

Molecular Pathogenesis of Genetic and Inherited Diseases

A Human Yeast Artificial Chromosome Containing the Multiple Endocrine Neoplasia Type 2B *Ret* Mutation Does Not Induce Medullary Thyroid Carcinoma but Does Support the Growth of Kidneys and Partially Rescues Enteric Nervous System Development in *Ret*-Deficient Mice

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We generated a line of transgenic mice using a yeast artificial chromosome containing the *Ret* mutation responsible for the multiple endocrine neoplasia type 2B syndrome (MEN 2B). The resulting animals did not develop any of the expected neoplasms associated with MEN 2B. Transgenic animals were then bred with animals lacking murine *Ret* (*Ret_M*) to further evaluate the function of human mutated *Ret* (*Ret_H^{2B}*) in the murine context. Whereas mice lacking *Ret_M* exhibit intestinal aganglionosis and the absence of kidneys with other genitourinary anomalies, expression of the *Ret_H^{2B}* transgene in *Ret_M*-deficient mice allowed significant renal development with a partial rescue of the enteric nervous system. These *Ret_H^{2B}*-positive/*Ret_M*-deficient mice exhibit normal *Ret* expression and survive longer than *Ret_M*-deficient mice, but still die at 3 to 5 days of age with evidence of enterocolitis. We conclude that the normal expression of a human *Ret* proto-oncogene with the MEN 2B mutation does not cause any features of MEN 2B in

mice. Although the gene is normally expressed in the appropriate target tissues, there is incomplete phenotypic rescue in mice lacking murine *Ret*. These results suggest important interspecies differences between humans and mice in the function of the *Ret* oncogene. (Am J Pathol 2005, 166:265–274)

The *Ret* proto-oncogene encodes a receptor tyrosine kinase that regulates the growth and development within the neurological and excretory systems.^{1–3} The gene was discovered using the NIH3T3 transfection assay and the name *Ret* derives from this discovery (rearranged during transfection).⁴ More recently, it was discovered that certain germ line mutations in the *Ret* proto-oncogene are associated with the multiple endocrine neoplasia (MEN) syndromes types 2A and 2B and familial medullary thyroid carcinoma (FMTC).^{5–9} These syndromes are inherited in an autosomal dominant manner. Patients with MEN 2A develop medullary thyroid carcinomas (MTCs), pheochromocytomas, and hyperparathyroidism; similarly, those with MEN 2B also develop MTCs and pheochromocytomas, but additionally develop mucosal neuromas of the lips, oral mucosa, and alimentary tract. Finally, FMTC is characterized only by the development of medullary thyroid carcinoma.^{10–12}

To study the normal function of murine *Ret* (designated *Ret_M*), transgenic mice were created that lacked either *Ret*, the co-receptor *GFR- α 1*, or glial cell line-derived neurotrophic factor (*GDNF*), the cognate ligand for the receptor complex.^{13–18} The *Ret*, *GFR- α 1*, and *GDNF*

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knockout mice are all missing the enteric ganglion cells distal to the stomach and also exhibit varying degrees of renal agenesis or dysgenesis.¹⁸ The absence of intestinal ganglion cells is reminiscent of Hirschsprung's disease in humans, in which there is the congenital absence of enteric ganglia in the distal gut. Indeed, inactivating *Ret* mutations are also the most common defined cause of Hirschsprung's disease in humans. The importance of *Ret* in the development of the nervous system was further demonstrated when the other *Ret* ligands persephin, artemin, and neurturin were characterized and demonstrated to interact with *Ret* to support the growth of neurons in the peripheral, central, and enteric nervous systems (ENSs).^{19–23}

Recently, to study the dominant transforming activity of the MEN 2B mutation of *Ret*, investigators created a transgenic mouse line containing the equivalent codon 918 Met to Thr mutation in the mouse *Ret* gene.²⁴ Interestingly, these mice did not precisely model the human phenotype. Whereas they developed thyroid C-cell hyperplasia and pheochromocytomas, the mice did not develop MTC. Moreover, the animals did not possess the neuromas of the lips, oral mucosa, or gastrointestinal tract found in humans with MEN 2B. These results suggest that there are species differences between humans and mice that are responsible for the different phenotypes associated with the MEN 2B mutation of *Ret*. However, *Ret* with the MEN 2B mutation does support normal development of the kidney and ENS, even in mice homozygous for MEN 2B.²⁴ In contrast to the above mouse model, two other studies^{25,26} found that overexpression of a human MEN 2B transgene causes developmental renal malformations.

In an attempt to create a mouse model of MEN 2B that recapitulates all of the sequelae of the human disease, we created a line of transgenic mice possessing the human *Ret* gene with the codon 918 Met to Thr mutation responsible for MEN 2B. To assure faithful expression of the gene through embryonic development and in the appropriate tissues, we used a yeast-associated chromosome (YAC) containing the human *Ret* gene with the MEN 2B mutation (designated *Ret_H^{2B}*). This allows the inclusion of a considerable amount of genetic material upstream and downstream from the coding elements of the gene, to ensure the inclusion of genetic elements responsible for the regulation of *Ret* expression. These transgenic animals were followed to see whether they developed the features of the human MEN 2B syndromes. To further evaluate the functionality of the human transgene, we bred these *Ret_H^{2B}* transgenic mice to animals lacking one murine *Ret* allele (designated *Ret_M*), and then performed the genetic backcrosses to create mice that only express *Ret_H^{2B}*. Our aims in this portion of the study were to determine whether the *Ret_H^{2B}* gene corrects the phenotype associated with the *Ret_M* knockout and to investigate whether the human *Ret* gene with the MEN 2B mutation, expressed outside the context of the mouse *Ret* gene, impacts the development of tumors associated with MEN 2B.

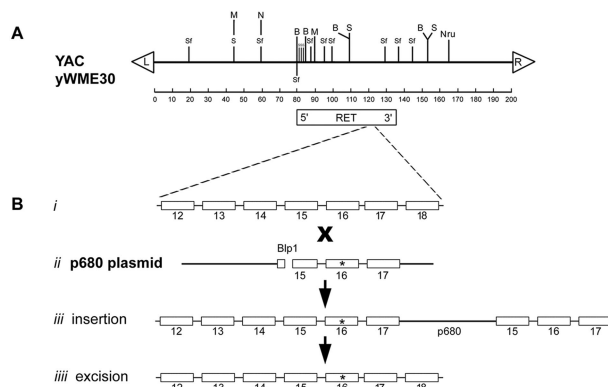


Figure 1. A: Restriction map of YAC yWME30 containing the *Ret* proto-oncogene. The **arrowheads** denote the left and right arms. *Ret* is ~50 kb in length and the 5' end is located ~80 kb from the left arm of the YAC. The restriction endonucleases are denoted: S, *Sac*I; B, *Bam*HI; SF, *Sfi*I; M, *Mlu*I; N, *Nof*I. **B:** Site-directed mutagenesis to introduce the 918Met→Thr mutation into *Ret* within the YAC. ii, The PCR was used to amplify the length of DNA containing *Ret* exons 15, 16, and 17, with the intervening introns, from DNA obtained from a patient with MEN 2B. The amplified DNA, containing the 918Met→Thr mutation in exon 16 (**asterisk**), was cloned into the p680 plasmid containing the *LEU2* gene (for positive selection) and *CYH2* gene (for negative selection). iii, The restriction endonuclease *B*l*p*I was used to linearize the plasmid, which then allowed targeted transformation of yeast cells containing the YAC by selecting in Leu+ medium for colonies containing the *LEU2* gene. iv, The resulting strains were grown in medium containing cycloheximide to select for the occasional colony in which the p680 plasmid was deleted by homologous recombination, leaving the MEN 2B point mutation in yWME30.

Materials and Methods

Introduction of the MEN 2B Mutation into the Human *Ret* Proto-Oncogene

The *Ret* proto-oncogene measures ~50 kb in length, and is contained within YAC yWME30, obtained from the Washington University Human Genome Sequencing Center. This YAC, measuring ~200 kb in length, was transferred from the native AB1380 yeast strain into the YPH985 strain by *kar-1* mating.²⁷ The location and orientation of *Ret* within the YAC was ascertained by Southern analysis. The restriction map is depicted in Figure 1 and is consistent with other published results.²⁸ To create the mutated gene that causes MEN 2B, the codon 918 T to C mutation responsible for the syndrome was introduced into the YAC by the two-step mutation method essentially as described.²⁷ Briefly, the polymerase chain reaction (PCR) was used to isolate a ~2-kb length of *Ret* from DNA obtained from a patient with MEN 2B. This genetic segment extended from the 5' end of exon 15 to the 3' end of exon 17, containing the intervening introns and the 16th exon, in which the point mutation resides. The PCR oligonucleotide names and sequences are 15F (5'TCATAAGCTTCATGGCCTGACGACTCGTCGATT) and 17R (5'TCATCTCGAGCAAGTGAGGCTGGC-CAAAGC). The exonic coding segments of the resulting PCR product were sequenced to ensure the absence of PCR fidelity errors, and the product was cloned into the yeast vector p680.^{27,29} This construct, containing the bp change of interest, was linearized with *B*l*p*I, and the mutated region of *Ret* was incorporated into the YAC using targeted homologous recombination as described.²⁷

Again, exons 15 through 17 were sequenced in the YAC clone to ensure that the mutation of interest had been introduced into the gene, and that no other abnormalities were present. Finally, Southern analysis was performed to document that no gross rearrangements in *Ret* or in the YAC had occurred. The final YAC genetic construct with the mutation associated with MEN 2B was designated *Ret_H^{2B}*.

Generation and Characterization of Transgenic Mice with the Mutated *Ret* Proto-Oncogene

To purify the YAC for pronuclear injection, the mutation-containing YAC was first separated from the other yeast chromosomes using pulsed-field gel electrophoresis.³⁰ The edges of the gel were removed and ethidium-stained to determine where the YAC band had migrated. This band was cut from the 1% agarose gel, cut in half, and the two gel slices were placed side-by-side within a 4% agarose gel in a direction 90° to the pulsed-field gel electrophoretic run. The YAC DNA was concentrated by running the DNA through the 1% agarose into the 4% gel. One of the bands was stained with ethidium bromide to localize the DNA. The unstained gel slice containing the YAC DNA was dialyzed in digestion buffer, and subjected to GELase (Epicenter, Inc., Madison, WI) digestion. Purified YAC DNA (concentration 4 ng/μl) was injected into male pronuclei of fertilized eggs from F₁ hybrid mice (C57BL/6 × B6C3H), and founder transgenic mice were generated using standard techniques.^{31,32} A total of 162 animals underwent tail-DNA screening for the human gene using PCR analysis to amplify the mutated 16th exon (oligonucleotides 16F 5'AGAGAGTAGAGTA-CTTCAATGTC, 16R 5'CTACATGTATAAGGGTGT),³³ followed by *FokI* digestion to document presence of the expected mutation.⁵ Three transgenic animals were generated, and in each of these lines, there was successful germline transmission of human *Ret* (*Ret_H^{2B}*). To ensure that the transgene contained the entire *Ret* coding sequence, PCR was used to amplify the first and 20th exons. We found that a genetic rearrangement had occurred in one of the transgenic lines, resulting in the deletion of exon 1. The intact human *Ret* proto-oncogene was present in two lines of transgenic mice (line numbers 7 and 24) but there was a deletion in the inserted YAC from transgenic line 7 that resulted in the inclusion of only a very short length of intact DNA upstream to the first exon. The animals from line 24 were bred, and evaluated histologically for the development of the expected thyroid and adrenal neoplasms associated with *Ret* mutations. These animals were also bred with mice heterozygous for murine *Ret* (*Ret_M^{+/-}*) to generate mice expressing *Ret_H^{2B}* and heterozygous for murine *Ret* (*Ret_H^{2B}/Ret_M^{+/-}*). These animals were then backcrossed with murine *Ret* heterozygotes (*Ret_M^{+/-}*) to generate mice with the genotype *Ret_H^{2B}/Ret_M^{-/-}* possessing only the mutated human YAC transgene. All of these experiments were performed with the prior approval of the Institutional Animal Care and Use Committees at Duke University and Washington University.

Histopathological Analysis

Anesthetized animals were sacrificed by transcardiac perfusion with phosphate-buffered saline, followed by 4% paraformaldehyde. In adult animals, tissues were dissected and embedded in paraffin before sectioning and staining with hematoxylin and eosin using standard procedures. Newborn pups were similarly perfused, and whole pups were paraffin-embedded and sectioned either longitudinally or transversely through the entire specimen before staining for histological analysis.

Evaluation of *Ret* Expression

RNA was purified from transgenic mouse tissues using Triazol (Life Technologies, Gaithersburg, MD) reagent, and reverse-transcribed into cDNA using a kit and protocol from Life Technologies. We assayed for expression of both the long (*Ret*51) and short (*Ret*9) transcripts of the human *Ret* proto-oncogene using species-specific oligonucleotides with the following sequences: upstream-human CCAGTTAAATGGACGGCAATTG (exon 16); downstream human *RET*9 AGACTTTGGTTTGTTCAGAC (exon 19); downstream human *RET*51 CGGTAGACTTTC-CATTCTCAG (exon 20); upstream mouse CCCGT-CAAGTGGATGGCAATTG; downstream mouse *RET*9 AAGCTTTGGTGTCTCGGTGGGAT; downstream mouse *RET*51 CGGTGGACTTGATGCTCTTGG. The locations of the mouse primers correspond to those of the human primers. *Ret* was amplified from mouse cDNA for 40 cycles using the following parameters: 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 45 seconds. The PCR products were then resolved on a 1.2% agarose gel.

Assays to quantitatively compare the transcription of the human and murine *Ret* transcripts were performed using the Clontech PCR MIMICS kit (B.D. Biosciences Clontech, Palo Alto, CA), essentially according to the manufacturer's instructions. Species-specific oligonucleotides were designed to anneal within the coding region of the *Ret* gene (exon 15, forward; exon 17, reverse), as well as the *v-erb* internal standard mimic oligonucleotide (murine: forward 5' TGGCACACCTCTGCTCTATGTGTT-ATACAGGGAGATGAAA; reverse 5' TGTTCCAGGAACTGTGGTCTCTGTCAATGCAGTTTGTAG; human: forward 5' GATTCGGCTTGTCCCGAGATGTTATACAGGGAGATGAAA; reverse 5' TCAGGAGGAATCCCAGGATAGTCTGT-CAATGCAGTTTGTAG).

Acetylcholinesterase Histochemistry

Acetylcholinesterase staining was performed on the alimentary tract of transgenic animals as described.¹⁴ Briefly, the alimentary tract was removed and fixed in 4% paraformaldehyde for 1 to 2 hours at 4°C. After overnight storage in saturated sodium sulfate, the gut was incubated in buffer [0.2 mmol/L ethopromazine HCl (Sigma Chemical Co., St. Louis, MO), 4 mmol/L acetylthiocholine iodide (Sigma), 10 mmol/L glycine, 2 mmol/L cupric sulfate, 65 mmol/L sodium acetate, pH 5.5] for 2 to 4 hours. Finally, staining for acetylcholinesterase was developed

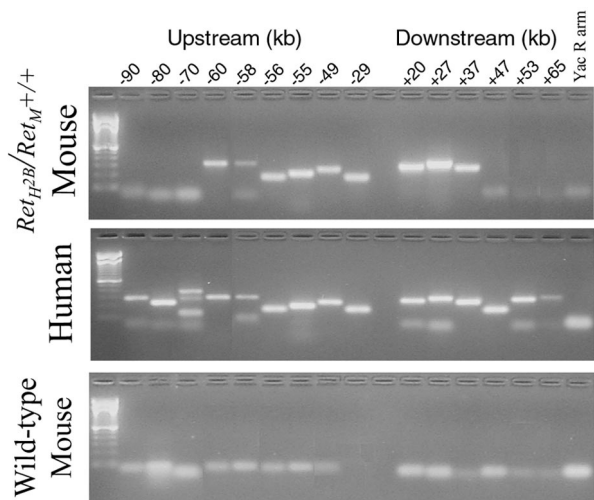


Figure 2. Genetic mapping to determine the regions of YAC yWME30 integrated into the transgenic mouse genome. Oligonucleotides were designed for use in PCR to selectively amplify human DNA in the region of the *Ret* proto-oncogene. Markers were located at variable distances 5' and 3' to *Ret* at the positions noted, and resolved by agarose gel electrophoresis. The **top** photograph demonstrates the results obtained from transgenic mouse DNA, the **middle** photograph demonstrates the results from normal human DNA, and the **bottom** photograph demonstrates the results from wild-type mouse DNA, to demonstrate the human specificity of the PCR reactions. The YAC was integrated from a region of 60 kbp upstream from *Ret* to 37 kbp downstream.

by incubating for 1.5 minutes in sodium sulfide (1.25%, pH 6.0), and the specimens were photographed under a dissecting microscope.

Genetic Mapping of the Integrated YAC

To verify the amount of human genetic material upstream and downstream from *Ret*, and to investigate whether genetic material was lost from the YAC during the manipulations required to create the transgenic mouse line, genetic mapping was used to investigate which portions of the YAC were incorporated into the transgenic mice. Oligonucleotides for PCR analysis were synthesized corresponding to human sequences adjacent to *Ret* at intervals of 3 to 5 kb along chromosome 10 upstream and downstream from the upstream and downstream ends of the gene. As demonstrated in Figure 2, we found that a total of ~150 kb of the YAC were successfully transferred into the transgenic mouse. There were ~60 kb 5' to *Ret* and 37 kb 3' to the gene. The oligonucleotide sequences are listed in Table 1.

Results

The *Ret_H^{2B}* Transgene Does Not Cause MEN 2B Symptoms in Mice

In humans, the visible phenotypic features associated with the MEN 2B syndrome include facial pigmentation spots and submucosal neuromas of the tongue and lips, and a marfanoid appearance.³⁴ Patients with MEN 2B also develop mucosal neuromas in the intestine and exhibit optic nerve hypertrophy. The first generation of

Table 1. Oligonucleotides for YAC Mapping

| Position relative to <i>Ret</i> (kb) | Primer sequence |
|--------------------------------------|---|
| -90 | F-TCTCTCCATTTATGGCTGGG R-TGCAAGGTGAAGCAAGAATG |
| -80 | F-CAGACTGCTGACCTCCACAA R-GGGCCTGCAGAGACTAAG |
| -70 | F-AGGAGAATGGCATGAACCTG R-ACAAAACCAGTTCCTGGTGC |
| -60 | F-AGACTCCGTCTTCTACAAAA R-AAGAGAGGAAGAGCGATACGTCC |
| -58 | F-TCTAATTCCTTCCAGAAATGCT R-TGGACCGACAAAAATAACCAGT |
| -56 | F-TTGCTCCATGGAGAAGTGGG R-GGCAATGAGGGAAGTGGAAA |
| -55 | F-GGGAGCTAATTTCTCCCTGG R-GAGCCTCGACTAAGGGAATA |
| -49 | F-GCCTGCTCATTAGAGGATTAT R-TTCTTCAAATTCAGAGTCACAG |
| -29 | F-TTAAAGCCTCTGTTTCAGCT R-AGAAAAGCCAGAAGTCCCATT |
| +20 | F-TGAAAAGTCCAGGCCAAGAG R-AGGTCGAGAGTTCAAGACCA |
| +27 | F-AATGGGCAATGCCCATACT R-GGGAGCTGTGCCAGTTTTAT |
| +37 | F-AATTACTTTTTGGGGTGGGA R-GTTGGATGAGAAGTCAGGTT |
| +47 | F-TGAGTGAAAAGAAGCCAGTCATA R-TAGAGCATTTACCCCCAAAAAG |
| +53 | F-CCATCCCTTTTGAAGCAGAA R-GGAGTGAGAGGCCCTCATTCA |
| +65 | F-TTTCACGCATCAACCTTTGG R-CCAAACCACAGAGCAGGAAG |
| YAC right arm | F-ACGTCCTGTGAATGTTGCCG R-TGCTTGGGAGTTGGGAATTGAAG |

transgenic mice with the human *Ret_H^{2B}* proto-oncogene YAC construct also contained both normal mouse *Ret* alleles, and were designated genotypically *Ret_H^{2B}/Ret_M^{+/+}*. Standard anatomical and histological analysis of more than 25 transgenic animals revealed none of the phenotypic features seen in affected humans.³⁴ Moreover, our breeding studies did not find any adverse effects of the transgene on fetal survival. The various genotypes were present in newborn mice at the expected frequency. We followed the animals longitudinally to determine whether they developed any of the expected neoplasms associated with MEN 2B. Anatomical analysis of more than 100 animals as old as 24 months revealed no neoplasms or other histological abnormalities in the thyroid or adrenal glands.

To exclude the possibility that our findings were related to the presence of three *Ret* alleles (the mutated human *Ret_H^{2B}* and two normal mouse *Ret* genes), instead of the normal genetic complement of two alleles, we bred the F1 transgenic animal with mice lacking one *Ret_M* allele to generate animals possessing the *Ret_H^{2B}* gene in the context of only one murine *Ret* allele, designated genotypically as *Ret_H^{2B}/Ret_M^{+/-}*.¹⁸ Again, there were no mucosal neuromas in the face or in the intestine, nor were there any obvious abnormalities in the central nervous system. Moreover, the animals did not develop any of the thyroid or adrenal neoplasms characteristic of the human MEN 2B syndrome throughout 24 months of follow up (more than 100 animals examined). Thus, we found no pheno-

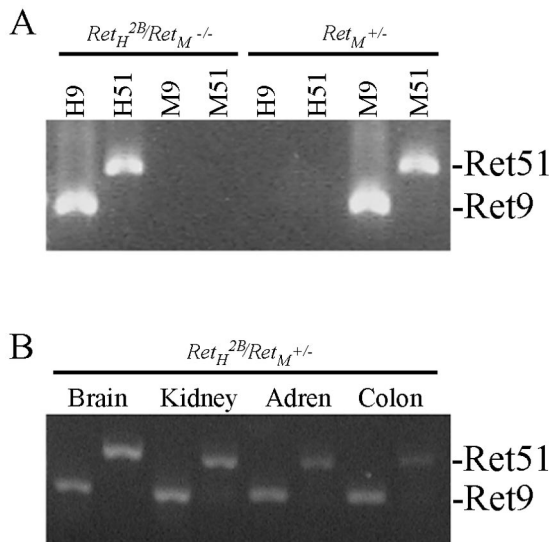


Figure 3. PCR products amplified from whole transgenic mice and selected mouse tissues. **A:** Using species-specific primer sets, cDNA was amplified from a whole *Ret_H^{2B}/Ret_M^{-/-}* mouse expressing only the human *Ret_H^{2B}* transgene or the *Ret_M^{+/-}* mouse expressing only the mouse *Ret* allele. Both primer sets from human and mouse correctly amplified the *Ret* splice variants from the corresponding cDNA. Importantly, the mouse primers did not amplify either splice variant of human *Ret* nor did the human primers amplify mouse *Ret* demonstrating species specificity of the primer sets. Each lane is labeled with the primer set used in the amplification: H9 and H51 amplify human *Ret9* and *Ret51*; M9 and M51 amplify the corresponding murine products. The genotype of the source cDNA is labeled above the corresponding lanes. **B:** cDNA from mouse tissues with a *Ret_H^{2B}/Ret_M^{+/-}* genotype was amplified using the human primer sets for *Ret9* and *Ret51*. The source tissue is labeled above the corresponding lanes and the PCR products are labeled on the **right**. In the transgenic mouse brain, kidney, adrenal gland, and colon, both human *Ret_H^{2B}* splice variants are amplified.

typic effect in transgenic mice possessing the human *Ret* proto-oncogene with the mutation associated with MEN 2B, irrespective of whether the animals had the mutated gene in the context of either one or two wild-type murine *Ret* alleles.

Expression of *Ret_H^{2B}* from the YAC Transgene Was Similar to that of Normal Murine *Ret*

To determine whether the absence of MEN 2B-associated neoplasms was the result of improper expression of the *Ret_H^{2B}* transgene in the target tissues, we used reverse transcriptase-PCR to evaluate expression of the human *Ret* transgene in *Ret_H^{2B}/Ret_M^{+/-}* mice (ie, in animals possessing one each of the human and mouse alleles). In humans, there are multiple *Ret* transcripts that encode two distinct *Ret* proteins that differ in the C-terminal region.³⁵ These two moieties are termed *Ret9* and *Ret51* and demonstrate different downstream signaling characteristics.^{36–39} Using human-specific primers, we evaluated the expression of *Ret9* and *Ret51* by reverse transcriptase-PCR in transgenic mice in the brain, kidney, adrenal gland, and colon. The results are presented in Figure 3, and, although not quantitative, demonstrate that both isoforms of the human *Ret_H^{2B}* oncogene are expressed in the transgenic mice. To determine whether the human YAC-transgene is expressed at physiological levels, we used the PCR mimics technique to

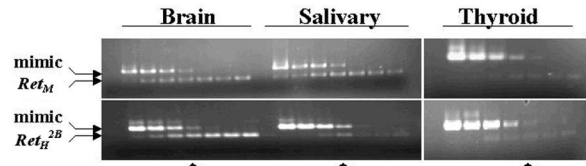


Figure 4. Comparison of expression levels for human and murine *Ret* from brain, salivary gland, and thyroid glands obtained from transgenic mice possessing the human *Ret_H^{2B}* YAC transgene. Species-specific primers were used to amplify *Ret* from cDNA at varying dilutions, and compared to the mimic product by agarose gel electrophoresis. The **arrowheads** denote the dilution at which the human and murine transcripts are expressed at equivalent levels compared to the PCR mimic, demonstrating that human and murine *Ret* are expressed at the same levels in these tissues.

quantitate *Ret* expression. As shown in Figure 4, the human gene was expressed at approximately the same level as the native murine gene in these animals in the brain, salivary gland, and thyroid gland. Also tested were the kidney and adrenal (not shown). Although we have not tested every tissue in which *Ret* might be expressed, in those tissues examined, the human *Ret* oncogene appears to be appropriately expressed at physiological levels.

The *Ret_H^{2B}* Transgene Partially Rescues the *Ret_M^{-/-}* Kidney and ENS Phenotype

We further evaluated the function of the YAC transgene *Ret_H^{2B}* by backcrossing the heterozygous *Ret_H^{2B}/Ret_M^{+/-}* transgenic mice with the *Ret* heterozygotes *Ret_M^{+/-}* to generate mice with the genotype *Ret_H^{2B}/Ret_M^{+/-}* to generate mice with the genotype *Ret_H^{2B}/Ret_M^{-/-}* possessing only the mutated human YAC transgene. If the human gene were able to compensate for the absence of murine *Ret*, then these animals would be expected to exhibit the same phenotype as *Ret_M^{+/-}* animals or as mice described by Smith-Hicks and colleagues²⁴ in which normal murine *Ret* was replaced by two copies of *Ret* with the MEN 2B mutation (*Ret_M^{2B/+}*) (ie, normal kidneys and normal abundance of enteric ganglion cells). The genotypes of the litters were distributed as predicted, demonstrating no increased intrauterine mortality in any of the genotypes. Interestingly, the newborn pups generated from our backcross breeding experiments having the *Ret_H^{2B}/Ret_M^{-/-}* genotype were normal at birth, but all died within 3 to 5 days after birth. In evaluating these animals shortly after death, the stomachs were distended with milk, and there was a striking presence of air in the wall of the intestine as demonstrated in Figure 5. This pneumatosis intestinalis is similar to that which occurs in human newborns with necrotizing enterocolitis or enterocolitis related to congenital aganglionosis. The animals apparently succumbed to either dehydration or overwhelming infection resulting from enterocolitis.

Histological analysis of cross sections of the bowel from the *Ret_H^{2B}/Ret_M^{-/-}* transgenic animals demonstrated the absence of enteric ganglion cells in the small bowel and colon of these mice. This phenotype is very similar to that found in mice lacking both *Ret* alleles. Because bowel cross-sections provide a limited view of

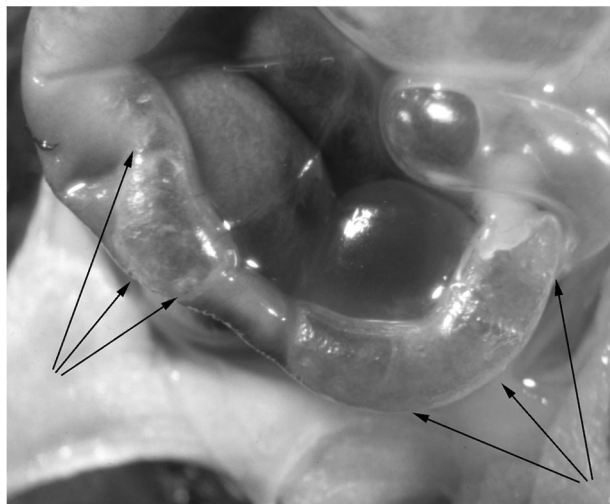


Figure 5. *Pneumatosis intestinalis* in transgenic mice possessing only the human *Ret* transgene with the MEN 2B mutation (genotype $Ret_H^{2B}/Ret_M^{-/-}$). Mouse pup was dissected shortly after death at 5 days of life. The **arrowheads** indicate the obvious bubbles of gas within the intestinal wall.

the ENS and could miss a subtle rescue of ENS development, we next performed whole mount acetylcholinesterase staining to examine the ENS. The staining patterns were compared between wild-type animals, $Ret_M^{-/-}$ mice, and $Ret_H^{2B}/Ret_M^{-/-}$ animals and are shown in Figure 6. Whereas the $Ret_M^{-/-}$ mice had no enteric neurons in the small bowel or colon and exhibited severe hypoganglionosis of the stomach as previously reported,¹⁸ the $Ret_H^{2B}/Ret_M^{-/-}$ mice showed considerable ENS development in the stomach and a few chains of enteric ganglia extending for several centimeters down the proximal small bowel. The $Ret_H^{2B}/Ret_M^{-/-}$ mice were still missing most of the neurons in the small bowel and all of the enteric neurons in the colon. These chains of enteric ganglia were strikingly different from the $Ret_M^{-/-}$ phenotype in which the ganglia are not observed in the small

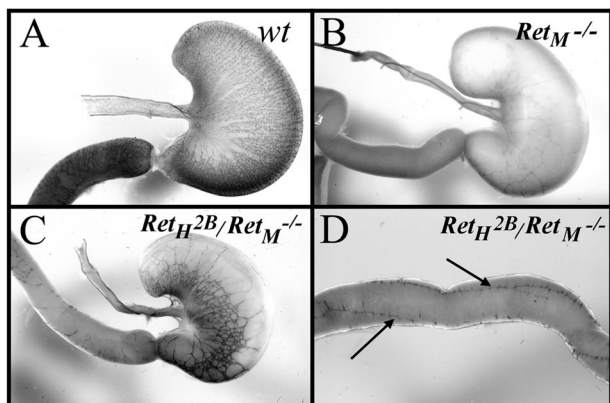


Figure 6. Acetylcholinesterase staining of ENS in the stomach and duodenum. **A:** Wild-type stomach and duodenum (**arrow**) showing the fine network of nerves of the ENS. **B:** Stomach and duodenum (**arrow**) of mouse lacking *Ret* (genotype $Ret_M^{-/-}$) showing aganglionosis of the ENS. **C:** Stomach and duodenum (**arrow**) of transgenic mouse with genotype $Ret_H^{2B}/Ret_M^{-/-}$ showing partial rescue of the ENS by expression of human Ret_H^{2B} . **D:** Distal to the stomach in the $Ret_H^{2B}/Ret_M^{-/-}$ mouse, acetylcholinesterase staining is limited to a small number of longitudinal ENS bundles (**arrows**) in the proximal small intestine.

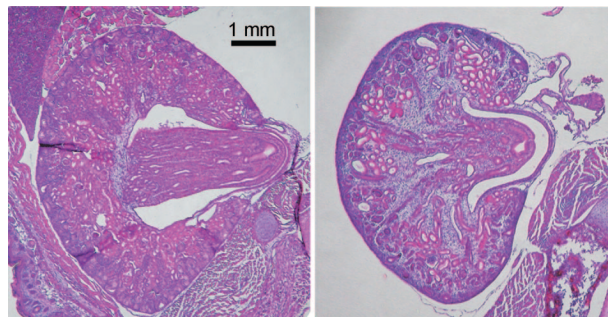


Figure 7. Photomicrographs of kidneys from newborn mice. Image on **left** is from a wild-type animal, and the **right** image is from a pup with genotype $Ret_H^{2B}/Ret_M^{-/-}$, demonstrating mild cortical dysplasia. Original magnifications, $\times 40$.

bowel except for a very few scattered nuclei in the first millimeter of the duodenum.¹⁸ These results suggest that the human YAC Ret_H^{2B} transgene partially rescues the function of native mouse *Ret* in the ability to support the development of the ENS.

The other significant finding in $Ret_H^{2B}/Ret_M^{-/-}$ mice is the frequent absence of kidneys, along with other anomalies of the urinary tract. We evaluated the excretory system in 25 animals with the $Ret_H^{2B}/Ret_M^{-/-}$ genotype. In most cases, there were two kidneys, but in five (20%) of the transgenic mice, there was only one kidney present. The extra-renal excretory tracts were unremarkable. Histological analysis of the kidneys demonstrated some dysplasia in transgenic animals, histologically shown in Figure 7. Thus, whereas *Ret* knockout mice have renal aplasia and other abnormalities in the genitourinary tract, mice having the human Ret_H^{2B} allele generally had grossly normal excretory systems with varying degrees of mild renal dysplasia.

Discussion

The *Ret* proto-oncogene is critical for the formation of the ENS and kidneys in mice. *Ret* activation also is essential for normal development of the parasympathetic and sympathetic nervous system.¹⁸ By activating both the MAPK and PI-3 kinase pathways, *Ret* tyrosine kinase signaling in many parts of the body regulates cell survival, migration, and proliferation during development.⁴⁰⁻⁴² Inadequate *Ret* signaling causes intestinal aganglionosis in both humans and mice and renal hypoplasia/aplasia in mice.^{18,43} In fact, inactivating *Ret* mutations are the most frequent identifiable cause of Hirschsprung's (intestinal aganglionosis) in humans.^{44,45}

Increased *Ret* activation causes cancer in humans. There are three inherited cancer syndromes (FTMC, MEN 2A, and MEN 2B) that result from excessive and inappropriate *Ret* activation.^{5-9,46} Of these syndromes, MEN 2B is the most severe and results from a single point mutation in the *Ret* kinase domain changing Met918 to Thr918. This mutation increases *Ret* activation and may also change the intracellular signal transduction pathways activated by *Ret*.⁴⁷ Humans with the MEN 2B mutation uniformly develop medullary carcinoma of the thyroid by

age 20. They also have abnormalities of bone development leading to a marfanoid body habitus, and develop pheochromocytomas and mucosal neuromas.^{10–12}

We generated a transgenic mouse expressing the human *Ret* gene with a Met918 to Thr918 mutation, expecting to develop a mouse model for MEN 2B. To ensure that appropriate regulatory signals were present in the transgene to recapitulate the normal *Ret* expression patterns, we elected to express *Ret* in the mouse from a human YAC clone with the M918T mutation. This 150-kb clone was expected to include the regulatory signals necessary for physiological *Ret* expression in the mouse. Indeed, human YAC transgenes have successfully rescued absent murine genes in a number of mouse knockout models of human disease.^{48–50} In other cases, however, human YACs have been unable to completely correct for the absence of a mouse gene.^{51,52} Our data demonstrate that this human YAC *Ret_H^{2B}* clone apparently resulted in normal levels of both *Ret* splice isoforms in several target tissues including the kidney, brain, thyroid, and salivary and adrenal glands. Further, human RET protein exhibits an overall 83% identity with murine RET protein, and exhibits a 95% identity over the C-terminal 400 amino acids, which encompasses the intracellular signaling domain. Thus, we expected that the *Ret_H^{2B}* transgene would create a good model for MEN 2B in mice.

To our surprise, none of the *Ret_H^{2B}* mice developed cancer even when maintained for up to 2 years. Careful analysis of the thyroid and adrenal glands, tissues in which humans with MEN 2B typically develop cancer, did not reveal any evidence of neoplasia, despite normal expression pattern of the human transgene. This was true both in mice with two copies of wild-type murine *Ret* in addition to the transgene and in mice with a single wild-type murine *Ret* and a single human YAC *Ret_H^{2B}* transgene. So why do these mice not develop MTC, the life-threatening neoplasm that occurs in virtually every human with MEN 2B³⁴ or any of the other *Ret*-associated neoplasms for that matter? To answer this question, one must consider what previous studies have shown: 1) expression of physiological levels of human *Ret* with the MEN 2B mutation in mice will not induce tumor formation (MTC, pheochromocytoma, or intestinal ganglioneuromas); 2) expression of physiological levels of murine *Ret* with the MEN 2B mutation in mice will induce C-cell hyperplasia, pheochromocytomas, and certain ganglioneuromas, but not MTC;²⁴ and 3) expression of supra-physiological levels of human *Ret* with the MEN 2B mutation (or the MEN 2A mutation) in mice will induce MTC and other associated tumors.^{53–57} It seems possible that intrinsic differences in the human and mouse RET proteins exist such that oncogenic signals from physiological levels of mutant human RET in a mouse are insufficient to drive tumor formation (and that this insufficiency can be overcome by overexpressing mutant human RET). Alternatively, modifier genes may exist that must also be expressed in addition to activated Ret for the development of MTC and that this requirement for modifier genes can be overcome by overexpressing mutant human RET. Further, these modifier genes may or may not be present in any particular strain of inbred mouse, which could

explain the observed penetrance of MTC.⁵⁴ Lastly, it is possible that subtle, undetected differences in *Ret* expression from the *Ret_H^{2B}* transgene are ultimately responsible for the lack of tumor formation. This would take further detailed expression studies to confirm.

To test the biological activity of the human YAC *Ret_H^{2B}* transgene, we bred the transgenic mice with *Ret_M^{+/-}* heterozygous animals to generate *Ret_H^{2B}/Ret_M^{-/-}* mice. Whereas *Ret_M^{-/-}* mice have no kidneys, the *Ret_H^{2B}/Ret_M^{-/-}* mice have essentially normal sized kidneys. Microscopic examination of the kidneys, however, demonstrates abnormalities in the kidney architecture. The cause of these developmental renal anomalies is not yet clear. Interestingly, Gestblom and colleagues²⁶ observed renal dysgenesis in their *Ret^{MEN2B}*-transgenic mice. They found that sympathoadrenal hyperplasia caused the renal malformations by locally competing for ligand during renal development. However, we did not observe any gross sympathoadrenal hyperplasia in our *Ret_H^{2B}/Ret_M^{-/-}* mouse pups. In our mice, we expected that the *Ret_H^{2B}* transgene would have increased activity compared to the native *Ret_M* gene. If, however, the *Ret_H^{2B}* transgene had increased activity compared to the native mouse *Ret_M* gene, we would not have expected to see any mice with only a single kidney. The unilateral renal agenesis and dysplastic kidney phenotype in the *Ret_H^{2B}/Ret_M^{-/-}* mice is reminiscent of the renal abnormalities observed in *GDNF^{+/-}* mice suggesting that despite the mutation, the *Ret_H^{2B}* transgene exhibits less downstream signaling activity in the mouse than the wild-type murine *Ret* gene.¹⁶ This overall reduction in *Ret* activity of the *Ret_H^{2B}* transgene compared to the native murine *Ret*, might also explain the absence of tumors in our mice. In fact, the extracellular portion of human Ret exhibits only a 77% identity with the same portion of murine Ret (versus a 95% identity within the intracellular portion). Thus, reduced Ret-related signaling during kidney development may be a function of reduced interaction with murine GDNF.

If the *Ret_H^{2B}* transgene is really less active than wild-type *Ret_M*, defects might also be expected in the ENS of *Ret_H^{2B}/Ret_M^{-/-}*. Whereas *Ret_M^{-/-}* mice have essentially no enteric neurons distal to the pylorus, and have a reduced density of neurons within the stomach, heterozygous *Ret_M^{+/-}* mice have normal numbers of enteric neurons in the colon and small bowel.⁵⁸ Thus, unlike in humans, haploinsufficiency in mice for *Ret_M* does not seem to cause Hirschsprung's disease (intestinal aganglionosis) or hypoganglionosis. The ENS phenotype in the *Ret_H^{2B}/Ret_M^{-/-}* mice is unusual because single chains of enteric ganglia with thick interconnecting nerve fiber bundles extend down the proximal small bowel. Interestingly, although there are thick bundles of nerve fibers that connect ganglia along a chain that extends down the bowel, there are very few connections between individual chains of ganglia. An explanation for this finding would be that single ENS precursors actually give rise to linear arrays of interconnected ganglia as they migrate down the bowel. In this case, the chains of ganglia would represent the progeny of a single ENS precursor cell. This intestinal hypoganglionosis would be comparable to

the piebaldism or coat spotting phenotype that occurs in endothelin receptor B or endothelin-3-deficient mice, in which inadequate proliferative signals for developing melanocytes result in regions of the mouse coat that are devoid of melanocytes, instead of a diffuse reduction in melanocytes spread over the surface of the skin.^{59,60} An alternative explanation is that the few surviving and proliferating ENS precursors are attracted to each other and stay bundled in ganglia rather than spreading out into the bowel. As the proliferating ENS precursors migrate down the bowel, they also stay connected by neuronal projections. The observation that there are few connections between the chains of ganglion cells present in the same region of the bowel suggests that extending neuronal fibers find it more difficult to reach distant chains of enteric ganglia, leading to limited interactions between the separate chains of enteric ganglia. Regardless of how these ganglia of the ENS develop, it is clear that the partial rescue of the ENS is mediated by human Ret encoded by the *Ret_H^{2B}* transgene. However, some defect in development of the ENS still exists. Because murine Ret with the MEN 2B mutation has been shown to be sufficient to direct the normal development of the ENS,²⁴ it seems that our phenotype could be explained by either a lack of proper *Ret_H^{2B}* expression in the distal ENS or a lack of proper downstream signaling from mutant human Ret within the ENS. Thus, although the *Ret_H^{2B}* transgene supports kidney development, it may lack those genetic elements required for the proper temporal expression of Ret in the ENS. Alternatively, there may exist intrinsic differences between human and murine Ret such that defects in Ret-related signaling results in only a partial rescue of the ENS.

One incompletely characterized aspect of Ret function is the contribution of the gene to the regulation of respiratory function. This connection is suggested by the coincidence of Hirschsprung's disease with the congenital central hypoventilation syndrome in 22% of cases.^{61,62} Alterations in *Ret* have been described infrequently in patients having both Hirschsprung's disease and central and congenital central hypoventilation syndrome.^{63,64} However, it is likely that mice lacking *Ret* have central hypoventilation, because they all die within 24 hours of birth, which is sooner than one might expect if such mice only had renal agenesis and total intestinal aganglionosis.¹⁸ To study this, investigators evaluated the effect of hypercapnea on mice lacking either one or both *Ret* alleles.⁶⁵ Mice lacking *Ret* demonstrated depressed ventilatory response to inhaled CO₂, leading to the conclusion that the *Ret* gene is an important factor in the development of the neurological regulation of ventilation. In contrast to mice lacking *Ret*, animals possessing one human *Ret* allele having the mutation associated with MEN 2B (genotypically *Ret_H^{2B}/Ret_M^{-/-}*) live until the age of 4 to 5 days. We believe this time of death is most consistent with the development of enterocolitis and/or dehydration related to the pseudo-obstruction of the intestine from their hypoganglionosis, rather than a hypoventilation syndrome. Whereas we did not formally measure the response of the transgenic animals to hypercapnea, these results suggest that the introduction of

the human *Ret* proto-oncogene into mice lacking *Ret* is able to correct in part the neurological phenotype present in the knockout animals, despite the limited ability to support the development of the enteric nerves.

To summarize our findings, we introduced into mice a YAC transgene containing the human *Ret* oncogene with the mutation associated with MEN 2B. Surprisingly, the resulting transgenic mice did not develop any of the neoplasms associated with MEN 2B in humans. Moreover, introduction of the transgene into mice lacking *Ret* resulted in only partial correction of the murine knockout phenotype consisting of renal aplasia and partial intestinal aganglionosis. In particular, whereas the *Ret_H^{2B}/Ret_M^{-/-}* transgenic mice were able to develop kidneys, albeit slightly dysplastic ones, they were unable to develop a complete ENS. Moreover, whereas knockout mice possess dysregulation of their response to hypercapnea and ventilation, and presumably die of apnea within the first day of life, this phenotypic feature was corrected by the introduction of the human transgene. These results are in contrast to the case in which a normal murine *Ret* allele is present; such mice exhibit complete correction of the knockout phenotype.

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