Neural Injury, Repair, and Adaptation in the GI Tract III. Role of the RET signal transduction pathway in development of the mammalian enteric nervous system*

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Pachnis, V., P. Durbec, S. Taraviras, M. Grigoriou, and D. Natarajan. Neural Injury, Repair, and Adaptation in the GI Tract. III. Role of the RET signal transduction pathway in development of the mammalian enteric nervous system. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G183–G186, 1998.—The enteric nervous system (ENS) in vertebrates is derived from the neural crest and constitutes the most complex part of the peripheral nervous system. Natural and induced mutagenesis in mammals has shown that the tyrosine kinase receptor RET and its functional ligand glial cell line-derived neurotrophic factor (GDNF) play key roles in the development of the ENS in humans and mice. We have developed and briefly describe here a number of assays that analyze the specific function of the RET receptor and its ligand. Our data suggest that the RET signal transduction pathway has multiple roles in the development of the mammalian ENS.

RET tyrosine kinase receptor; glial cell line-derived neurotrophic factor; enteric nervous system

ONE OF THE FUNDAMENTAL PROBLEMS in developmental biology today is to understand the genetic and molecular mechanisms by which a single cell, the fertilized egg, generates a mature organism containing a very large number of diverse cell types. Applied to developmental neurobiology, this problem is translated into the question of understanding the molecular control of neuronal and glial cell determination and differentiation. Work over the last 50 years has clearly established that the interaction of cells with their environment plays a critical role in cellular differentiation and pattern formation in both the central and peripheral nervous systems (CNS and PNS) (8). Furthermore, one of the principles that has emerged over the last several years is that signals required by one cell type at a given time can be used by different cells and at different stages of embryogenesis to perform very different functions. Therefore, understanding the mechanisms of cellular differentiation and pattern formation in relatively simple parts of the nervous system is likely to identify molecules that have critical functions in other more complex parts of the nervous system, such as the mammalian brain.

The enteric nervous system (ENS) in vertebrates consists of two ganglionic plexuses (myenteric and submucosal), which are composed of several types of neurons and glia and are arranged as two concentric rings in the wall of the bowel (4, 7). As part of the PNS, the ENS is derived from the neural crest (NC). Grafting experiments in avian embryos and lineage studies in mammalian embryos have shown that the ENS is derived from NC cells emigrating from the dorsal aspect of the neural tube at somite levels 1–7 (vagal NC of the postotic hindbrain and anteriormost region of the spinal cord) (2, 9, 17). The ENS is the most complex part of the PNS. Its functional complexity is reflected in the number and phenotypic diversity of its neurons, which are subdivided into distinct classes producing every type of neurotransmitter that has been identified thus far in other parts of the PNS or CNS (7). The combined expression of specific neurotransmitters and the enzymes responsible for their synthesis have served as a means of identifying various subsets of enteric neurons (15). A striking peculiarity of the ENS is that the neurons of the myenteric and submucosal plexuses form local reflex circuits that function largely independently of the CNS to regulate the contractility of the gut wall musculature and the secretion of its glands (7). It is therefore not surprising that establishment of the correct pattern of synaptic connectivity among the various groups of neurons and between neurons and their terminal targets (such as smooth muscle) during embryogenesis is essential for the normal function of the ENS.

NC cells colonizing the embryonic bowel differentiate under the influence of signals encountered along the migratory pathway and at their final destination (6). Such signals, produced by the gut mesenchyme, control a complex set of cellular interactions required for normal ENS development and organization. In addition to environmental signals, intracellular signaling molecules and transcription factors also control the differentiation of ENS progenitors along the glial and neuronal pathways. Although the full complement of such signaling molecules and their intracellular mediators remain unknown, recent genetic studies have

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identified some of the key regulators of ENS development. For example, mutations in the loci encoding endothelin-3 (ET-3) or its receptor [endothelin β-receptor (EDNRB)] in mammals lead to aganglionosis (i.e., absence of enteric ganglia) of the terminal colon. Also, mutations in the loci encoding the ciliary neurotrophic factor receptor (CNTFR-α) or leukemia inhibitor factor receptor (LIFR-β) result in the loss of specific subsets of motoneurons from enteric ganglia. Finally, mutations in the loci encoding the ciliary neurotrophic factor necessary for the development of sympathetic neurons, eliminate the transient catecholaminergic cells of the bowel, precursors to the serotonergic lineage of the mammalian ENS (5).

A characteristic of the phenotypes resulting from the above mutations is that they affect only specific subpopulations of enteric neurons and ganglia. However, mutations in the loci encoding the tyrosine kinase receptor RET and its functional ligand glial cell line-derived neurotrophic factor (GDNF) have been shown to have “global” effects on the development of the mammalian ENS (5). Humans heterozygous for germ-line mutations in the c-RET locus often suffer from congenital megacolon (Hirschsprung's disease), a condition characterized by the absence of enteric ganglia from varying lengths of the colon (3, 13). By generating a mouse strain carrying a functional deletion of the c-RET locus, we have shown that animals homozygous for such a mutation die within the first day of birth and have severe hypoplasia or aplasia of the kidneys and lack the superior cervical ganglia and all enteric ganglia posterior to the cardiac stomach (2, 16). Consistent with the functional interaction between GDNF and RET in vivo, elimination of the gdnf locus from mice results in a phenotype identical to that of Ret mutant animals, including the absence of enteric ganglia (11, 12, 14).

The NC cells destined to populate the embryonic bowel and form the ENS emigrate from the dorsal aspect of the posterior hindbrain/anterior spinal cord and migrate ventrally and posterior to the branchial arches prior to their entry into the foregut mesenchyme. From the foregut, the ENS progenitors migrate in a rostrocaudal direction until they populate the entire length of the gastrointestinal tract. Simultaneously with their migration, the ENS precursors receive signals that allow them to proliferate and survive in the gut mesenchyme. Finally, they differentiate into a large variety of neuronal cell types and glial cells, which are organized into ganglionic complexes occupying characteristic positions around the periphery of the gut wall (6). Because formation of the mammalian ENS depends on all of these interrelated processes, it is likely that misregulation of any of them can lead to a phenotype similar to that observed in RET and GDNF-deficient animals, i.e., elimination of enteric ganglia from the gut wall. We have studied the expression pattern of c-RET during mouse embryogenesis extensively, and our findings indicate that the gene is expressed from very early stages of ENS formation, at the time of entry of the NC-derived ENS progenitors into the foregut mesenchyme (Fig. 1) (2). This suggests that the RET signal transduction pathway functions at an early stage of ENS neurogenesis and is required for an essential function of the early NC cells that populate the embryonic bowel. This hypothesis is consistent with extensive phenotypic analysis of the Ret mutant embryos, which has shown that the progenitors of the neurons and glia of the ENS are eliminated at early stages of ENS organogenesis (2). To study further the role of the RET receptor tyrosine kinase in the development of the mammalian ENS, we have examined the degree of apoptotic cell death of the ENS progenitors in mutant embryos in situ and compared it to their wild-type littermates. Our findings indicate that the majority of ENS precursors undergo apoptosis as they enter the foregut of mutant embryos and shortly after induction of expression of the RET receptor. Despite the extensive programmed cell death observed in the foregut of RET-deficient embryos, the majority of tyrosine hydroxylase-expressing cells, which are transiently observed in the murine bowel (1), survived and populated parts of the esophagus and cardiac stomach. These findings are consistent with lineage studies indicating that a distinct subpopulation of vagal NC cells (most likely those emigrating from the anterior spinal cord corresponding to somites 6–7) are capable of colonizing the foregut of Ret-mutant embryos and are independent of a functional RET receptor. These data also provide strong support for the hypothesis that one of the critical roles of the RET receptor is to provide...
survival signals to a large proportion of the NC progenitors of the ENS (2). Given the expression of GDNF in the mesenchyme of the gut and the functional interaction between RET and GDNF, we conclude that one of the critical components of the mesenchyme-derived survival signals is GDNF itself.

The early elimination of the ENS progenitors in RET mutant embryos precludes the study of the potential role of the RET receptor on other aspects of ENS histogenesis using the available mutant mouse strains. To bypass this caveat, we have developed a culture system in which the response of the mammalian ENS progenitors to various growth factors can be studied in vitro. For this, immunoselection is used to isolate the RET-expressing population of NC derivatives from the gut of wild-type mouse or rat embryos (using a cocktail of anti-RET monoclonal antibodies and fluorescence-activated cell sorting), which is subsequently cultured in a defined medium in the presence or absence of GDNF (10). Consistent with our in vivo studies, absence of growth factors from the culture medium resulted in the elimination of all RET-expressing ENS progenitors. However, addition of GDNF promoted the survival, proliferation, and morphological differentiation of these cells. These findings show that, in addition to the early function of the RET receptor on the survival of the ENS progenitors, it is likely that the activation of the RET signal transduction pathway has additional functions in these cells, such as promotion of proliferation and differentiation. Stage-specific mutagenesis of the c-RET locus in mice will be necessary to study such potential roles in vivo. Finally, the elimination of all ENS progenitors in vitro in the absence of growth factors indicates that even the RET-independent subpopulation of ENS progenitors required the presence of growth factors that prevent programmed cell death in vivo.

It has been suggested that correct differentiation and morphogenesis in the vertebrate ENS require the normal three-dimensional organization of the gut wall observed in vivo. Therefore, in addition to the cell culture studies described above, we have developed an in vitro explant culture system in which the gut of a mouse embryo is placed in culture under conditions that maintain the integrity and normal spatial relationship of the various cell types of the gut wall. At the time of isolation (E10.5–11.5 mouse embryos), the bowel has only partially been populated by NC cells, which are negative for mature neuronal or glial markers. However, culture of this explant for 7 days in a defined medium results in extensive neuronal and glial differentiation. Furthermore, culture of a gut explant isolated from RET-deficient embryos fails to generate any neurons or glial cells consistent with the in vivo phenotype of the mutation. Interestingly, introduction of wild-type RET-expressing ENS precursors into the aganglionic gut in vitro leads to colonization of the entire gut wall and generation of the majority of the cell types normally present in the adult ENS. These data indicate that the aganglionic phenotype of RET-deficient animals is a cell-autonomous defect due to absence of the RET receptor from the cell surface of ENS precursors and that the mutant gut mesenchyme is capable of supporting the proliferation, migration, and differentiation of wild-type NC derivatives. The ability of wild-type RET-expressing cells to generate neurons and glial cells when introduced in vitro into the “empty” gut of RET-deficient embryos provides us with an experimental system in which the parameters that play a critical role in the reconstitution of a functional ENS in the aganglionic bowel can be studied in vitro. The study of such parameters can be valuable for any future attempts of fetal therapy in patients with Hirschsprung's disease.

Overall, the studies summarized here indicate that the murine ENS is a system amenable to genetic, molecular, and cellular analysis and therefore constitutes a useful experimental system for studying the biological processes underlying cellular differentiation and morphogenesis in the mammalian nervous system.

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REFERENCES


