Animal Model

Sympathoadrenal Hyperplasia Causes Renal Malformations in *Ret*^{MEN2B}-Transgenic Mice

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The tyrosine kinase receptor Ret is expressed in the ureteric bud and is required for normal renal development. Constitutive loss of Ret, its co-receptor *gfra-1*, or the ligand glial cell line-derived neurotrophic factor results in renal agenesis. Transgenic embryos that express a constitutively active form of Ret (*Ret*^{MEN2B}) under the control of the dopamine- β -hydroxylase (*D β H*) promoter develop profound neuroglial hyperplasia of their sympathetic ganglia and adrenal medullae. Embryos from two independent *D β H-Ret*^{MEN2B}-transgenic lines exhibit renal malformations. In contrast with *ret*^{-/-} embryos, renal maldevelopment in *D β H-Ret*^{MEN2B}-transgenic embryos results from primary changes in sympathoadrenal organs extrinsic to the kidney. The ureteric bud invades the metanephric mesenchyme normally, but subsequent bud branching and nephrogenesis are retarded, resulting in severe renal hypoplasia. Ablation of sympathoadrenal precursors restores normal renal growth *in vivo* and *in vitro*. We postulate that disruption of renal development results because *Ret*^{MEN2B} derived from the hyperplastic nervous tissue competes with endogenous renal Ret for *gfra-1* or other signaling components. This hypothesis is supported by the observation that renal malformations, which do not normally occur in a transgenic line with low levels of *D β H-Ret*^{MEN2B} expression, arise in a *gdnf*^{+/-} background. However, renal maldevelopment was not recapitulated in kidneys that were co-cultured with explanted transgenic ganglia *in vitro*. Our observations illustrate a novel pathogenic mechanism for renal dysgenesis that may explain how putative activating mutations of the *RET* gene can produce a phenotype usually associated with *RET* deficiency. (*Am J Pathol* 1999, 155:2167–2179)

The tyrosine kinase receptor RET is a central component in the pathogenesis of several human diseases. Translocations that involve *RET* are frequently associated with

papillary carcinoma of the thyroid.^{1–3} In addition, the inherited cancer syndromes multiple endocrine neoplasia (MEN) types 2A and 2B and familial medullary thyroid carcinoma are caused by missense mutations in the *RET* gene that confer constitutive activity.^{4–9} In contrast, loss of function mutations of *RET* produce Hirschsprung disease and/or congenital central hypoventilation syndrome.^{10–15} Mice homozygous for loss-of-function mutations in *ret* have neuronal deficiencies, including intestinal aganglionosis, and agenic/dysgenic kidneys.¹⁶ It is likely that altered RET activity underlies some cases of human renal agenesis/dysgenesis as well, but no examples have been reported.

Ret functions as one component of a more complex receptor system for members of the glial cell line-derived neurotrophic factor (*gdnf*) family.¹⁷ Ligands that activate ret include *gdnf*, neurturin, artemin, and persephin. The ligands bind to one or more *gdnf* family receptors (*gfra-1* to *-4*), which are glycosyl-phosphatidyl inositol-linked proteins. Ligand binding to *gfra* probably elicits a conformational change in the latter that affects its interaction with Ret and thereby promotes Ret activation.¹⁸

Formation of the metanephric kidney and enteric nervous system depends on interaction between *gdnf*, Ret, and *gfra-1*.^{19–21} The kidney is formed by reciprocal interaction of two tissues, the metanephric mesenchyme and the ureteric bud. In murine embryos, the ureteric bud begins to form on embryonic day (E) 10.5 as an outgrowth of mesonephric duct cells, which express Ret and *gfra-1*, in response to *gdnf* produced by adjacent metanephric mesenchyme. The ureteric bud invades the metanephric mesenchyme and goes through a series of Ret-dependent branching events that culminate in formation of the entire renal collecting system by E17.²² Concomitant with invasion and branching by the ureteric bud, metanephric mesenchymal cells respond to bud-derived

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inductive signals with a complex series of morphogenetic events that result in glomerulus and proximal nephron formation.^{19,23,24} Ablation of *Ret*, *gfra-1*, or *gdnf* impairs growth and/or branching of the ureteric bud and results in renal agenesis or severe dysgenesis.^{20,21,25–28} *Ret*^{–/–}, *gdnf*^{–/–}, and *gfra-1*^{–/–} mice also lack intestinal ganglion cells, indicating a function for this signaling pathway in development of a second organ system.

MEN2B is characterized by medullary carcinoma of the thyroid, pheochromocytoma, enteric ganglioneuromas, dysmorphic facies, and skeletal abnormalities. The clinical syndrome is caused by a point mutation (M918T) in the tyrosine kinase-coding domain of *RET*.^{6,7,9} The receptor derived from the mutant allele has constitutive activity which may be further amplified by *gdnf*/*gfra-1* stimulation.²⁹ The MEN2B mutation also changes the substrate specificity of the activated receptor, but how this affects downstream cellular events is not fully understood.⁹

To model aspects of the MEN2B phenotype, transgenic mice were produced that overexpress *Ret*^{MEN2B} under control of the dopamine- β -hydroxylase (*D β H*) promoter.³⁰ The promoter is active in adrenal chromaffin cells, catecholaminergic neurons, and their precursor cells.^{31,32} Three lines of transgenic mice were created. All developed benign neuroglial tumors in sympathetic ganglia and adrenal medullae consistent with *Ret*'s presumed role as a proto-oncogene, although malignant transformation never occurred. Unexpectedly, the majority of the transgenic pups from the intermediate- and high-copy lines displayed renal agenesis or renal dysgenesis, reminiscent of *ret*^{–/–} mice. Insertional mutagenesis was not the underlying mechanism for the renal phenotype, because it occurred in two independent transgenic lines. Because the *D β H* promoter is not known to be active within developing kidneys, the transgenic phenotype raised the question of whether extra-renal *Ret*^{MEN2B} overexpression can disrupt renal organogenesis. In this paper, we describe renal development in *D β H-Ret*^{MEN2B} embryos in detail and present evidence that malformation of the kidney is secondary to *Ret*^{MEN2B} expression by hyperplastic sympathoadrenal tissues.

Materials and Methods

Mice

Production and initial characterization of *D β H-Ret*^{MEN2B} and *D β H-nlacZ* transgenic mice are described elsewhere.^{30,31} The genetic backgrounds of the founder animals for these transgenic lines were hybrid C57Bl/6J $\times$ SJL, but the mice were backcrossed to C57Bl/6J animals for more than four generations. We used two lines of *D β H-Ret*^{MEN2B} transgenic mice, designated the intermediate-copy line (*D β H-Ret*^{MEN2B}, 70 copies) and the low-copy line (*D β H-Ret*^{MEN2B}_{low}, 4 copies). Mice were hemizygous for transgenes unless otherwise noted. *D β H-Ret*^{MEN2B}_{low}, *gdnf*^{+/–} compound heterozygotes were generated by crossing *D β H-Ret*^{MEN2B}_{low} mice with mice that carry a null mutation in one *gdnf* allele (kindly pro-

vided by Heiner Westphal, National Institutes of Health).²⁶ Similarly *D β H-Ret*^{MEN2B}, *Sox10*^{Dom} compound heterozygotes were obtained from crosses of the intermediate-copy line with *Dominant megacolon* (*Sox10*^{Dom},^{33,34} Jackson Laboratory, Bar Harbor, ME) heterozygotes. For prenatal studies, overnight matings were conducted, with the morning of vaginal plug detection defined as E0.5.

Genotype Analysis

Heads from prenatal mice or 1-cm tail segments from postnatal mice were digested overnight at 37°C in 1 $\times$ SET buffer (1% sodium dodecyl sulfate, 10 mmol/L Tris-HCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0) containing 20 mg/ml proteinase K and 1.4 mmol/L NaCl. DNA was extracted with phenol/chloroform, precipitated in ethanol, and dissolved in 10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0. Polymerase chain reaction (PCR) analysis was performed with 100 ng of DNA as described previously.³⁵ For detection of the *D β H-Ret*^{MEN2B} transgene primers, 5'-GCA TCT TCA CGG CCA CCG TGG TG-3' and 5'-CGT GGA TGC CTT CAA GAT C-3' were used. PCR analyses to genotype *gdnf* alleles and the *Sox10*^{Dom} mice were performed as described by Pichel et al²⁶ and Kapur et al,³⁶ respectively.

Histology, Electron Microscopy, and Immunohistochemistry

Embryos were fixed overnight in 10% buffered formalin, dehydrated, embedded in paraffin, and sectioned. The sections (5 μ m) were either stained with hematoxylin and eosin (H&E) or analyzed by immunohistochemistry. For ultrastructural studies, the caudal portion of E12.5-stage embryos were transected just rostral to the lower limb bud, fixed in 3% glutaraldehyde–0.1 mol/L sodium cacodylate (pH 7.4) for 2 hours, and postfixed with osmium tetroxide. The samples were dehydrated, embedded in plastic, and thin-sectioned.

For immunohistochemical staining, sections were deparaffinized, microwave-pretreated for 12 minutes while submerged in 10 mmol/L citrate, pH 6.0, incubated with 0.01% pronase for 15 minutes at 37°C, and blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS) for 10 minutes. Incubation with primary antibody, anti-tyrosine hydroxylase (anti-TH; 1:1000, Eugene Tech International, Ridgefield Park, NJ), or anti-peripherin (1:1000, Chemicon International Inc, Temecula, CA) was performed overnight in a moist chamber at 4°C. Bound antibody was visualized with biotinylated anti-rabbit secondary antibody (90 minutes, 1:250, Boehringer Mannheim, Indianapolis, IN), followed by horseradish peroxidase-conjugated streptavidin (NEN Life Science Products, Boston, MA) and 0.05% diaminobenzidine (Sigma Chemical, St. Louis, MO) in PBS. Retroperitoneal organs from E12.5 and E13.5 *D β H-lacZ* transgenic embryos were analyzed for transgene expression by whole-mount X-gal staining as described previously.³²

The average number of ureteric bud branches per kidney was calculated from histological counts (three sections per embryo). To determine average kidney size in E14.5 embryos, histological sections of wild-type and transgenic embryos were scanned into Adobe Photoshop 4.0. The image histogram function was then used to calculate the single largest cross-sectional renal area from five different levels. The largest renal cross-sectional area was set at 100%, and the comparative sizes of kidneys from littermates were calculated. Student's *t*-tests were applied to evaluate the significance in mean values between different groups.

Organ Culture

Metanephric kidneys were dissected from E12.5 embryos in PBS. Because embryos carrying the *DβH-Ret^{MEN2B}* transgene could not be distinguished grossly from wild-type embryos at this gestational age, cranial tissue was genotyped by PCR analysis. Isolated kidneys were transferred to Millipore filters (0.45-μm pore size). The filters were placed on metal grids in the central wells of 60- × 15-mm tissue culture dishes (Becton Dickinson, San Jose, CA) that contained 1 ml of Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY), supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The organs were cultured at 37°C in 5% CO₂, and the medium was changed every third day. For serum-free cultures, equal volumes of DMEM and Ham's F-12 supplemented with 6.8 nmol/L selenium, 2 nmol/L triiodothyronine, 0.83 μmol/L insulin, 62 nmol/L transferrin, 71 nmol/L prostaglandin E₁, 100 U/ml penicillin, and 100 μg/ml streptomycin were used. After 4 days the organs were either fixed in 10% buffered formalin and embedded for histology or fixed in ice-cold methanol for whole-mount immunohistochemistry.

For celiac ganglion/renal co-cultures, metanephric kidney explants were juxtaposed with preaortic neuroglial tissue from E12.5 embryos and incubated in medium that included serum for 4 days. The embryos used in the co-culture experiments also carried the *DβH-nlacZ* transgene, expression of which served as a marker of sympathetic neurons. After fixation and X-gal staining, digital images of the explants were captured with a video camera and dissection microscope and analyzed with the image histogram function, as described above. Histological sections of the explants were also prepared and stained with hematoxylin and eosin or immunostained for peripherin immunoreactivity.

Whole-Mount Immunohistochemistry

Pan-cytokeratin whole-mount immunohistochemistry was performed as described by Sainio et al.³⁷ Cultured kidneys were harvested in 80% ice-cold methanol, washed in 11% sucrose–1% bovine serum albumin in PBS, and incubated overnight with an anti-pancytokeratin antibody (1:400, Sigma) at 4°C. After three 2-hour washes in 11% sucrose–1% BSA in PBS, kidneys were incubated over-

Figure 1. A: The *DβH-nlacZ* transgene is not expressed within embryonic kidneys. X-gal staining of an E13.5 *DβH-nlacZ* embryo reveals transgene expression in preaortic sympathetic ganglia (c), and in chromaffin cells of the adrenal gland (ad), whereas no stained cells are seen within the kidneys (k). **B:** RT-PCR detects transcripts of the *DβH-Ret^{MEN2B}* transgene (MEN), as well as endogenous *DβH* (DBH), in preaortic sympathetic tissue (Lanes c) isolated from an E12.5 transgenic embryo. In contrast neither the transgene nor endogenous *DβH* mRNAs are detected within isolated E12.5 kidneys (Lanes k). Amplification of dihydrofolate reductase (DHFR) mRNA is an internal control, which confirms the presence of cDNA in all lanes.

night with a fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. After three 2-hour washes in PBS, kidneys were examined with a fluorescence microscope.

Reverse Transcriptase (RT)-PCR

Metanephric kidneys and intervening soft tissue (celiac ganglia) from E12.5 *DβH-Ret^{MEN2B}*-transgenic and wild-type embryos were harvested individually in 150 μl of lysis buffer, and RNA was isolated with the RNeasy Mini Kit (Qiagen, Santa Clarita, CA), according to the manufacturer's instructions. Contaminating DNA was digested with 10 U of DNase I, followed by cDNA synthesis with the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim, Indianapolis, IN). Fifty nanograms of cDNA were used per PCR reaction. Primers 5'-GGA GGA GAT GTG TGT CAA CTA TGT GC and 3'-GGG CTC TGA GTC TGT CGG CAT GG were used to amplify endogenous *DβH* cDNA (350 bp), and primers 5'CCT CTC TGA GGT CCA GGA GG-3' and 5'-GGA GGC TTG AAC AGT GGG ACA TG-3' were used to amplify *DHFR* cDNA (450 bp). PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Results

The *DβH-Ret^{MEN2B}* Transgene Is Not Expressed in Developing Kidneys

The *DβH* promoter drives expression of β-galactosidase in *DβH-nlacZ*-transgenic mice, thereby providing a histochemical marker for nuclei of *DβH*-expressing cells.^{31,32} To verify that the *DβH* promoter is not active within kidneys during early organogenesis, E12.5 and E13.5 *DβH-nlacZ* embryos were examined with X-gal staining. *lacZ* expression was confirmed in sympathetic neuroblasts of the preaortic ganglia and within chromaffin cells of the adrenal gland. However, no blue nuclei were within the

Figure 2. A-D: H&E-stained sections of E12.5 wild-type (wt) and transgenic (tg) embryos. The ureteric bud has branched several times in the metanephric mesenchyme of the wt embryo (**A**). In contrast, branching is reduced in the right tg kidney, and in the left tg kidney the ureteric bud has not branched at all (**arrow** in **B**). Although very few sympathetic neuroblasts are located between the developing kidneys of wt embryos (**C**), a mass of preaortic neuronal precursors (**arrowhead** in **D**) is found in tg embryos at the same anatomical level (**D**). **E** and **F:** Adjacent sections are immunostained with anti-peripherin antibody to localize neuronal (**arrowheads**) cells. A few, scattered peripherin-positive neurons are located by the aorta in the wt embryos at this anatomical level. However, abnormal sympathetic hyperplasia fill the midsection of the tg embryo. Scale bars, 180 μ m (**A** and **B**) and 90 μ m (**C-F**).

developing kidneys (Figure 1A). RT-PCR analysis of mRNAs isolated from kidneys and sympathoadrenal tissues of E12.5 transgenic embryos confirmed that the same was true for the *D β H-Ret*^{MEN2B} transgene. Although endogenous *D β H* and transgene-derived *Ret*^{MEN2B} mRNAs were both detected in preaortic sympathetic tissue, neither of the genes was expressed within kidneys (Figure 1B).

Most *D β H-Ret*^{MEN2} Mice Die before Weaning

D β H-Ret^{MEN2B}-transgenic fetuses survive to term with Mendelian frequency ($n = 173$), but the postnatal survival rate of transgenic pups was very low. Only 13% survived

to weaning (21 days). Twenty transgenic mice that survived to adulthood were sacrificed and examined for renal abnormalities, and three of these had unilateral renal agenesis and a normal contralateral kidney. The remaining 17 had normal kidneys bilaterally.

Most transgenic pups died within the first few days after birth. The frequency of renal anomalies was examined in embryos collected before birth (E17.5) because many dead pups were consumed by their mothers. The vast majority (47 of 62) of transgenic pups had abnormal kidneys, easily distinguished from normal. Most had bilateral renal hypoplasia (38 of 62), whereas unilateral (6 of 62) or bilateral agenesis (2 of 62) was rare. In 6 of 10 cases of renal agenesis, the ureter was present.

Figure 3. The mean number of ureteric bud branches/kidney in E12.5 embryos. Transgenic kidneys (open bars) vary from 0 branches (ie, the ureteric bud has not invaded the metanephric mesenchyme) to 7 branches per kidney. Wild-type kidneys (hatched bars) contain a ureteric bud that branched at least four times.

Kidney Growth Is Reduced in $D\beta H$ -Ret^{MEN2B}-Transgenic Embryos

Kidney development in wild-type and $D\beta H$ -Ret^{MEN2B}-transgenic embryos was investigated by histological analysis of serially sectioned embryos. Kidneys begin to develop at E10.5 to E11, when the ureteric bud grows toward the metanephric mesenchyme, and by E11 the ureteric bud reaches the metanephric mesenchyme. We could not distinguish renal sections of $D\beta H$ -Ret^{MEN2B}-transgenic ($n = 8$) from wild-type ($n = 3$) embryos at E11.5 (not shown). By E12.5, when the ureteric bud had branched several times within wild-type kidneys ($n = 8$; Figures 2A and 3), branching was reduced in most (14 of 20) $D\beta H$ -Ret^{MEN2B}-transgenic kidneys (Figures 2B and 3). In many transgenic embryos, kidneys appeared normal on one side, but exhibited reduced or no branching of the ureteric bud on the opposite side. The ureteric bud almost always invaded the metanephric mesenchyme (38 of 40 cases), in contrast with *ret*^{-/-} embryos, in which the ureteric bud fails to invade the metanephric mesenchyme (18 of 22).¹⁶

Over the following 2 days of embryogenesis, wild-type kidneys grow rapidly, with repeated branching of the ureteric bud and concomitant nephrogenesis. Although renal growth occurred in $D\beta H$ -Ret^{MEN2B}-transgenic embryos, the overall growth rate was reduced. The mean size of kidneys of transgenic embryos was 65% (range, 39–100%) that of their wild-type littermates at E14.5 ($P < 0.05$). Small size was due to a general reduction in the number of tubules (Figure 4, C and D). At later embryonic stages the size difference was even more pronounced

(Figure 4, E and F). Histological examination of newborn kidneys confirmed that hypoplastic kidneys had renal cortices that contained glomeruli, proximal, and distal tubuli and renal medullae with collecting tubules. Thus, nephrogenesis occurred, but too few complete nephrons were formed. Dysplastic features (chondrogenesis, fibrosis, and cysts) were not observed, although occasional dilated tubules were present in adults.

Preaortic Sympathetic Tumors Develop Concomitant with Reduced Kidney Growth

The $D\beta H$ promoter is active in sympathetic neuroblasts from E10.5 onwards. In wild-type E12.5 embryos, preaortic sympathetic neuroblasts form a solid mass at the level of the developing adrenal glands. This mass is much larger in $D\beta H$ -Ret^{MEN2B}-transgenic embryos, and extends down to the kidney level, where transverse histological sections of wild-type embryos contain very few neuronal precursors at this stage (Figure 2, C and E). Hence, sympathoadrenal cells that express the Ret^{MEN2B} receptor were located in close proximity to the developing kidneys (Figure 2, D and F), although no direct contact with either the ureteric bud or the metanephric mesenchyme was observed.

Electron microscopic studies of the celiac region and adjacent tissues were conducted with E12.5 transgenic and nontransgenic embryos. The preaortic ganglia from normal embryos and corresponding tumors from transgenic embryos were morphologically similar. Each was composed of closely packed cells with abundant cytoplasm that contained numerous dense-core vesicles, which are typically associated with catecholamine production (Figure 5A). Processes from these cells were found within the ganglion/tumor, but did not project into the kidney, even in transgenic embryos with obvious renal anomalies (Figure 5B). Aside from a paucity of ureteric bud-derived tubules, no renal ultrastructural differences were evident between the two genotypes (non-transgenic not illustrated).

From E12.5 onwards, transgenic preaortic sympathetic ganglia grew dramatically so that, by E13.5, hyperplastic ganglia formed massive celiac tumors. By E14.5 the celiac tumors were bigger than the adjacent kidneys (Figure 4, A and B). At this stage, anti-peripherin immunohistochemistry revealed nerve fibers from the celiac tumor, which projected in some, but not all, embryos into the developing kidneys (Figure 6, A and B). The nerve bundles were very prominent at P1 (Figure 6, C and D) and formed thick bundles in the renal hilum and cortex. However, distal branches were lacking, because neither small nerves nor individual fibers were observed intermixed with surrounding renal tissue.

Kidney Growth Is Restored in Renal Explants

Given that the earliest features of abnormal renal development in $D\beta H$ -Ret^{MEN2B}-transgenic embryos were apparent by E12.5, but the transgene was not expressed within E12.5 kidneys, we investigated whether hypoplasia

Figure 4. Histological sections of E14.5 wild-type (wt; **A** and **C**) and transgenic (tg; **B** and **D**) embryos. The preaortic sympathetic ganglia (c) are fused to form a large tumor in the *DβH-Ret^{MEN2B}*-transgenic embryo. Although nephrogenesis occurs in wt and tg kidneys (k), the overall renal growth rate is reduced in transgenic embryos. Higher magnifications of the left kidneys in **A** and **B** show that the reduction in size in tg kidneys is accompanied by a reduced number of tubules. By E16, a wt kidney (**E**) is several times bigger than a representative tg kidney (**F**). In the tg embryo, the adrenal gland has fused with the celiac tumor, but is outside the photographic field. Abbreviations: sc, spinal cord; ad, adrenal gland. Scale bars, 100 μ m (**A** and **B**); 260 μ m (**C** and **D**); 920 μ m (**E** and **F**).

was an intrinsic renal defect by examining development of explanted kidneys *in vitro*. E12.5 kidneys from transgenic embryos and their nontransgenic littermates were explanted and cultured *in vitro* for 4 days. No difference in overall growth between wild-type ($n = 11$) and transgenic ($n = 14$) kidneys occurred in serum-containing or defined culture medium. Histology showed similar nephrogenesis in both wild-type and transgenic explants (Figure 7, **A** and **B**), and anti-cytokeratin whole-mount immunohistochemistry showed no difference in ureteric bud branching (Figure 7, **C** and **D**). Thus, it seems unlikely that a defect intrinsic to the kidney accounts for the renal malformations in *DβH-Ret^{MEN2B}*-transgenic mice. Instead, an extrinsic influence, possibly the nearby hyperplasia of *Ret^{MEN2B}*-expressing sympathoadrenal precursors, might be involved.

The Sox10^{Dom} Mutation Modifies the Renal Phenotype of DβH-Ret^{MEN2B} Embryos

To investigate whether hyperplasia of sympathoadrenal precursors was required for transgene-induced renal

malformations, we exploited the *Sox10^{Dom}* mutation as a tool to eliminate prevertebral ganglia *in vivo*, because homozygous *Sox10^{Dom}/Sox10^{Dom}* mice are missing abdominal sympathetic ganglia. The *Sox10^{Dom}* allele resulted from a spontaneous missense mutation of the *Sox10* gene and is associated with absent enteric ganglia and melanocyte deficiencies in heterozygous mice.^{33,34,36,38} Embryos homozygous for the *Sox10^{Dom}* mutation die prenatally with severe deficiencies in their sympathetic nervous system. Preaortic sympathetic ganglia are virtually absent in *Sox10^{Dom}/Sox10^{Dom}* embryos.³⁹ Introduction of the *DβH-Ret^{MEN2B}* transgene did not alter any of these phenotypic features, nor did heterozygosity for a single *Sox10^{Dom}* allele affect the renal malformation rate associated with the *DβH-Ret^{MEN2B}* transgene (Figure 8). However, in *DβH-Ret^{MEN2B}, Sox10^{Dom}/Sox10^{Dom}* embryos, preaortic sympathetic ganglia did not form (Figure 9, **B** and **C**), and renal malformations did not occur (8/8; Figures 8 and 9). Thus, when dissociated from hyperplastic sympathoadrenal cells induced by the *DβH-Ret^{MEN2B}* transgene, kidney growth was normalized *in vitro* and *in vivo*.

Figure 5. Ultrastructure of the preaortic tumor (**A**) and kidney (**B**) from an E12.5 *DβH-Ret^{MEN2B}*-transgenic embryo. The tumor is composed of neuronal cell bodies (CB) and their processes (p), which contain numerous dense-core vesicles (arrows). No neural elements were identified in the kidney, despite a careful search of the interstitial areas between tubules (T). Aside from a reduction in mature tubules, the transgenic kidneys were not different ultrastructurally from wild-type embryos (not shown). Scale bars, 3 μ m (**A**) and 7.5 μ m (**B**).

gdnf Haploinsufficiency Elicits Renal Malformations in the *DβH-Ret^{MEN2B}* Line

In *DβH-Ret^{MEN2B}*-transgenic embryos, hyperplastic, preaortic sympathetic neurons form in close proximity to and later innervate developing kidneys. These neurons presumably express endogenous Ret and *gfra-1*, overexpress the *Ret^{MEN2B}* receptor, and possibly have increased cellular capacity to bind *gdnf*. Because kidney formation is dependent on proper *gdnf-ret* interaction, a possible mechanism by which *Ret^{MEN2B}* interferes with renal growth could be disruption of *gdnf-ret* signaling in the kidneys. It is possible that preaortic sympathetic neurons compete with endogenous Ret receptors on branches of the ureteric bud for signaling components, reducing the amount of available *gdnf* for renal morphogenesis. If so, a more dramatic phenotype might be achieved experimentally by disrupting one or both *gdnf* alleles in *DβH-Ret^{MEN2B}* embryos. However, because the frequency of renal malformations already is very high in *DβH-Ret^{MEN2B}* mice (only 13% survive to weaning), a worsened phenotype would be difficult to assess.

Instead, we conducted a similar experiment using a low-copy line of *DβH-Ret^{MEN2B}* (*DβH-Ret^{MEN2B}low*). *DβH-Ret^{MEN2B}low*-transgenic mice express less transgene, have sympathetic tumors similar to the intermediate-copy line, but develop normal kidneys.³⁰ We crossed *DβH-Ret^{MEN2B}low*-transgenic mice with mice that carry a null

mutation in the *gdnf* gene.²⁶ As originally described, mice heterozygous for the null allele sometimes display unilateral agenesis or, rarely, dysgenesis that does not affect pup viability.²⁶ In our colony, which has a different genetic background, newborns heterozygous for the *gdnf* mutation ($n = 16$) or hemizygous for the *DβH-Ret^{MEN2B}low* all had normal kidneys ($n = 26$; Figure 10). However, 5 of 12 compound hemizygous pups (*DβH-Ret^{MEN2B}low*, *gdnf*+/-) had malformed kidneys (Figure 10). Three embryos had bilaterally small kidneys, and two embryos had unilateral renal agenesis accompanied by a contralateral hypoplastic kidney. Three of the compound heterozygotes were found dead at postnatal day 1. Thus, *gdnf* haploinsufficiency exposed a renal phenotype in *DβH-Ret^{MEN2B}low* embryos, which was previously associated only with higher levels of transgene expression in other lines.

Co-Culture with *DβH-Ret^{MEN2B}*-Transgenic Sympathoadrenal Tissue Does Not Promote Renal Maldevelopment in Vitro

The preceding data suggest that renal malformations in *DβH-Ret^{MEN2B}*-transgenic mice are secondary to sympathoadrenal neuroglial hyperplasia and are influenced by *gdnf* expression levels. We suspect that overexpression of Ret by the neuroglial tumors competes with endogenous renal Ret for signaling components (*gdnf* or *gfra-1*). To test this hypothesis, the effects of transgenic and

Figure 6. Histological sections showing abnormal nerve fibers in E14.5 (**A** and **B**) and newborn (**C** and **D**) transgenic kidneys. **A:** An H&E-stained, hypoplastic E14.5 kidney (k), is innervated by a thick nerve fiber (arrow). **B:** The nerve is stained by an anti-peripherin antibody. **C:** Anti-peripherin immunostaining of a section from a newborn transgenic pup shows a large preaortic celiac tumor (c) that invades the adjacent kidney (k). **D:** Giant abnormal nerve fibers, which fail to give rise to smaller interstitial branches, are present in the cortex of a newborn transgenic pup. Scale bars, 90 μ m (**A** and **B**) and 180 μ m (**C** and **D**).

nontransgenic sympathoadrenal precursors on the development of explanted kidneys from E12.5 embryos were compared. After four days *in vitro*, no significant differences in growth (Figure 11) or histological differentiation (not shown) were evident between kidneys, regardless of their genotype or proximity to transgenic neuroglial tissue. A uniform distribution of differentiated nephron units, derived from metanephric mesenchyme (glomeruli and proximal tubules) and ureteric bud (collecting tubules), was present throughout the grafts, including areas in contact with neuroglial tissue. Thus, the co-culture conditions used in this study failed to reproduce the renal malformations observed *in vivo*.

Discussion

The Ontogeny and Pathology of Renal Malformations in $D\beta H\text{-Ret}^{\text{MEN2B}}$ Embryos Differ from $ret^{-/-}$, $gdnf^{-/-}$, and $gfra-1^{-/-}$ Models

The results of this study suggest that renal malformations in $D\beta H\text{-Ret}^{\text{MEN2B}}$ transgenic embryos are secondary to sympathoadrenal hyperplasia and represent a distinct pathogenic process from that which underlies renal defects in $ret^{-/-}$, $gfra-1^{-/-}$, or $gdnf^{-/-}$ embryos. The

difference is evident in the renal pathology of $D\beta H\text{-Ret}^{\text{MEN2B}}$ pups *versus* the knockout models. All of the latter are characterized predominantly by renal agenesis with severe dysgenesis in only a small fraction of pups. Renal agenesis was rarely observed in $D\beta H\text{-Ret}^{\text{MEN2B}}$ pups, in which variable renal hypoplasia, ranging from severe to none, was the most common finding. Ontogeny of renal agenesis has been investigated in $ret^{-/-}$ embryos and usually results from failure of ureteric bud formation that is evident by E11.²⁵ When cells of the metanephric mesenchyme do not receive required bud-derived signals, they die by apoptosis before E13.5, and no kidneys form. Renal agenesis and/or dysgenesis in $gdnf^{-/-}$ and $gfra-1^{-/-}$ mice are also caused by primary failure of the ureteric bud to form or invade metanephric mesenchyme.^{20,21,26–28} In contrast, the first anatomical indication of defective renal growth in $D\beta H\text{-Ret}^{\text{MEN2B}}$ embryos appears significantly later (E12.5), after successful invasion of the metanephric blastema by ureteric bud. Beginning at this stage, a subset of embryos exhibits reduced branching of the ureteric bud and organ hypoplasia without overt dysplasia (cyst formation, fibrosis, or chondrogenesis). Thus, the $D\beta H\text{-Ret}^{\text{MEN2B}}$ transgene rarely interferes with the initial formation and growth of the ureteric bud, but frequently affects later branching events.

Figure 7. **A** and **B:** H&E-stained sections of E12.5 wild-type (wt) and transgenic (tg) kidneys after 4 days of development *in vitro*. Nephrogenesis occurred in both wt and tg kidneys, with developing glomeruli (**short arrows**), proximal tubules (**arrows**), and collecting ducts (**asterisk**) visible. **C** and **D:** Whole-mount cytokeratin immunohistochemistry stains the branching ureteric bud (ub). Scale bars, 40 μ m (**A** and **B**) and 250 μ m (**C** and **D**).

Extrinsic Expression of *Ret*^{MEN2B} Interferes with Renal Development

The human *DBH* promoter in the *DBH-Ret*^{MEN2B} transgene was used to regulate expression of many other cDNAs in transgenic mice, and transgene expression has been restricted consistently to catecholaminergic cells.^{31,32,40,41} The only exception is a subset of mesenchymal cells in the midface, which transiently express reporter genes fused to the promoter.³² Rare (5–24 cell soma per postnatal kidney) intrinsic neurons have been described in the rat kidney. These neurons are always associated with extrinsic nerves that do not enter the kidney until E16, are primarily located in the renal hilum or adjacent to the pelvis, and co-express TH and *DBH* with other neurotransmitters.^{42–45} However, we never observed TH immunoreactivity in the developing murine kidney, except in cell processes of older embryos. Furthermore, no endogenous *DBH*, *DBH-nlacZ*, or *DBH-Ret*^{MEN2B} transcripts were detected in E12–E14 renal samples by RT-PCR. Therefore, we believe that the effects of *DBH-Ret*^{MEN2B} on renal development are secondary to gene expression in nonrenal tissues. This hypothesis is supported by the fact that transgenic kidneys exhibit growth and differentiation indistinguishable from nontransgenic kidneys, when cultured as isolated organs *in vitro*.

The earliest phenotypic change in *DBH-Ret*^{MEN2B} embryos is profound hyperplasia of sympathoadrenal precursors, particularly in the celiac region. The proximity of the primitive neural tumor that arises in this region and the fact that ganglion cells in the normal celiac ganglion innervate the kidneys suggested that sympathoadrenal hyperplasia may be the primary lesion responsible for renal malformations. As predicted by this hypothesis, renal development in *Sox10*^{Dom}/*Sox10*^{Dom}, *DBH-Ret*^{MEN2B} embryos, which lack celiac ganglia, was not impaired. Although unanticipated

molecular or cellular effects associated with the *Sox10*^{Dom}/*Sox10*^{Dom} genotype, other than absent sympathoadrenal precursors, might underlie the rescue phenomenon, we believe that the most straightforward interpretation is that

Figure 8. *DBH-Ret*^{MEN2B}, *Sox10*^{Dom}/+ mice were crossed with mice carrying only the *Sox10*^{Dom} mutation, and the kidneys of embryos at E12.5 were examined for abnormalities. *DBH-Ret*^{MEN2B}-transgenic embryos homozygous for the *Sox10*^{Dom} mutation all had normal kidneys.

Figure 9. Absence of renal malformations in $D\beta H\text{-Ret}^{\text{MEN2B}}$ -transgenic embryos that are homozygous for the *Sox10*^{Dom} mutation. An H&E-stained histological section of the pre-aortic region (**A** and **B**) from an E12.5 $D\beta H\text{-Ret}^{\text{MEN2B}}$ -transgenic embryo shows very few sympathetic neuroblasts, as confirmed by scanty peripherin-positive cells (**arrowhead** in **C**). Compare with Figure 2. k, kidney; **arrows**, ureter. Scale bars, 170 μm (**A**) and 85 μm (**B** and **C**).

the renal malformations are secondary to sympathoadrenal hyperplasia.

Another approach to test this hypothesis was to co-culture E12.5 celiac ganglia from $D\beta H\text{-Ret}^{\text{MEN2B}}$ -transgenic and nontransgenic embryos with E12.5 renal tissue from transgenic or nontransgenic mice. Experiments of this type failed to demonstrate any influence of transgenic sympathoadrenal precursors on renal development *in vitro*. It seems likely that either critical *in vivo* variables (eg, levels of expression of the transgene, endogenous ret, gdnf, *gfra1*, or other signaling components) were not recapitulated *in vitro* or hyperplastic pre-aortic ganglia act indirectly *in vivo*

Figure 10. *Gdnf* haploinsufficiency elicits renal malformations in $D\beta H\text{-Ret}^{\text{MEN2B}low}$ -transgenic pups. In contrast with the normal renal development that occurs in $D\beta H\text{-Ret}^{\text{MEN2B}low}$ pups with two intact *gdnf* alleles and non-transgenic *gdnf*^{+/−} pups, more than 50% of $D\beta H\text{-Ret}^{\text{MEN2B}}$ -transgenic, *gdnf*^{+/−} embryos have hypoplastic or absent kidneys.

via a mechanism that could not be exercised in the co-cultures (see below).

Competition with Endogenous Ret for *gfra-1* or Other Signaling Components Probably Causes Renal Malformations in $D\beta H\text{-Ret}^{\text{MEN2B}}$ Embryos

Several mechanisms might be envisioned by which primary overgrowth of the celiac ganglion could interfere with kidney growth. The tumors might exert a mass effect on the kidneys or their vasculature. Direct physical deformation of renal tissue is unlikely because the body of the tumor does not actually contact the kidney, except through nerve processes, which do not enter the kidney until after renal malformation is evident. Compression of the renal arteries or veins is easier to envision because the tumor abuts these vessels near the embryonic midline. However, our histological studies showed no evidence for arterial narrowing, venous congestion, or thrombosis. Furthermore, structures served by other vessels in the same vicinity (eg, spleen, liver, or lower extremities) exhibited no signs of ischemic injury.

Alternatively, products of the tumor might impair renal development by paracrine, endocrine, or neurosecretory pathways. At E12.5, when retarded branching of the ureteric bud is first detectable, abundant dense-cored neurosecretory granules are present in the celiac

Figure 11. Kidney growth is unaffected by co-culture with celiac ganglia from *DBH-Ret^{MEN2B^{low}}*-transgenic embryos. Explanted kidneys from E12, transgenic (Tg) embryos and their nontransgenic (Ntg) littermates were cultured either alone or apposed to celiac ganglia. The embryos also carried the *DBH-nlacZ* transgene such that nuclei of the celiac ganglia (c) are dark after X-gal staining. Growth and morphogenesis of the explants after 4 days *in vitro* were not significantly different as assessed grossly (A), histologically (not shown), and by measurements of cross-sectional area (B). The arrowheads indicate ureteric buds. Scale bar, 1 mm.

masses. Presumably, these contain norepinephrine, which is known to reduce renal blood flow and cell replication in neonatal kidneys and may have comparable effects in developing kidneys.⁴⁶ Renal hypoplasia in human fetuses has been associated with exposure to pharmacological agents that exhibit vasoactive properties comparable with norepinephrine.^{47–50} However, the renal pathology differs from *DBH-Ret^{MEN2B}* kidneys in that proximal tubules are markedly reduced or absent, collecting ducts are preserved, and cortical cysts are common.

The preceding mechanisms imply that the role of *Ret^{MEN2B}* in the pathogenesis of renal malformations is merely to promote hyperplasia of preaortic sympathoadrenal precursors. However, the existing data suggest that tumor formation alone is not sufficient. Rather, high expression of *DBH-Ret^{MEN2B}* by the tumors appears necessary. In part, this conclusion is based on the fact that equivalent or greater degrees of preaortic sympathoadrenal overgrowth occur in two other transgenic models, which do not exhibit renal defects. One example is the

low-copy line of *DBH-Ret^{MEN2B^{low}}* mice. The latter carry fewer copies of the *DBH-Ret^{MEN2B}* transgene and express significantly less *Ret^{MEN2B}* protein, but they develop tumors that are ontogenically and pathologically identical to those associated with renal anomalies in the higher-copy line. Another example is mice that carry the *DBH-ras^{val12}* transgene, which uses the *DBH* promoter to target expression of an oncogenic *ras* cDNA.⁴⁰ *DBH-ras^{val12}* mice develop pathologically similar ganglioneuromas, but their masses are larger, on average, than those of *DBH-Ret^{MEN2B}* mice (R. P. Kapur, unpublished observation). Despite comparable tumors, neither the *DBH-Ret^{MEN2B^{low}}* nor *DBH-ras^{val12}* embryos have kidney malformations. Thus, in addition to sympathoadrenal hyperplasia, something associated with high levels of *DBH-Ret^{MEN2B}* expression appears to impair renal development.

We believe that *Ret^{MEN2B}* protein produced by the preaortic neural tumors of *DBH-Ret^{MEN2B}* transgenic embryos competes for components of the *Ret*-signaling complex with endogenous *Ret* made by cells that line

branches of the ureteric bud. The effect could be mediated by intact Ret^{MEN2B} receptor on the surface of post-ganglionic nerve fibers or by soluble truncated receptor fragments, which might be secreted by these cells due to receptor overexpression or cDNA truncations within the transgene array. This competitive mechanism could account for transgene-induced renal malformations because outgrowth and branching of the ureteric bud are Ret-dependent. Our conclusion is based partly on the fact that kidney defects do not occur in *DβH-Ret^{MEN2B}* embryos, even though they develop pre-aortic tumors similar in size to the two higher-expressing transgenic lines with renal agenesis. Stronger support for this model is the observation that identical renal anomalies occur in the low-copy line, when embryos are haploinsufficient for *gdnf*. Thus, the quantity of *gdnf* produced in *gdnf*^{+/−} embryos is sufficient to ensure normal renal development in nontransgenic mice, but insufficient to mask renal effects of low levels of *DβH-Ret^{MEN2B}* expression.

Based on this model, it is understandable why the earliest Ret-dependent event in renal development, outgrowth of the ureteric bud, is not compromised in most *DβH-Ret^{MEN2B}*-transgenic embryos, as it is in *gdnf*^{−/−}, *ret*^{−/−}, or *gfra-1*^{−/−} embryos. Ureteric bud formation and initial invasion of the metanephric mesenchyme occur when sympathoadrenal precursors are just colonizing the pre-aortic region, well before a sizable tumor has developed and high levels of transgene expression are present. Other embryonic events mediated by *gdnf*/*gfra-1*/Ret interaction (eg, enteric neurodevelopment) were not affected in our embryos, presumably because the *DβH* promoter is not active in the embryo until after Ret-dependent events in enteric neurodevelopment have concluded.⁵¹

A recent independent study led to a similar model to explain renal malformations that result from *Ret* overexpression in the ureteric bud and other embryonic tissues under control of the *Hoxb7* promoter.⁵² The ontogeny and pathology of renal malformations observed in the latter embryos are similar to *DβH-Ret^{MEN2B}* embryos, but, in contrast with our system, renal maldevelopment occurs *in vitro* when *Hoxb7/RET*-transgenic kidneys are explanted. The difference between the two transgenic models strengthens our impression that extrarenal *DβH-Ret^{MEN2B}* transgene expression is responsible for the kidney malformations in our mice.

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