993) at all but one base pair, which appears to be either a polymorphism or an error in the published sequence¹. Second, co-expression of the RET9 and RET51 isoforms might be important for kidney development. Overexpression of RET9 (encoded by the transgene) would alter the ratio of the two isoforms, which might somehow interfere with normal signal transduction. This possibility has been ruled out by producing *Hoxb7/RET51* transgenic mice and breeding this transgene together with the *Hoxb7/RET9* transgene. The doubly transgenic mice showed no improvement in kidney development compared to the *Hoxb7/RET9* transgenic mice (unpublished data).

We propose an alternative model to explain the dominant defect, in which the ectopically expressed RET receptors on UB trunk cells can bind and sequester GDNF, thus reducing the amount available to the UB tip cells, for whose growth the level of GDNF is critical (Fig. 9). It is possible that the UB trunk cells, which are phenotypically distinct from tip cells (Bard et al., 1994; Sariola and Sainio, 1997; Vainio and Muller, 1997), may be unable to respond to RET signaling with the same biological response as UB tip cells and so cannot overcome the defect. Several observations are consistent with this model. First, GDNF is diffusible, as localized sources of the factor can act at a distance to stimulate UB outgrowth from the Wolffian duct (Durbec et al., 1996; Sainio et al., 1997). It is therefore plausible that GDNF produced by the peripheral mesenchyme cells may bind to receptors in more proximal regions of the UB. Second, GDNF is produced in limiting quantities, as *GDNF* knockout heterozygotes display reduced kidney size (Fig. 6) and occasional renal agenesis (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Therefore, a reduction in the level of available GDNF could account for the observed growth inhibition, as well as the ability of added GDNF to partially rescue this defect. Third, *GFR α -1* is expressed not only in the UB tip cells but also

in the immediately proximal UB trunk cells (Baloh et al., 1997; Pachnis et al., 1993; Sainio et al., 1997), which may become a sink for GDNF when they co-express RET. Alternatively, soluble *GFR α -1* whose GPI anchor has been cleaved might also mediate the binding of GDNF to RET receptors in more medullary regions of the UB in the transgenic mice. Fourth, at early stages (E10.5-E12.0) when RET is normally expressed throughout the UB (Pachnis et al., 1993; Pepicelli et al., 1997), the transgene has no deleterious effect. Only at the stage when RET expression normally becomes restricted to the UB tips (E12.5-E13.5) does the transgene begin to inhibit kidney development. A similar mechanism has been invoked to explain the dominant effects of several naturally occurring mutations that cause the abnormal

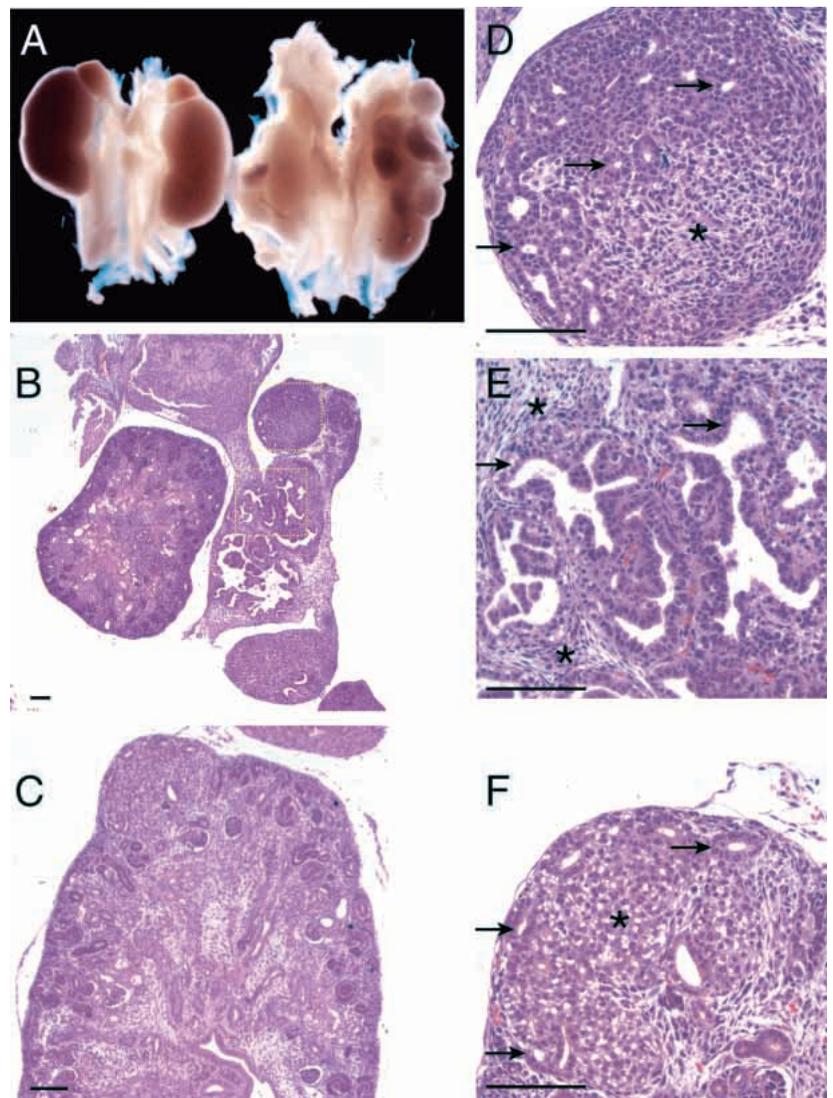


Fig. 8. Uncontrolled growth of the ureteric bud in *Hoxb7/RET-PTC2* transgenic mice at E16.5 and E18.5. (A) Kidneys from a wild-type mouse (left) and a transgenic mouse (right) analyzed at E18.5, the latter showing multiple extra-renal nodules. (B) Section of kidney and attached nodules shown in A, H&E stain. (C) Section of kidney from an E16.5 *Hoxb7/RET-PTC2* transgenic mouse, showing an abnormal nodule at the periphery of the kidney. (D,E) Higher magnification views of two areas (yellow boxes) in B, showing branched epithelial tubules (arrows) surrounded by undifferentiated mesenchyme (asterisks). (F) Higher magnification view of the nodule in C, containing ureteric bud-like tubules and undifferentiated mesenchyme. Scale bars, 0.1 mm.

¹Nucleotide 521 of RET was a T, whereas in the published sequence it is a C. Several RET cDNA clones were sequenced in this region, and all had a T at position 521. This would encode a Phe at amino acid 174, instead of the Ser in the published mouse RET sequence. However, human and chicken RET both have a Phe at this position (Iwamoto et al., 1993; Schuchardt et al., 1995; Takahashi et al., 1989; Takahashi et al. 1988).

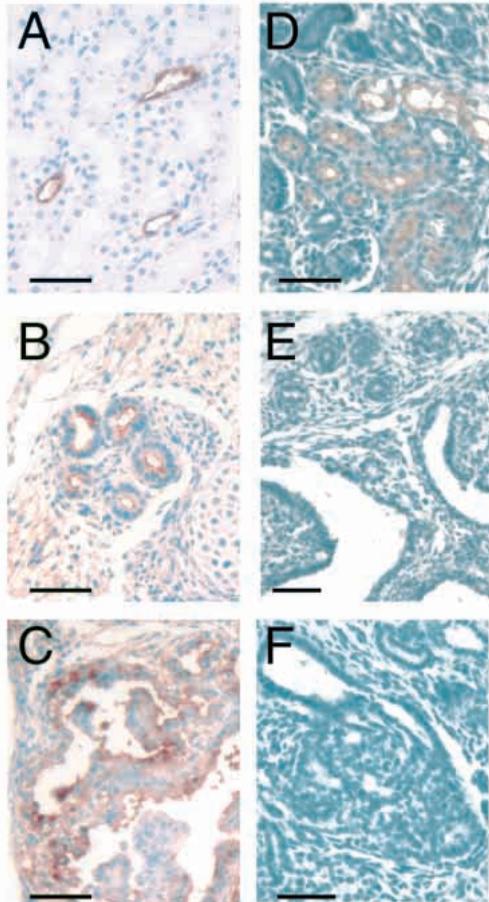


Fig. 9. Lectin staining of abnormal nodules in *Hoxb7/RET-PTC2* transgenic kidneys. (A) *Dolichos biflorus* lectin (DB) specifically stains the collecting ducts in a normal kidney. (B,C) *Dolichos biflorus* lectin staining of epithelia in nodules from an E18.5 kidney (shown in Fig. 8A and B) confirms their origin from the ureteric bud. (D) *Tetragonolobus lotus* lectin specifically stains the maturing proximal tubules in the inner cortex of an E16.5 kidney. (E,F) Epithelia in abnormal nodules are negative with *Tetragonolobus lotus* stain. Hematoxylin counterstain. Scale bars, 0.05 mm.

expression of c-kit, a tyrosine kinase receptor for the Steel factor, in somites or other ectopic sites. In these mutants, which include *W^{sash}*, *W^{banded}* and *Patch*, ectopically expressed c-kit is believed to sequester Steel, which normally promotes the survival or proliferation of migrating melanoblasts, resulting in pigmentation defects (Duttlinger et al., 1995, 1993; Kluppel et al., 1997; Wehrle-Haller et al., 1996).

One question we initially posed was why the normal expression of RET is so tightly restricted to a small population of cells at the tips of the UB. We initially hypothesized that this pattern of gene regulation might be part of a mechanism to limit the sites of UB branching. However, in the cultured transgenic kidneys, either in normal medium or with added GDNF, we observed no signs of abnormal branching or unusual growth of UB trunks. Furthermore, expression of a constitutively active form of RET throughout the UB did not visibly perturb the overall histoarchitecture of the kidney, which might have been expected if it induced ectopic branching. Therefore, while our data do not rule out the initial hypothesis, they suggest another

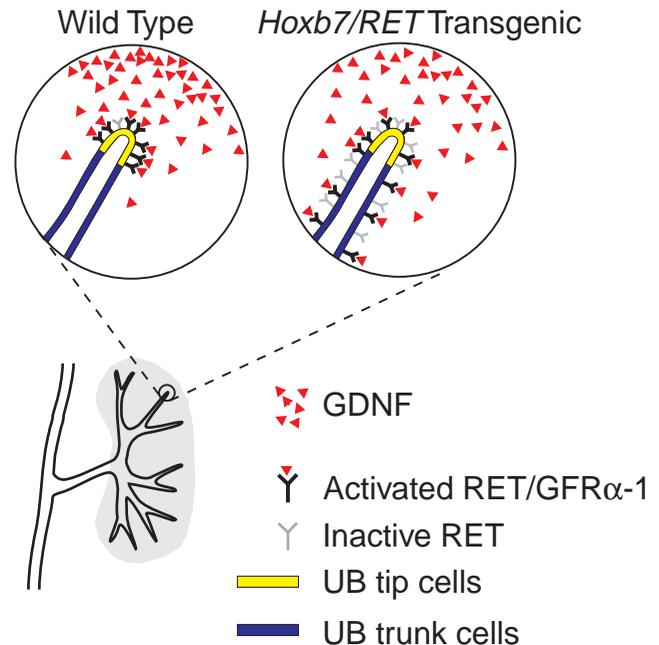


Fig. 10. A model for the growth-inhibitory effect of ectopic RET expression in the developing kidneys of *Hoxb7/RET* transgenic mice. In the wild type, the RET receptor tyrosine kinase (Y symbols) is expressed only at the distal tips of the UB branches (yellow regions), while GDNF (red triangles) is expressed by the surrounding mesenchyme cells at the periphery of the metanephros. GFR α -1, a co-receptor required for binding of GDNF to RET (not indicated in diagram), is co-expressed with RET in the UB tip cells, but is expressed in more proximal regions of the UB as well, and may also be present in a soluble form. In the presence of normal levels of GDNF, many of the RET receptors bind GDNF and are activated (black Y symbols). In the transgenic kidney, RET is no longer restricted to the UB tips, and some of the GDNF molecules bind to RET receptors in more proximal regions of the UB (UB trunks, shown in blue). This reduces the amount of GDNF available to bind to RET receptors on the UB tip cells, with the result that many more RET receptors on the UB tips fail to be activated (grey Y symbols), and growth stimulation of the UB is sub-optimal. Evidence in support of this model is described in the text.

explanation. Because the RET ligand GDNF is produced in limiting quantity and is diffusible, the expression of RET in a less tightly regulated pattern (e.g., throughout the UB) would result in binding and sequestering of the ligand by UB trunk cells at the expense of the UB tip cells.

The *Hoxb7/RET* transgene can support kidney development in *RET* knockout mice

When *Hoxb7/RET* transgenic mice were bred with *RET* knockout mice, the transgene was able to rescue kidney development in some of the newborn *RET*^{-/-} mice (Figs 3 and 7). Fifteen percent of the kidneys in these mice were normal in size and histology, and an additional 55% were partially rescued (i.e., smaller than normal but larger than *RET*^{-/-} kidneys, displaying mild to moderate dysplasia). The incomplete rescue in some animals may be due to two competing effects of the transgene: the ability of exogenous RET to substitute for endogenous RET in the UB tips, and the incompletely penetrant, inhibitory effect of the ectopically expressed RET. The *Hoxb7/RET* transgene did not

rescue the other defects in the *RET*^{-/-} mice (e.g., intestinal aganglionosis), presumably because it is not expressed in many of the normal sites of *RET* expression in the developing nervous system (Kress et al., 1990; Vogels et al., 1993).

Two important conclusions can be drawn from this result. First, it formally demonstrates that the renal agenesis phenotype in *RET* knockout mice is due to the *RET* mutation, and not to an inadvertently introduced mutation in another gene linked to *RET*. Second, it demonstrates that the RET9 isoform is sufficient to support normal kidney development. RET9 and RET51 differ only at the C terminus, where the last 9 amino acids of RET9 are replaced by a different 51 amino acid tail in RET51. One difference between the isoforms is that only RET51 contains binding sites for Grb2, an adaptor protein that links RET to Ras and the MAP kinase pathway (Borrello et al., 1994; Liu et al., 1996; Lorenzo et al., 1997). Apparently, the ability to bind Grb2 is not essential for the role of RET signaling in kidney development. This may be because both RET isoforms have binding sites for Shc, another adaptor protein through which RET signaling can activate Ras (Arighi et al., 1997; Asai et al., 1996; Lorenzo et al., 1997; Ohiwa et al., 1997). Our observation is consistent with previous findings that RET9 and RET51 are both biologically active in a number of systems that depend on Ras activation, including fibroblast transformation and induction of neuronal differentiation in PC12 cells (Asai et al., 1995; Iwashita et al., 1996; Rossel et al., 1997; Santoro et al., 1995).

A ligand-independent form of RET uncouples ureteric bud growth from the growth of the nephrogenic zone

Expression of RET-PTC2 under the *Hoxb7* promoter resulted in abnormal growth of the UB in localized nodules at the periphery of the kidney, and in one case, in multiple nodules outside the kidney. Surprisingly, with the exception of these nodules, the kidneys of newborn transgenic mice were histologically normal, and their size was unaffected. This suggests that throughout the early stages of kidney development, the expression of a constitutively active RET kinase had little or no effect, perhaps because there is already sufficient GDNF to maintain RET signaling in the UB tips. As noted above, this result also suggests that ectopic RET signaling in the UB trunk cells is inconsequential. The ability of the UB to eventually outgrow the rest of the kidney, forming nodules that protrude through the nephrogenic zone, suggests that at later stages of kidney development, the centripetal growth of the UB tips is normally restricted by the availability of GDNF. GDNF is expressed by a thin layer of mesenchymal cells near the periphery of the kidney, and any UB tips that grow through this layer would normally outgrow their source of ligand. In transgenic mice expressing a ligand-independent RET kinase, this level of regulation of UB growth is apparently circumvented. This indicates that RET signaling is not only *necessary* for UB development (as shown by the *RET* and *GDNF* knockouts), but that RET signaling actively stimulates the growth of the UB. In previous *in vitro* studies, addition of GDNF to the culture medium was observed to stimulate UB cell proliferation within intact metanephroi (Pepicelli et al., 1997), but not in isolated ureteric buds (Sainio et al., 1997). While our observations are consistent with a mitogenic role for GDNF/RET signaling, other factors produced by the kidney or surrounding tissues may also be required for the stimulation of UB growth.

It is interesting that the tubular UB elements in the nodules were surrounded only by undifferentiated mesenchymal or stromal cells, lacking mature glomeruli or immature nephrogenic vesicles. One likely explanation is that the UB has outgrown the nephrogenic stem cells at the periphery of the kidney, or (in the animal with extra-renal nodules) has grown into regions of mesenchyme lacking any nephrogenic potential. Alternatively, the abnormally growing UB may have lost the ability to induce nephrogenesis.

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