**ABSTRACT**

Multiple endocrine neoplasia type 2B (MEN2B) syndrome is caused by a missense mutation in the RET gene, which replaces Met918 by Thr in the intracellular kinase domain of the protein. This single amino acid substitution transforms the receptor into a constitutively active monomeric kinase (RETMen2B) and produces an autosomal dominant syndrome characterized by medullary thyroid carcinoma, pheochromocytomas, musculoskeletal anomalies, and mucosal ganglioneuromas. The ligand, GDNF, stimulates RET activity through a coreceptor, GFRα-1. In vitro studies have shown that the kinase and mitogenic properties of RET Men2B are enhanced by GDNF/GFRα-1 stimulation. A relevant clinical question is whether ablation of either GDNF or GFRα-1 could alter penetrance or severity of the MEN2B syndrome. We report that ganglioneuromatous tumors caused by a RETMen2B transgene in mice are not affected grossly or microscopically by the absence of gdnf or gfrα-1. Loss-of-function mutations in ret, gdnf, or gfrα-1 cause pan-intestinal aganglionosis in mice. We find that expression of the RETMen2B transgene in enteric neural progenitors, after they colonize the gut, does not prevent intestinal aganglionosis associated with gdnf or gfrα-1 deficiency.

**Key words:** Hirschsprung disease, ganglioneuroma, RET receptor, animal model, transgenic mice, multiple endocrine neoplasia

**INTRODUCTION**

RET is a receptor tyrosine kinase that is expressed by a subset of neural crest derivatives, including sympathoadrenal and enteric neural and chromaffin cell precursors. De novo mutations of the RET receptor tyrosine kinase cause at least four human disorders. Gain-of-function mutations result in multiple endocrine neoplastic syndromes 2A (MEN2A) or 2B (MEN2B) and familial medullary thyroid carcinoma (reviewed in reference [1]). In contrast, loss-of-function mutations are associated with Hirschsprung disease (reviewed in reference [2]). In addition, chromosomal rearrangements involving the RET gene are found in many papillary thyroid carcinomas [3].

Intracellular signaling via the RET tyrosine kinase receptor is important for survival, proliferation, and differentiation of several cell types including neurons of the central and peripheral nervous systems (reviewed in reference [4]). The secreted proteins that activate RET belong to the GDNF family of ligands (GFLs), which include glial cell line–derived neurotrophic factor (GDNF) [5], neurturin (NRTN) [6], artemin (ARTN) [7], and persephin (PSPN) [8]. GFLs are unable to bind or activate RET directly, but instead act indirectly by binding to a co-receptor in the GFRα family of
four glycosyl-phosphatidylinositol anchored proteins (GFRα-1, -2, -3, and -4) [9]. Each of the four GFLs binds with high affinity to a particular GFRα receptor, the principle ligand for GFRα-1 being GDNF [4]. GDNF preferentially binds GFRα-1 and thereby recruits RET into specialized plasma membrane domains to form a signaling complex in which RET molecules dimerize and activate downstream effectors including Akt and MAP kinases [10,11]. In vitro, the formation of the signaling complex in the lipid rafts is important for normal signal transduction via RET and consequent neuronal survival and differentiation [10]. In vivo, null mutations of gdnf [12–14], gfrα-1 [15,16], or ret [17] produce nearly identical murine phenotypes characterized by intestinal aganglionicosis, agenesis/ hypoplasia of the superior cervical ganglia, and renal agenesis. These results suggest that RET activation by GDNF and GFRα-1 is essential for the development of kidneys, the enteric nervous system, and parts of the sympathoadrenal system.

The RETMen2B protein results from a mutation that alters Met 918 to Thr in the intracellular kinase domain of the receptor [18]. The substitution transforms the receptor into a monomeric, constitutively active kinase in contrast to proto-RET, which is activated by ligand-induced homodimerization [19–21]. RETMen2B, however, is also capable of ligand-induced dimerization, and GDNF stimulation elicits enhanced mitogenic and kinase activities along with increased phosphorylation of Shc substrates in RETMen2B-expressing cell lines [22,23]. Like proto-RET, the enhanced transforming ability of RETMen2B by GDNF specifically requires the presence of the co-receptor GFRα-1 [22]. However, the importance of GDNF/GFRα-1 in the pathogenesis of RETMen2B-induced neoplasia has not been investigated in vivo.

We hypothesized that lack of ligand stimulation via GFRα-1 could significantly alter the phenotypic effects of RETMen2B expression. To test this hypothesis, we introduced the ΔβH-RETMen2B transgene into the genomes of gdnf−/− or gfrα-1−/− mice. The transgene makes use of the human dopamine β-hydroxylase (ΔβH) promoter to target expression of human RETMen2B in sympathetic neurons, adrenal chromaffin cells, and enteric neurons and their precursors [24]. Wild-type mice carrying the transgene develop neuroglial tumors in the sympathetic nervous system and adrenal medulla. Here we report that gdnf or gfrα-1 deficiency does not alter the incidence or gross or microscopic features of ΔβH-RETMen2B transgene-induced tumors.

Loss-of-function mutations in RET have been associated with Hirschsprung disease, and analogous mutations in ret, gdnf, or gfrα-1 cause total intestinal aganglionicosis in mice. Gene-targeting experiments have shown that in the developing enteric nervous system, RETMen2B completely compensates for proto-RET, such that homozygous RETMen2B mice have normal enteric nervous systems [25]. On this basis, we predicted that expressing the RETMen2B transgene in gdnf−/− or gfrα-1−/− mice might prevent aganglionicosis. However, we find that transgene-derived RETMen2B is not sufficient to rescue the aganglionic phenotype associated with either mutation mice.

**METHODS**

**Mice**

Production and characterization of ΔβH-RETMen2B-transgenic mice were described previously [24]. Two independent transgenic lines, designated the “intermediate-copy line” (ΔβH-RETMen2B with 70 transgene copies) and the “low-copy line” (ΔβH RETMen2B with four copies), were used for this study. ΔβH-RETMen2B, gdnf−/− mice were generated by crossing the F1 progeny of ΔβH-RETMen2B intermediate copy line with mice carrying a null mutation in one gdnf allele [13] (provided by Heiner Westphal, National Institutes of Health). Similarly, ΔβH-RETMen2B, gfrα-1−/− null mice were produced by mating the F1 progeny of a cross between ΔβH-RETMen2B low-copy line animals with mice carrying a null mutation in one gfrα-1 allele [15] (provided by Arnon Rosenthal, Genetech). All experiments in this study were conducted with postnatal day 1 pups of two independent parental crosses. A minimum of three animals of each genotype were examined.

**Genotype analysis**

One-centimeter segments of distal tail were digested overnight at 37°C in 1× SET buffer (1% sodium dodecyl sulphate, 10 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) containing 20 mg/ml proteinase K and 1.4 mM