C-cell hyperplasia, pheochromocytoma and sympathoadrenal malformation in a mouse model of multiple endocrine neoplasia type 2B

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Dominantly inherited multiple endocrine neoplasia type 2B (MEN2B) is characterized by tumors of the thyroid C-cells and adrenal chromaffin cells, together with ganglioneuromas of the gastrointestinal tract and other developmental abnormalities. Most cases are caused by substitution of threonine for Met918 in the RET receptor tyrosine kinase, which is believed to convert the RET gene to an oncogene by altering the enzyme's substrate specificity. We report the production of a mouse model of MEN2B by introduction of the corresponding mutation into the *ret* gene. Mutant mice displayed C-cell hyperplasia and chromaffin cell hyperplasia progressing to pheochromocytoma. Homozygotes did not develop gastrointestinal ganglioneuromas, but displayed ganglioneuromas of the adrenal medulla, enlargement of the associated sympathetic ganglia and a male reproductive defect. Surprisingly, homozygotes did not display any developmental defects attributable to a loss-of-function mutation. Thus, while our results support the conclusion that the Met918Thr substitution is responsible for MEN2B, they suggest that the substrate specificity of the RET kinase does not interfere with its normal role in the development of the kidneys and enteric nervous system.

Keywords: C-cells/MEN2B/pheochromocytoma/RET receptor tyrosine kinase/sympathetic ganglia

Introduction

The *RET* proto-oncogene encodes a receptor tyrosine kinase (Takahashi *et al.*, 1988, 1989), which, in conjunction with GFR α co-receptors, serves as the receptor for the GDNF family of neurotrophic factors (reviewed in Airaksinen *et al.*, 1999; Rosenthal, 1999). Heterozygous loss-of-function mutations in *RET* result in Hirschsprung's disease in humans (Edery *et al.*, 1994; Romeo *et al.*, 1994), while in mice a kinase-deficient *ret* mutant was recessive, causing intestinal aganglionosis, renal agenesis and a defect in the superior cervical ganglia (Schuchardt *et al.*, 1994, 1996; Durbec *et al.*, 1996). Major sites of *RET* expression during development (in addition to the excretory and central nervous systems) are the neural crest and many of its derivatives, including the peripheral

nervous system (PNS), enteric nervous system (ENS) and neuroendocrine cells, such as the C-cells of the thyroid and chromaffin cells of the adrenal gland (Pachnis *et al.*, 1993; Tsuzuki *et al.*, 1995). Several of these tissues are affected in a class of dominantly inherited syndromes caused by mutations in the *RET* gene, termed multiple endocrine neoplasia type 2 (MEN2). The three clinical subtypes of MEN2, called MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC), all include the C-cell tumor medullary thyroid carcinoma (MTC), while MEN2A and MEN2B also include pheochromocytoma, a chromaffin cell tumor. Some MEN2A patients also develop parathyroid hyperplasia, while MEN2B is characterized by a variety of developmental anomalies including neuromas of the lips, tongue and conjunctivas, intestinal ganglioneuromas, marfanoid skeletal changes and a male reproductive defect (Takahashi, 1995; Pasini *et al.*, 1996; Ponder and Smith, 1996).

Molecular genetic studies have shown that FMTC and MEN2A are caused by an overlapping set of germline mutations, the great majority of which result in substitutions involving one of six conserved cysteine residues in the RET extracellular domain (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993; Edery *et al.*, 1997). Each of these substitutions is believed to disrupt intramolecular Cys–Cys disulfide bonds, allowing the unpaired cysteine residue to form an aberrant intermolecular disulfide bond with a second RET molecule, thus resulting in ligand-independent dimerization and constitutive signaling (Asai *et al.*, 1995; Santoro *et al.*, 1995). In contrast, ~95% of MEN2B cases as well as most cases of spontaneous MTC result from a substitution of threonine for Met918 within the catalytic core of the RET kinase domain (Carlson *et al.*, 1994; Hofstra *et al.*, 1994; Smith *et al.*, 1997). Unlike the mutations associated with MEN2A, this substitution does not promote ligand-independent dimerization, but confers oncogenicity through a different mechanism. Both RET–MEN2A and RET–MEN2B are oncogenic in fibroblast transformation assays (Asai *et al.*, 1995; Borrello *et al.*, 1995; Santoro *et al.*, 1995) and act as dominant oncogenes in humans, as the wild-type RET allele is usually retained and expressed in tumor tissue (Landsvater *et al.*, 1996; Pegoraro *et al.*, 1998).

Clues to the transforming mechanism of the *RET*^{Met918Thr} mutation initially came from comparison with other tyrosine kinases, which revealed that this mutation converts the substrate-binding pocket of RET to resemble those of non-receptor src-related tyrosine kinases (Carlson *et al.*, 1994; Hofstra *et al.*, 1994; Smith *et al.*, 1997). This prediction was confirmed by a number of studies showing that this mutation results in increased activity toward the optimal peptide substrates of src and abl (Songyang *et al.*, 1995; Pandit *et al.*, 1996), and alters the pattern of RET autophosphorylation as well as its phosphorylation of

cellular substrates in NIH 3T3 cells (Santoro *et al.*, 1995). Among the changes reported for $RET^{Met918Thr}$ were decreased binding to the adaptor Grb2 (Liu *et al.*, 1996), novel tyrosine phosphorylation of several proteins that interact with the adaptors Shc and Nck (Bocciardi *et al.*, 1997) and increased levels of phosphatidylinositol 3-kinase activation (Murakami *et al.*, 1999). While these observations suggested a basis for the gain-of-function nature of the Met918Thr mutation, they also raised the possibility that it might create concomitant loss-of-function effects by failing to phosphorylate some of its normal substrates. Because humans with this mutant allele always carry a wild-type *RET* allele, it was not clear whether this mutation interfered with one or more normal developmental functions of *RET*.

To produce an animal model with which to study the dominant activity of the MEN2B mutation in tumorigenesis and tumor progression, as well as to investigate the potential loss-of-function effects, we used gene targeting techniques in conjunction with Cre/loxP site-specific recombination to introduce the corresponding codon alteration (Met919Thr) into the murine *ret* gene. Heterozygous mutant mice displayed several features of the human disease, including C-cell hyperplasia and chromaffin cell hyperplasia/pheochromocytoma, while homozygotes displayed more severe thyroid and adrenal disease as well as male infertility. While the mutant mice did not develop ganglioneuromas of the intestinal tract or mucosae, homozygotes displayed ganglioneuromas of the adrenal medulla and enlargement of the associated sympathetic ganglia. Surprisingly, ret^{MEN2B} homozygotes did not display any developmental abnormalities that could be attributed to a loss-of-function mutation.

Results

Introduction of a Met919Thr mutation into the mouse *ret* gene

Codon 919 of murine *ret*, the equivalent of human codon 918, was mutated *in vitro* to encode threonine rather than methionine (Figure 1A and B), and a targeting vector was constructed to insert the mutant exon into the mouse genome, together with a *neo* gene in the adjacent intron to allow positive selection (Figure 1C). To permit subsequent removal of the *neo* gene with Cre recombinase, it was flanked by loxP sites (Dale and Ow, 1991; Sauer, 1993). The linearized construct was electroporated into W9.5 embryonic stem (ES) cells, and correctly targeted ES cell clones were identified (Figure 1C and D). When injected into C57BL/6J blastocysts, one clone produced chimeric animals that transmitted the mutation through the germline (Figure 1E).

Mice heterozygous for the targeted allele ($ret^{MEN2B-neo}$) were viable and normal, whereas homozygotes died within the first 24 h of birth, displaying a phenotype (i.e. renal agenesis, failure of intestinal peristalsis presumably due to aganglionosis) indistinguishable from mice homozygous for *ret-k⁻*, a kinase-deficient allele (Schuchardt *et al.*, 1994). Thus, $ret^{MEN2B-neo}$ acts as a severe loss-of-function allele. Because we suspected that this was due to the presence of *neo* in an intron (Moens *et al.*, 1992), the *neo* was removed by mating $ret^{MEN2B-neo}/+$ mice to animals homozygous for a β -actin promoter/*Cre* transgene, which

is active throughout early development (Lewandoski and Martin, 1997). In 100% of the progeny inheriting the targeted *ret* allele, the *neo* gene had been excised (Figure 1E, lane 3) leaving a single loxP site in the *ret* intron. Mice heterozygous for this modified allele (ret^{MEN2B}) were intercrossed to produce ret^{MEN2B}/ret^{MEN2B} homozygotes (Figure 1E, lanes 4 and 6) which, unlike the $ret^{MEN2B-neo}/ret^{MEN2B-neo}$ homozygotes, were viable. These mice had normal kidneys and ENS, indicating that ret^{MEN2B} does not behave as a loss-of-function mutation for the development of either the kidneys or the ENS. Although morphometric studies of the skeleton have not been performed, neither heterozygotes or homozygotes displayed any gross malformations resembling marfanoid habitus.

To test whether the remaining 70 bp insertion (a single loxP site and polylinker sequences) in the ret^{MEN2B} intron had any adverse effect on *ret* gene expression, we used an RT-PCR assay to examine the level of mRNA from the ret^{MEN2B} allele relative to the wild-type *ret* allele in adult brain of $ret^{MEN2B}/+$ mice. This analysis demonstrated that the mutant allele was expressed as efficiently as the wild-type allele, and thus that the remaining loxP site does not interfere with gene transcription or RNA processing (Figure 1F).

Germline MEN2B mutation causes thyroid C-cell hyperplasia

In humans, the earliest effect of the MEN2B mutation on the thyroid is C-cell hyperplasia (CCH), the presumed precursor to carcinoma (Wolfe *et al.*, 1973). Diffuse CCH (DCCH), an increase in the number and size of calcitonin-positive cells, progresses to nodular CCH (NCCH), which is characterized by solid aggregates of C-cells that gradually replace the pre-existing follicles (Matias-Guiu *et al.*, 1995). Thirty-one percent of young (4–7 months) ret^{MEN2B} heterozygotes displayed DCCH, while 41% of older heterozygotes (8–12 months) had DCCH, and 14% had the more advanced NCCH (Table I and Figure 2). ret^{MEN2B} homozygotes showed an earlier onset of both DCCH and NCCH, and a more advanced disease state at any given age: at 6–10 months, 26% displayed DCCH and 60% NCCH (Table I). The increase in penetrance and reduction in latency displayed by homozygous mice are likely to result from a dosage effect of the dominant mutation. Medullary thyroid carcinoma was not observed in any of the animals examined up to 10–12 months of age.

Pheochromocytomas induced by the germline MEN2B mutation

A second characteristic feature of MEN2B in humans is the presence of pheochromocytoma, a usually benign tumor of adrenal chromaffin cell origin. Therefore, adrenals from ret^{MEN2B} heterozygotes and homozygotes were examined histologically at various ages. A small fraction (16–17%) of ret^{MEN2B} heterozygotes up to 12 months of age displayed nodular chromaffin cell hyperplasia, which may represent an intermediate stage in the development of pheochromocytoma (Figure 3b), but only rarely had it progressed to pheochromocytoma (Figure 3c, Table II). Homozygotes exhibited both qualitative and quantitative differences in adrenal pathology compared with their heterozygous littermates. Nodular

chromaffin cell hyperplasia was observed as early as 4 months, compared with 7 months in heterozygotes, while pheochromocytomas were apparent as early as 5 months and were found in every animal examined by 6 months (Table II, Figures 3 and 4). Increased numbers of mitotic figures were observed in both the hyperplastic nodules and the pheochromocytomas (not shown). Both types of lesion were always bilateral, as in most cases of human MEN2B syndrome. Also consistent with the human disease, the murine pheochromocytomas appeared to be benign, since gross examination of the lung, liver and gastrointestinal tract revealed no metastases.

Immunocytochemical analyses were employed to infer the functional characteristics of the adrenal medullary lesions. As expected, the cells comprising both nodular chromaffin cell hyperplasia and pheochromocytomas expressed tyrosine hydroxylase (TH), consistent with a capacity for catecholamine production (Figure 4c and d). This characteristic confirmed their development from the sympathetic lineage and distinguished them from adrenal cortical cells. They also stained strongly for chromogranin

A (CGA) (Figure 4e and f), which reflects the number of intracytoplasmic secretory granules and thus the degree of differentiation, as mature chromaffin cells contain numerous granules. Although the mouse adrenal medullary lesions resembled human pheochromocytomas seen in MEN2 syndromes in that they were TH positive and CGA positive, they differed from their human counterparts, and also from the majority of chromaffin cells in the normal mouse adrenal, by the absence of phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme required for conversion of norepinephrine to epinephrine (Figure 4g and h).

Dosage-dependent expression of *ret*^{MEN2B} is associated with ganglioneuromas of the adrenal and sympathetic ganglia

A third feature of MEN2B in humans, and one that distinguishes it from MEN2A, is the development of multiple oral mucosal neuromas and ganglioneuromas of the gastrointestinal tract. Surprisingly, neither of these features was detected in *ret*^{MEN2B} heterozygous or

Table I. The frequency of C-cell hyperplasia as a function of genotype and age

Genotype	Age (months)	<i>n</i>	Normal (%)	Diffuse CCH (%)	Nodular CCH (%)
Wild-type	6–12	25	96	4	0
<i>MEN2B</i> /+	4–7	16	69	31	0
	8–12	49	45	41	14
	2–5	16	56	19	25
<i>MEN2B</i> / <i>MEN2B</i>	6–10	27	14	26	60

Table II. The frequency of adrenal medullary pathology as a function of genotype and age

Genotype	Age (months)	<i>n</i>	Normal (%)	Nodular hyperplasia (%)	Pheochromocytoma (%)	Ganglioneuroma-like areas (%)
Wild-type	6–12	25	100	0	0	0
<i>MEN2B</i> /+	4–7	12	83	17	0	0
	8–12	57	82	16	2	0
	2–5	18	62	33	5	100
<i>MEN2B</i> / <i>MEN2B</i>	6–10	27	0	0	100	100
	2–6	18	100	0	0	0

Fig. 1. Introducing a MEN2B mutation into the *ret* locus. (A) Structure of the normal protein encoded by *ret*. The location of Met919 (equivalent to human Met918) is indicated. (B) Site-directed mutagenesis to introduce the Met919Thr mutation. In addition, two silent base changes were introduced to destroy a *MunI* restriction enzyme site, so that the mutant allele could be detected easily in ES cells and mice. (C) Schematic representation of the targeting strategy. (i) A segment of the normal *ret* gene including exons 15–17 (black boxes). (ii) The targeting vector included two segments of genomic *ret* DNA, one of which includes exon 16 (with the Thr919 mutation), separated by the *loxP-neo-loxP* gene. *LoxP* sites are indicated by triangles. (iii) Homologous recombination between the *ret* genomic locus and the targeting construct introduced the mutant exon 16 and the *loxP-neo-loxP* gene in the adjacent intron, to produce the *RET*^{MEN2B-neo} allele. (iv) The *ret*^{MEN2B} allele produced by Cre-mediated excision of *loxP-neo-loxP*. The restriction enzyme sites shown are M, *MunI*; X, *XbaI*; B, *BamHI*; K, *KpnI*; S, *ScaI*; H, *HindIII*; and P, *PstI*. (D) Southern blot analysis of targeted ES cell clone #10 and the parental ES cell line W9.5. Left: DNA digested with *MunI* and hybridized with probe A. A 15 kb mutant band and a 6.5 kb wild-type band were observed. Right: DNA digested with *XbaI* and hybridized with probe B to check the integrity of the 3' end. A 12 kb wild-type band and a 7.4 kb mutant band resulted. (E) Southern blot (left) and PCR (right) analysis of mouse tail DNAs after germline transmission and Cre-mediated excision of *loxP-neo-loxP*. Left: lanes 1 and 2, two progeny from a mouse chimeric for *ret*^{MEN2B-neo/+} ES cells. Lane 1, wild-type; lane 2, *ret*^{MEN2B-neo/+}. Lane 3, a *ret*^{MEN2B-neo/+} mouse derived from *ret*^{MEN2B-neo/+} crossed with a β -actin/Cre transgenic. Lane 4, a *ret*^{MEN2B-neo/+} homozygote. The DNAs were digested with *XbaI* and hybridized with probe A. The 12 kb band was derived from the wild-type allele, the 6 kb band from the *ret*^{MEN2B-neo} allele and the 4 kb band from the *ret*^{MEN2B} allele. Right: PCR analysis on three newborn pups from a *ret*^{MEN2B} intercross, using primers p5 and p6. The 280 bp amplification product was generated from the wild-type allele and the 350 bp product from the *ret*^{MEN2B} allele (the difference, 70 bp, is due to a 34 bp *loxP* site and 36 bp of polylinker sequence). Lane 5, heterozygote; lane 6, *ret*^{MEN2B} homozygote; lane 7, wild-type. M, molecular weight markers. (F) RT-PCR analysis of the expression of the *ret*^{MEN2B} allele. Left: schematic representation of the mutated locus, indicating exons 16 and 17. Primers p9 and p10 were used to amplify a 632 bp cDNA fragment spanning the two exons. The product of the wild-type *ret* allele is cleaved by *MunI* to yield two fragments of 300 and 332 bp, while the product of the mutant allele is not cleaved by *MunI*. Right: total brain cDNA from adult wild-type, *ret*^{MEN2B/+} and *ret*^{MEN2B-ret} was amplified and the product analyzed before and after *MunI* digestion. In *ret*^{MEN2B} heterozygotes, half of the PCR product is cleaved, indicating that the wild-type and mutant alleles produce similar amounts of mRNA.

Fig. 2. Nodular and diffuse C-cell hyperplasia in the $ret^{MEN2B/+}$ mutant thyroid. Calcitonin-stained sections through the thyroid of an 8-month-old wild-type mouse (**a** and **b**) showing the normal follicular pattern; intrafollicular C-cells are indicated by an arrow and f represents the follicular space. (**c**) Diffuse C-cell hyperplasia (DCCH) and (**d** and **e**) nodular C-cell hyperplasia (NCCH) in 8-month-old heterozygous mice. Magnification bars = 120 μ m.

homozygous mice, either by gross examination or in tissue sections stained with hematoxylin and eosin (H&E) or antibodies against peripherin, a neuron-specific marker (data not shown). On the other hand, the ret^{MEN2B} homozygotes consistently displayed neuromatous enlargement of the sympathetic ganglia along the medial aspect of the adrenal glands, a feature that can co-exist with mucosal neuromas in human MEN2B (Carney *et al.*, 1978).

In addition, a striking finding in adult homozygous mice was incomplete enclosure of the adrenal medulla by the adrenal cortex. Enlarged sympathetic ganglia were contiguous with ganglioneuroma-like areas extending into the adrenal medulla (Figure 5c). These areas were composed of mature ganglion cells in a bed of nerve fibers, as shown at higher magnification in Figure 5d. In wild-type mouse adrenals, the medulla was always completely enclosed by the cortex (e.g. Figure 3a).

As all adult homozygous mice exhibited ganglioneuromatous areas, we examined 12 ret^{MEN2B} homozygote newborns to determine whether this phenomenon was a pre- or postnatal event. All 12 newborn mice revealed bilateral

malformation of the adrenal glands, which appeared to be secondary to massive enlargement and invasion of the sympathetic ganglia into the adjacent adrenal (Figure 5b).

Because ganglioneuromas were observed in homozygous but not heterozygous mutant mice (Table II), it was possible that they resulted from a partial loss of function due to the altered RET substrate specificity caused by the Met919Thr mutation. We were able to test this hypothesis by generating compound heterozygous mice carrying the ret^{MEN2B} allele and the $ret-k^{-}$ loss-of-function allele, and comparing them with age-matched mice homozygous for ret^{MEN2B} . The presence of ganglioneuromas in the compound heterozygous animals would imply that the defect was due to a partial loss of function of the ret^{MEN2B} allele. However, of 18 compound heterozygous animals examined between the ages of 2 and 6 months, none exhibited ganglioneuromas (Table II), indicating that this defect was a dosage effect of the dominant ret^{MEN2B} allele. Nodular chromaffin cell hyperplasia and pheochromocytomas were also absent in all 18 animals, whereas 38% of ret^{MEN2B} homozygotes displayed adrenal pathology at

Fig. 3. Histological sections of *ret*^{MEN2B} mutant adrenals: (a) wild-type adrenal; c, cortex; m, medulla; (b) heterozygous mutant adrenal with nodular chromaffin cell hyperplasia (nh); (c) heterozygous mutant with pheochromocytoma (p) replacing the medulla. (d) A homozygous mutant with a ganglioneuroma-like area (g) extending into and abutting the pheochromocytoma (p). (e) A higher magnification view of a pheochromocytoma in a homozygous mutant, in which the normal medulla tends to be replaced by sheets and wide cords of cells. (f) Wild-type medulla, showing the nested or 'alveolar' pattern that predominates in the normal mouse adrenal medulla. Magnification bars = 120 μ m.

similar ages. The absence of any adrenal disease in the 18 compound heterozygotes examined at 2–6 months of age, in contrast to the low frequency (17%, or 2/12) in *ret*^{MEN2B}/+ heterozygotes at 4–7 months, is probably due to the fact that the former group was slightly younger, as well as to the small sample sizes. We conclude that the earlier onset of adrenal disease in *ret*^{MEN2B} homozygotes compared with heterozygotes also resulted from the increased dosage of a gain-of-function allele.

***ret*^{MEN2B} homozygous male mice display a reproductive defect**

While female homozygous mutants showed normal fertility, 83% ($n = 29$) of the male homozygotes failed to produce copulation plugs when housed with female mice for 10 days. In contrast, 50 heterozygous mutant males tested were all fertile. Although all the homozygous males exhibited mounting behavior, no pregnancies resulted when they were housed with females for >4 months. Gross and histological analysis revealed normal gonads and accessory organs and normal spermatogenesis, as indicated by the presence of mature sperm in the testis and epididymis (data not shown). Since this reproductive defect was only observed in the homozygotes, it could

result from either a partial loss-of-function effect or a dosage effect of a semi-dominant mutation. To distinguish between these possibilities, 18 compound heterozygote male mice carrying *ret*^{MEN2B} over the *ret*-*k* allele were examined. All 18 compound heterozygous males were fertile, suggesting that the defect is a dosage effect of a gain-of-function mutation.

Discussion

By introducing a site-directed mutation, causing a Met919Thr substitution in the murine RET receptor tyrosine kinase, we produced a mouse model for the dominantly inherited cancer syndrome MEN2B. Mice heterozygous for the mutant allele, *ret*^{MEN2B}, displayed two of the predicted disease features (hyperplasia of thyroid C-cells and of adrenal chromaffin cells), although they were less severely affected than humans with MEN2B. While humans with this syndrome are heterozygous for the *RET* mutation, with the mouse model we were able to examine the phenotype of mutant homozygotes. These animals modeled the human syndrome more closely, in that they displayed an earlier incidence and increased severity of C-cell and chromaffin cell hyperplasia (including invari-

Fig. 4. Nodular hyperplastic adrenal cells and pheochromocytoma are noradrenergic. (a, c, e and g) Sections of heterozygous *ret*^{MEN2B} adrenal medulla with a region of nodular chromaffin cell hyperplasia (nh) occupying part of the medulla (m); (b, d, f, and h) a pheochromocytoma. Distinction of nodular hyperplasia from pheochromocytoma in the veterinary pathology literature is arbitrary and is based on small size (<50% of medullary volume) and absence of significant compression or invasion of the cortex (Longeart, 1996). (a and b) H&E staining; (c and d) tyrosine hydroxylase; (e and f) chromogranin A; (g and h) PNMT. PNMT is not expressed in cells of the hyperplastic nodule or the pheochromocytoma. Small islands of PNMT-negative cells in (g) are normal norepinephrine cells. c, cortex.

able progression to pheochromocytoma) as well as a male reproductive defect. The murine homozygotes differed from humans with MEN2B in two respects: they did not develop medullary thyroid carcinoma (at least up to 12 months of age) or ganglioneuromas of the gastrointestinal tract and mucosa. However, they did display neuro-matous enlargement of sympathetic ganglia, a feature that can accompany mucosal neuromas in the human syndrome. Ganglioneuroma-like areas that extend into the adrenal gland from adjacent enlarged ganglia and cause

gross anatomic malformation are not a characteristic of human MEN2B, but this might reflect the size disparity between human and mouse adrenals. The sensitivity of the *ret*^{MEN2B} phenotype to gene dosage, both in our knock-in model and in a transgenic model (discussed below; Sweetser *et al.*, 1999), might also explain why this malformation is not generally observed in human MEN2B heterozygotes.

An important question we addressed by generating *ret*^{MEN2B} homozygotes concerned the mechanism of action

Fig. 5. Enlarged sympathetic ganglia and ganglioneuroma-like areas in *ret*^{MEN2B}/*ret*^{MEN2B} mice. (a and b) H&E-stained sections through the adrenal medulla of newborn wild-type (a) and homozygous mutant (b) newborn mice. (c) Tyrosine hydroxylase-stained section through the adrenal medulla of a 4-month-old homozygous mutant, showing the continuity between cells of the medulla (m), the enlarged sympathetic ganglia (sg) and the ganglioneuroma-like areas (g). (d) A representative ganglioneuroma-like area (g) is shown at a higher magnification (arrows point to nerve fibers while the arrowhead points to a ganglion cell). c, adrenal cortex; m, adrenal medulla; sg, enlarged sympathetic ganglia; g, ganglioneuroma. Magnification bars = 80 μ m.

of the Met919Thr substitution, which was predicted (Carlson *et al.*, 1994; Eng *et al.*, 1994; Hofstra *et al.*, 1994) and confirmed in several studies (Santoro *et al.*, 1995; Songyang *et al.*, 1995; Liu *et al.*, 1996; Pandit *et al.*, 1996) to alter the substrate specificity of the RET tyrosine kinase. Although the dominant nature of this mutation in humans, as well as the *in vitro* transforming activity of the mutant RET allele (Santoro *et al.*, 1995), indicated that it is a gain-of-function mutation, it might also possess loss-of-function characteristics if it prevented binding or phosphorylation of some of the normal RET substrates while increasing the activity toward other substrates. In that case, *ret*^{MEN2B} homozygotes might have displayed some of the lethal developmental defects seen in homozygotes for the kinase-deficient allele *ret*^{-k}, which include renal agenesis and intestinal aganglionosis. However, the kidneys and ENS of the *ret*^{MEN2B} homozygotes were apparently normal. Furthermore, when *ret*^{MEN2B}/+ mice were crossed to animals carrying the loss-of-function allele *ret*^{-k}, the *ret*^{MEN2B}/*ret*^{-k} compound heterozygotes showed none of the specific characteristics of *ret*^{MEN2B} homozygotes. Thus, the defects specific to *ret*^{MEN2B} homozygotes must all be due to dosage effects of a semi-dominant, gain-of-function allele, rather than to any loss-of-function. We conclude that while RET^{Met919Thr} might gain new substrates, it either retains all of the normal substrates, or those that it loses are not essential for development of the kidneys or ENS.

The hyperplasia of thyroid C-cells and adrenal chromaffin cells seen in both heterozygous and homozygous mutant mice is believed to represent an inter-

mediate state in the development of medullary thyroid carcinoma and pheochromocytoma, respectively (Wolfe *et al.*, 1973; Carney *et al.*, 1976; DeLellis *et al.*, 1976). It was therefore surprising that MTC was not observed, given the fact that in the human MEN2B syndrome, MTC has an earlier onset and greater penetrance than pheochromocytoma. The lack of tumor formation in the thyroids of *ret*^{MEN2B} mice (at least up to 12 months of age) may be due to the requirement for the accumulation of secondary genetic changes, and the relatively short lifespan of mice versus humans. It remains possible that older mice will develop MTC, or that breeding the mutation onto different genetic backgrounds might shorten the latency of tumor development, (i.e. alleles of modifier genes may act to inhibit the effect of the Met919Thr mutation or decrease the frequency of additional somatic mutations in the thyroid; McGregor *et al.*, 1999). A transgenic mouse model of the related syndrome MEN2A developed MTC at a high frequency (Michiels *et al.*, 1997). However, this model was produced by overexpression of a multi-copy, mutant *ret* transgene in the thyroid under a heterologous promoter, which is likely to account for the rapid development of malignancy.

The model we have described here is the first MEN2 mouse model to develop pheochromocytoma, and shows the highest frequency of pheochromocytoma yet reported for any mouse carrying a mutant gene implicated in human disease. One apparently anomalous characteristic both of the pheochromocytomas and of the hyperplastic nodules, which may be their precursors, is the absence of immunoreactive PNMT. Immunohistochemical staining for catechol-

amine-synthesizing enzymes permits the identification of cells with catecholamine-synthesizing ability and inference of what specific catecholamines they produced. TH, the first enzyme in catecholamine biosynthesis, is found in all catecholamine-producing cells, while PNMT, the final biosynthetic enzyme, is found only in cells that can convert norepinephrine to epinephrine. Almost all chromaffin cells in the human adrenal medulla are PNMT positive, while the mouse adrenal contains a minority population of clustered, PNMT-negative norepinephrine cells (Tischler *et al.*, 1996). Pheochromocytomas from humans (Lloyd *et al.*, 1986), at least some other genetically engineered mice (Tischler *et al.*, 1995) and spontaneously occurring mouse models (Tischler *et al.*, 1996) express PNMT. The PNMT-negative phenotype of hyperplastic chromaffin cell nodules and pheochromocytomas in the *ret*^{MEN2B} mice could suggest either loss of ability to maintain PNMT expression during neoplastic transformation or the existence of a selective growth or survival advantage to PNMT-negative cells, possibly imparted by the *ret* mutation.

An additional intriguing possibility is that the lesions might be closely related to the PNMT-negative 'extra-adrenal chromaffin cells', or 'small, intensely fluorescent cells', that persist along the pelvic sympathetic nerves and in the sympathetic ganglia of postnatal mice (Coupland, 1960). We observed several prominent nodules (up to ~50 cells) of these cells by chance in the portions of sympathetic chains that accompanied the adrenals from several homozygous mutant mice (data not shown). Although we did not conduct a systematic study to ask whether those nodules are abnormal for the mouse strain employed in this study, we encountered only one small aggregate of similar cells in samples from wild-type mice. During embryogenesis, trunk neural crest cells first populate the sympathetic ganglion anlage, and later undergo a second migration to invade the adrenal gland. A model hypothesizing origin of intra-adrenal lesions from ganglionic precursors that do not separate appropriately from the developing adrenal medulla might be supported by a recent report that *ret* is not expressed during mid to late development in the mouse adrenal, but is expressed in the sympathetic chains (Golden *et al.*, 1999). According to such a model, the development of macroscopic tumors within the adrenal, rather than in extra-adrenal locations, might be favored by the ability of corticosteroids to promote cell survival (Doupe *et al.*, 1985). A similar model has been posited recently by one of us to account for the PNMT-negative phenotype of intra-adrenal nodules in a rare human congenital disorder, Beckwith-Wiedemann syndrome (Tischler and Semple, 1996).

Ganglioneuromatous areas in the adrenal medulla and enlargement of the closely associated sympathetic ganglia have also been noted in transgenic mice in which either a *ret*^{MEN2B} cDNA (Sweetser *et al.*, 1999) or an activated *ras* cDNA (Sweetser *et al.*, 1997) was expressed under the promoter of dopamine β -hydroxylase (*DβH*), a gene active in the sympathoadrenal and ENS (but not C-cell) lineages. The mechanisms leading to these changes in all three cases could be equivalent because *ras* is a signal transducer for activated RET (Airaksinen *et al.*, 1999). However, our *ret*^{MEN2B} model differs from the *DβH/ret*^{MEN2B} and *DβH/ras* transgenic lines in that neither of

the *DβH* transgenic lines developed C-cell hyperplasia, chromaffin cell hyperplasia or pheochromocytoma. As suggested by Sweetser *et al.* (1999), the absence of chromaffin cell hyperplasia and pheochromocytoma could be due to differences in the expression pattern of the *DβH* and *ret* promoters, or to the fact that the *DβH/ret*^{MEN2B} transgene expressed only one of the two major RET isoforms (RET9), while the RET51 isoform of RET^{MEN2B} has a stronger activity in transformation or differentiation assays (Asai *et al.*, 1995; Rossel *et al.*, 1997). In contrast, the *ret*^{MEN2B} targeted allele retained the normal *ret* regulatory sequences as well as the ability to encode all the normal RET isoforms, two features that probably account for the many differences between these two mouse models.

Expression of *ret* is not required for the normal development of the sympathoadrenal lineage, as indicated by the presence of normal posterior sympathetic ganglia, adrenals and foregut ENS in *ret-k⁻* homozygotes (Schuchardt *et al.*, 1994; Durbec *et al.*, 1996). However, a gain-of-function *ret* mutation might be expected to affect the proliferation, migration, survival or maturation of multipotential sympathoadrenal progenitors. Thus, enlarged sympathetic ganglia may have resulted from abnormal proliferation or delayed differentiation of progenitors after migration to the ganglia, or an abnormally large number of cells might have migrated to the ganglia. Similarly, the abnormal presence of neurons within the adrenal medulla may be due to the ability of *ret*^{MEN2B} to alter migration of neuronal precursors to the adrenal, or to stimulate their survival or neuronal differentiation in the adrenal (Sweetser *et al.*, 1999). Alternatively, or in addition, discontinuity of the adrenal cortex occurring secondarily to sympathetic ganglion enlargement could allow sympathoadrenal precursors to escape the influence of corticosteroids that promote chromaffin cell differentiation (Tischler *et al.*, 1995). It is unlikely that ganglioneuromatous regions in the medulla are derived from neoplastic chromaffin cells for two reasons: first, these regions, as well as the enlarged sympathetic ganglia, are present at birth, before there is any sign of chromaffin cell neoplasia; and secondly, the *DβH/ret*^{MEN2B} transgenic mice display similar features in the absence of any chromaffin cell neoplasia (Sweetser *et al.*, 1999).

Another difference between our mouse model and human MEN2B was the absence of gastrointestinal tract neuromatosis. A similar observation was made in the *DβH/RET*^{MEN2B} transgenic mice, which, despite expression of the transgene in neonatal gut, showed no gastrointestinal abnormalities (Sweetser *et al.*, 1999). While the cells of the foregut ENS and sympathoadrenal lineage are both derived from the trunk neural crest and co-express *ret*, apparently in humans the ENS is more susceptible to the effects of the MEN2B mutation.

Eighty three percent of the *ret*^{MEN2B} homozygous male mice were incapable of impregnating females, although they exhibited normal mounting behavior, their reproductive organs were anatomically normal and they produced mature sperm. As erectile dysfunction with a neurological basis is common in humans with MEN2B (Ponder and Smith, 1996), a likely explanation is that the mutant mice also have an erectile defect. This defect is not caused by a hormonal defect secondary to pheochromocytoma, as many of the mice which failed to reproduce had not yet

developed these tumors. The pattern of *c-ret* expression is consistent with the hypothesis that a gain-of-function mutation might cause such a neurological defect, since *c-ret* transcripts have been localized to the PNS and CNS (Pachnis *et al.*, 1993; Golden *et al.*, 1999) including the major pelvic ganglia (V.Pachnis, personal communication).

In conclusion, targeted mutagenesis of the RET receptor tyrosine kinase resulted in a dose-dependent phenotype of C-cell hyperplasia, pheochromocytoma, adrenal gangliogliomas and male infertility. In addition to facilitating the elucidation of changes in signal transduction events which result in the MEN2B phenotype, this mouse model provides the opportunity to explore further the pathophysiological manifestations of this multiorgan disease.

Materials and methods

Gene targeting

A 16 kb mouse *ret* genomic clone including exon 16 (the exon encoding Met919; G.Romeo, personal communication) was obtained by screening a mouse 129/Sv bacteriophage library (a gift from Steven Tsang) using the *ret* cDNA containing exon 16 as a probe. A targeting vector was constructed using a 5.1 kb *XbaI*–*HindIII* 5' fragment and the contiguous 3.7 kb *HindIII*–*KpnI* 3' fragment, which contained exon 16. Between them was inserted a loxP-flanked *Pgk/neo* gene. Site-specific mutagenesis of exon 16 was performed using a mutagenic primer pCGGATTCCCGT-CAAGTGGACGGCTATAGAGTCCCTTTTCG (bold letters indicate the base changes) and the Stratagene Chameleon Site Directed Mutagenesis Kit. The vector was linearized and electroporated into W9.5 ES cells (a gift of Dr Colin Stewart, derived from strain 129/terSV). Out of 256 *neo*-resistant clones, three had the targeted insertion, and two of these had the MEN2B mutation. *ret*^{MEN2B-neo/+} ES cells were injected into C57BL/6J blastocysts to obtain chimeric mice. One of the two clones gave rise to highly chimeric mice (>95% ES cell derived), which transmitted the mutation through the germline. The genotypes of ES cells and mouse tails were analyzed by Southern blotting after enzymatic digestion with *MunI* and *XbaI*, and hybridized with a 444 bp *BamHI*–*ScaI* internal probe (probe A) and a 300 bp *KpnI*–*ScaI* external probe (probe B), respectively. In addition, genotyping was done using PCR primers p3 (5'-GCTGACCTCTTAGCCTGGG-3') and p4 (5'-GGTGTGACGAGCTGTATTGAAGC-3').

Excision of *neo*

Female F1 *ret*^{MEN2B-neo/+} mice were mated with male FVB mice homozygous for the β -actin/*Cre* transgene (Lewandoski and Martin, 1997). The resulting progeny were screened using PCR for the presence of the MEN2B mutation, using primers p5 (5'-CCTCTCACACACCAC-AACC-3') and p6 (5'-CGAGTCAGACTCTACGACCC-3'), and for the absence of *neo* by Southern analysis (Figure 1E). In subsequent generations, in which the β -actin/*Cre* gene segregated from the *ret*^{MEN2B} locus, the mice were screened for the absence of *Cre* by PCR using gene-specific primers p7 (5'-TGATGAGGTTTCGCAAGAAGAACC-3') and p8 (5'-CCATGAGTGAACGAACCTGG-3'). The *ret*^{MEN2B} analyzed herein were therefore on a mixed background of strains 129/terSV, C57BL/6J and FVB/N.

RT-PCR

RNA isolated from the brains of adult mice were used to generate first strand cDNA, and gene-specific primers p9 (5'-CCTCCFTFACAGCCG-CAAGC-3') and p10 (5'-CCCATCGTCATACAGCAGTG-3') were used to generate the amplification products, which were then digested with *MunI*.

Histological and immunohistochemical analyses

Adult animals of various ages were killed by cervical dislocation. The organs of interest (adrenal, thyroid and gastrointestinal tract) were fixed in 10% buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin. Serial sections 6 μ m thick were prepared on charged slides (Super-Frost Plus, Fisher Scientific) and de-paraffinized prior to immunostaining. C-cells of the thyroid were stained with polyclonal rabbit antibodies to calcitonin (Biogenetics, 1:200 dilution),

and enteric neurons with polyclonal rabbit anti-peripherin (Novoscastra Labs, 1:200 dilution). The adrenals were H&E stained and adjacent sections were immunostained with antibodies to TH, PNMT or rat CGA after microwave antigen retrieval (Shi *et al.*, 1992) as previously described (Tischler, 1999), with normal rabbit serum substituted for primary antibody as a negative control. Newborn mice were collected at birth and killed by asphyxiation in CO₂ followed by exposure to wet ice for 20 min. They were then fixed in 10% buffered formalin. The dorsal midline aorta with the adjacent sympathetic chain and the adrenals was dissected out, embedded in paraffin and serially sectioned at 6 μ m. The sections were prepared and stained in a similar fashion to the adrenals.

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