Published in final edited form as:
Neurogastroenterol Motil. 2009 February ; 21(2): 113–127. doi:10.1111/j.1365-2982.2008.01256.x.

Genetic Model System Studies of the Development of the ENS, Gut Motility and Hirschsprung's Disease

Grzegorz Burzynski[1], **Iain T. Shepherd**[1,*], and **Hideki Enomoto**[2,*]

[1]Department of Biology, Emory University, 1510 Clifton Road, Atlanta GA 30322 USA

[2]Laboratory of Neural Differentiation and Regeneration, RIKEN Center for Developmental Biology 2-2-3 Minatojima-Mnamimachi, Chuo-ku, Kobe, Hyogo 650-0047 JAPAN

Abstract

The enteric nervous system (ENS) is the largest and most complicated subdivison of the peripheral nervous system. Its action is necessary to regulate many of the functions of the gastrointestinal (GI) tract including its motility. Whilst the ENS has been studied extensively by developmental biologists, neuroscientists and physiologists for several decades it has only been since the early 1990's that the molecular and genetic basis of ENS development has begun to emerge. Central to this understanding has been the use of genetic model organisms. In this article we will discuss recent advances that have been achieved using both mouse and zebrafish model genetic systems that have led to new insights into ENS development and the genetic basis of Hirschsprung's Disease.

Keywords

Mouse; Zebrafish; Enteric neurons; neural crest; screens; transgenics

Introduction

The past 15 years has seen dramatic increase in our understanding of the cellular and molecular basis of Enteric Nervous System (ENS) development and gut motility. The focus of this review is to describe recent data that have been derived from genetic model organism studies, specifically mouse and zebrafish that have led to new insights into the molecular and cellular basis of ENS development as well as the establishment of intestinal gut motility. These studies have also led to new insights into the molecular basis and underlying causes of human diseases that affect gastrointestinal motility such as Hirschsprung's disease (HSCR).

The neurons and glia of the ENS form the intrinsic innervation of the gastrointestinal (GI) tract. Uniquely the ENS is the only part of the Peripheral Nervous System (PNS) that can function independent of central nervous system innervation [1]. While the ENS is primarily responsible for complex motility patterns, sub-populations of interstitial cells of Cajal (ICC) generate electrical slow waves, which organize the contractile activity of the gut muscle into

* Authors for correspondence.

phasic contractions [1-4]. ICCs arise from the intestinal mesenchyme and are essential for normal intestinal motility [5, 6]. In addition during development, “myogenic patterns” of intestinal motility are initiated by intestinal muscles themselves [1, 2]. These ENS independent gut contractions are believed to be critical for the development of coordinated gut motility and the development of the ENS. In adults, all main gut processes such as immune responses, absorption, secretion, blood flow and complex motility patterns such as mixing, peristalsis and migrating motor complexes are regulated or controlled by the ENS. Formation of the intestinal neural circuits that are necessary for these functions requires a number of different types of enteric neuron (around 20 in humans). These can be generally divided into excitatory and inhibitory motor neurons, primary intrinsic sensory neurons and inter-neurons. In avians and mammals the enteric neurons are primarily organized into the myenteric and submucosal ganglionated plexi. The myenteric plexus is located between longitudinal and smooth muscle layers and is present along the whole length of the GI tract while the inner, submucosal plexus is restricted to the small and large intestine [1].

Neural crest origin of the ENS

As with all parts of the PNS, the ENS is completely derived from the neural crest [7]. Lineage analysis studies indicate that the ENS is principally derived from vagal (postotic) neural crest in all species so far examined. In mammals and avians neural crest from the lumbo-sacral region also contributes to the ENS in the hindgut. Vagal neural crest cells enter the foregut and then colonize the whole length of the gut in a rostro-caudal direction [7, 8]. Sacral neural crest cells enter the distal hindgut, only colonizing this region of the gut in a caudo-rostral manner [7, 8]. Significantly, sacral crest only enter the hindgut after the arrival of vagal neural crest. Furthermore, chick quail chimera studies demonstrate that the sacral neural crest cannot compensate for a complete loss of vagal neural crest. While the basis of the neural crest induction is comparatively well established there is still no definitive data demonstrating whether there is a prespecification of the enteric neural crest within the premigratory vagal or lumbo-sacral neural crest. BMP, FGF, WNT and Notch/Delta signaling pathways have all been shown to be required for neural crest induction and the specification of different subtypes of crest derivatives in the premigratory crest [9]. However, what specifies and guides ENS precursors from the premigratory vagal neural crest to the foregut is still unknown. More defined roles have been established for specific molecules and signaling pathways necessary for enteric neural crest derived cell (ENCDC) migration proliferation and differentiation once they have reached the GI tract. These include the GDNF, Endothelin, BMP, Hedgehog (HH), Retinoic Acid (RA), Notch-Delta, Semaphorin, and Netrin pathways [10-32*]. In addition several transcription factors have been implicated as having a role in ENS development including Mash1, Pax3, Phox2b, Hand2, Hox11a and Hoxb5 [30, 33-47*].* In the following sections we will highlight how genetic model system studies have furthered our understanding of neuronal aspects of ENS development. We will highlight new avenues of research that will undoubtedly lead to novel insights into the molecular basis of ENS development and the development of gut motility.

*With respect to nomenclature molecules (proteins and genes) are shown in upper case letters and in lower case letters with the first letter capitalized, respectively. All gene names are italicized.

Animal models

The foundation of our understanding of the formation of the ENS has been established using animal models. Our focus in this review are studies that have been undertaken in the mouse and zebrafish model systems due to the genetic techniques available to manipulate and investigate gene expression in vivo in these systems as well as the ability to undertake forward genetic screens.

Significantly the use of model organisms has led to a clearer understanding of the multiple mechanisms by which mutations in specific genes can cause the complex human genetic disorder Hirschsprung's disease (HSCR) [48]. In the next section we will discus further the genetic basis of this condition.

Mouse studies

Nearly 40 mouse strains exhibiting spontaneous or genetically engineered mutations, either single or compound, have been reported to display ENS abnormalities with varying degrees of severity [49]. In this section we review the current state of knowledge of the molecular control of ENS development obtained from mouse and human genetic studies. We focus particularly on RET in the regulation of ENS development, as RET signaling controls multiple facets of ENS development and alterations in RET signaling are primarily implicated in HSCR. Interactions of RET signaling with other pathways will also be discussed, as will unresolved issues.

The Ret-GFL-GFRα system

The RET tyrosine kinase and the GFRα receptor, a family of GPI-anchored cell surface receptors, forms a receptor complex and mediates signals of the GDNF Family Ligands (GFLs), a neurotrophic factor family comprising of four members, glial-derived neurotrophic factor (GDNF), Neurturin (NRTN), Persephin (PSPN) and Artemin (ARTRN). There are also four members in the GFRα family, GFRα1, GFRα2, GFRα3 and GFRα4. Specific binding between GDNF-GFRα1, NRTN-GFRα2, ARTN-GFRα3 and PSPN-GFRα4, and subsequent RET activation regulates development of given neuronal populations and the urogenital system [50].

Pleiotropic actions of RET in ENS development

During development of the ENS, GDNF and GFRα1 are expressed in gut mesenchyme, whereas RET and GFRα1 are expressed in vagal neural crest cells colonizing the foregut. GFRα1 expression in mesenchymal cells is dispensable for ENS development [2], and GFRα1-mediated RET activation is thought to function in a cell-autonomous fashion in ENCDCs. In mice deficient for *Ret, Gdnf or Gfrα1* genes, the ENS fails to form in the gut below the stomach. Detailed examination of RET-deficient fetuses has revealed that ENCDCs are reduced in number and fail to enter the midgut as early as E10.5 [51]. This colonization failure is accompanied by abnormal cell death of ENCDCs in the foregut [52]. Separate studies have shown that GDNF is required for the promotion of cell migration and proliferation of ENCDCs [53]. Taken together, these findings suggest that the absence of the ENS in *Ret/Gfrα1/Gdnf* mutants is caused by compound deficits in cell survival, migration

and proliferation of ENCDCs. A recent study describes transient accumulation of *Ret*-deficient vagal neural crest cells at the dorsal aorta, indicating a possible novel role of RET signaling in vagal crest cell sorting [54]. Interestingly, the majority of neurons remaining in the esophagus of *Ret*-deficient fetuses fail to express the neuronal differentiation marker, NOS [55], suggesting that RET is required for neuronal differentiation as well. Moreover, a recent cell transplantation experiment showed that *Ret*-deficient fetal gut does not provide a permissive environment for grafted ENCDCs, suggesting a non-cell autonomous function of RET in this special context [54]. Collectively, these observations demonstrate the pleiotropic role of RET signaling in the early colonization of the gut by ENCDCs.

The *Gfrα1 and Ret* genes also play an important role in later ENS development. Conditional ablation of *Gfrα1* or *Ret* in E14.5-15.5 mice, a period after the completion of the vagal neural crest migration, causes massive death of ENCDCs in the colon, leading to colon aganglionosis [56, 57]. These observations indicate that RET and GFRα1 are crucial for the survival of postmigratory ENCDCs. It is thought that the GFL ligand supporting the ENCDC survival is GDNF as, among GFRαs, only GFRα1 and GFRα2 are expressed in the ENS and because intestinal aganglionosis does not occur in mice lacking neurturin (NRTN), the high affinity ligand for GFRα2. Interestingly, the ENS in the small intestine remains unaffected in *Gfrα1* or *Ret* conditional mutants, despite the severe degeneration of the ENS in the colon. The reason for this greater susceptibility of ENCDCs in the colon to cell death than those in the small intestine is unknown. Notably, cell death induced by *Gfrα1* or *Ret* inactivation is not associated with caspase activation, and dying ENCDCs lack the morphological and immunohistochemical hallmarks of apoptosis, indicating that the underlying death mechanism of *Ret*- or *Gfrα1*-deficient ENCDCs appears to be distinct from that of apoptosis. It remains unknown whether the non-apoptotic cell death observed in *Gfrα1*- or *Ret*- deficient colonic neurons also occurs during normal development of the nervous system including the ENS.

Signaling of NRTN, plays only a limited role in controlling enteric neuron numbers. However, this signaling is crucial for maintaining enteric neuron soma size and for the density of excitatory cholinergic nerve fibers in the ENS [18, 26].

HSCR genetics and the RET gene

Hirschsprung's disease is a congenital disorder in humans that affects the gastrointestinal tract and occurs in 1 in 5000 live births. The pathology of HSCR is characterized by the absence of enteric ganglia, resulting in tonic contraction of the aganglionic gut segment and intestinal obstruction. HSCR cases can be grouped by the length of the aganglionic segment: short-segment HSCR (S-HSCR; 80% cases) and long-segment HSCR (LHSCR; ~20%). Ultrashort aganglionic segment or total intestinal aganglinosis are also found in rare cases. Approximately 70% of HSCR cases occur as an isolated trait. In the majority of families exhibiting such non-syndromic HSCR, transmission of the disease displays a complex pattern of inheritance with incomplete penetrance and a male-preponderant sex bias: this form of HSCR is assumed to be a multifactorial disorder [48, 58].

Mutations in the *RET* coding sequences (CDS) account for 50% of familial and 15-20% of sporadic HSCR cases. However, most familial HSCR cases show association with the *RET*

locus by linkage analysis. Search for non-coding mutations has identified a SNP far more frequently associated with HSCR than CDS mutations [59]. The SNP lies in an enhancer element within intron 1, and its association to HSCR is dose- and gender-dependent. Although less common, a SNP lying 3'- UTR which is potentially involved in *RET* mRNA degradation has also been identified [60]. Mutation of the *Ret* gene is now considered as a requirement for most HSCR. Notably, in both non-coding SNPs, HSCR-associated alleles have been linked to changes in RET expression levels, suggesting that a failure to maintain RET dosage confers susceptibility to this disease. Chromosomal loci, 9q31, 3p21, 19q12 and 16q23 have been identified as *RET* modifier loci [61-63], accounting for the oligogenic inheritance pattern of HSCR. Finally, a *RET*-independent HSCR susceptibility locus, 4q31-q32, is found in a multigenerational HSCR family [64].

HSCR can also be found as a clinical symptom in various congenital syndromes. For instance, HSCR is found in approximately 20% of congenital central hypoventilation syndrome (CCHS) patients, the vast majority of whom carry *PHOX2B* mutations. Transmission of a *RET* hypomorphic allele in CCHS patients increases the risk for HSCR [65], indicating an epistatic interaction between *PHOX2B* and *RET* in HSCR expressivity. Importantly, a knockout mouse study showed that *Phox2b* is essential for *Ret* expression in the ENS, suggesting a significant contribution of RET dosage to the development of HSCR in this context as well. Human genetic studies have also identified overrepresentation of hypomorphic *RET* in Down syndrome and Bardet-Biedl syndrome patients presenting with HSCR [48]. Although the 21 trisomy and *MKKS/BBS6* gene mutations account for the majority of Down syndrome and BBS, respectively, how these genetic changes influence RET expression or function is unknown.

Ret mutants as mouse models for HSCR

Despite the high frequency of *RET* mutations in HSCR patients, how RET dysfunction leads to intestinal aganglionosis is not fully understood, and appropriate animal models are necessary to address this question. Various *Ret* mouse mutants are reported to display intestinal aganglionosis and so resemble HSCR to some extent [49]. However, most of them also display agenesis/dysplasia of the kidney and represent relatively rare forms of HSCR cases associated with kidney abnormalities, a population constituting approximately 5% of HSCR patients [48]. Interestingly, mice homozygous for the S697A mutation, in which serine 697 in RET is converted to alanine, display aganglionosis only in the distal colon and develop kidneys with almost normal histology [66]. $Ret^{S697A/S697A}$ ENS precursors fail to exhibit chemoattractive response to GDNF, but neurite outgrowth and proliferation of those cells remain unaltered, suggesting that impaired cell migration primarily contributes to distal colon aganglionosis. Similar conclusions about RET's function were also described in a previous study [67].

Genetic studies on HSCR strongly implicate reduced RET dosage in disease susceptibility, and one recent mouse genetic study revealed the physiological significance of RET dosage in ENS development. When *Ret* expression is reduced to approximately one-third of its normal level in mice ($Ret^{9/-}$ mice: the *Ret9* allele has *RET* cDNA knocked into the *Ret* locus and expresses RET at lower than normal levels), approximately 50% of the embryos develop

distal colon aganglionosis [57]. Interestingly however, other *Ret*-dependent organogenesis including that of kidneys and motor neuron development proceeds normally in $Ret^{9/-}$ mice. These observations reveal that ENS development is more sensitive to RET dosage than other forms of organogenesis. Notably, $Ret^{9/-}$ alleles exhibit not only incomplete penetrance but also a gender bias with males being more prone to develop aganglionosis than females. Taken together, $Ret^{9/-}$ mice faithfully represent clinical and genetic features of HSCR as an isolated trait and serve as a suitable model for studying the etiology of the most common form of HSCR.

Examination of $Ret^{9/-}$ fetal gut shows that early ENS development proceeds relatively normally, but colonization of the colon by ENCDCs is substantially delayed. Surprisingly, this colonization delay is associated with progressive cell death of ENCDCs in the colon. Interestingly, morphological attributes of the dying ENCDCs in $Ret^{9/-}$ colon are quite similar to those dying ENCDCs in the colon of conditional *Gfrα1* or *Ret* mutants. Importantly, the abnormal death in $Ret^{9/-}$ fetuses does not appear to be a secondary effect of delayed colonization of ENCDCs, because massive death of ENCDCs can also be triggered even when *Ret* expression levels are conditionally reduced after the completion of the ENCDC migration. This indicates that *Ret* reduction directly affects cell survival during late ENS development and suggests that intestinal aganglionosis is established by two non-interdependent processes, impaired cell migration and abnormal cell death. Since decreased RET expression is strongly implicated in HSCR, this study underscores the significance of cell death as one of developmental events linked to the etiology of HSCR [57].

It is noteworthy that intestinal aganglionosis develops simply as a result of the reduction of *Ret* expression to slightly lower than half of its normal levels in mice. In a multifactorial model for HSCR, *Ret* mutations and the other genetic factors were shown to act synergistically in the pathogenesis of intestinal aganglionosis. It is therefore reasonable to speculate that the functions of those factors lie on pathways regulating expression and/or stability of RET expression. Accordingly, *Ret*-heterozygous mice ($Ret^{+/-}$) will be an invaluable tool to identify the factors controlling *Ret* expression.

Interaction with RET signaling

Endothelin pathway

Endothelin-3 (ET-3) signaling via its G-protein-coupled receptor, EDNRB, constitutes another important signaling pathway for ENS development. Heterozygous mutations in *ET-3*, *EDNRB* or *ECE-1* (endothelin-converting enzyme) are found in both isolated and syndromic HSCR cases; the latter includes Waardenburg syndrome, characterized by colon aganglinosis, hypopigmentation and deafness. *ET-3* is highly expressed in mesenchyme of the caecum and colon at the developmental time-point immediately prior to the colonization of these gut regions by ENCDCs [68]. Consistent with this expression pattern, mice deficient for the *Et-3*, *Ednrb* or *Ece-1* genes display distal colon aganglionosis [11, 20, 31]. They also display pigmentation defects. Although *Ednrb* is expressed in both ENCDCs and gut mesenchyme, neural crest-specific inactivation of the *Ednrb* gene results in colon aganglionosis and hypopigmentation [69], a phenotype almost identical to that in conventional

Ednrb-null mice, suggesting that ET-3 acts primarily on neural lineages. The role of EDNRB in gut mesenchyme remains unknown.

A genome-wide study of HSCR in the Mennonite population shows a statistically significant co-transmission of *EDNRB* and *RET* alleles (harboring missense and presumptive non-coding mutations, respectively) in affected individuals. Moreover, mice with hypomorphic endothelin mutant alleles display higher incidence of intestinal aganglionosis on *Ret*-heterozygous ($Ret^{+/-}$) than on wildtype background [62]. The observations demonstrate the existence of a genetic interaction between *EDNRB* and *RET*. In vitro, ET-3 and GDNF synergistically promote ENCDC proliferation, a crucial driving force for ENCDC advancement [70]. ET-3 however has also been shown to inhibit GDNF-induced chemoattractive response in ENCDCs in a collagen gel assay [71]. Another study showed that ET-3 signaling is required in the maintenance of progenitor state of ENCDCs [72]. ET-3 signaling thus appears to regulate ENS development by modulating the action of GDNF and other signals in a context-dependent fashion.

Pathways controlling RET expression in ENS

Given the existence of critical threshold levels in RET expression for the manifestation of intestinal aganglionosis, it is crucial to understand how RET expression is controlled in cells. Induction and maintenance of RET expression involve multiple steps, including transcription of *Ret* mRNA, mRNA stabilization, translation, protein folding, receptor trafficking and degradation.

Among transcription factors, whose deficiency leads to intestinal aganglionosis in mice, Mash1, Phox2b, Sox10 and Pax3 are potential regulators of *Ret* expression [42-44, 72]. Regulation of *Ret* expression by Mash1, however, appears to be limited in vivo, as *Mash1*-deficiency affects only a small population of ENCDCs [73]. In *Phox2b*-deficient fetuses, ENCDCs fail to express *Ret* and are only present in the foregut at E10.5 [44]. A genetic interaction between *RET* and *PHOX2B* is also shown in CCHS patients (see above). Sox10 and Pax3 synergistically activate a *Ret* enhancer lying in genomic regions upstream of the *Ret* CDS. However, mouse genetic studies have failed to detect significant interactions between *RET* and *SOX10* [74]. Neural crest-specific ablation of FoxD3, a transcriptional repressor of the winged helix or Forkhead family of proteins, leads to the complete absence of ENS throughout the entire gut [75]. This phenotype is reminiscent of that of Sox10 mutants [40]. In *Foxd3*-deficient fetuses, Sox10 expression is down-regulated, and it is assumed that FoxD3 is involved in the maintenance of the progenitor state in neural crest cells, at least partly by regulating Sox10 expression. Finally, HoxB5 enhances the *RET* promoter activity, and genetic perturbation of HoxB5 activity leads to down-regulation of *RET* in ENCDCs and causes hypoganglionosis in mice [76]. Interestingly, some SNPs identified in a potential regulatory element of the *HOXB5* gene are overrepresented in HSCR patients, implying a potential contribution of *HOXB5* to HSCR susceptibility.

Extrinsic signals including BMP, retinoic acid (RA) and GDNF elevates *Ret* expression in some neuronal populations, and thus are potentially regulators of *Ret* expression in the developing ENS. BMP2 and BMP4 are expressed in gut epithelium and mesenchyme, and recombinant BMPs can upregulate *Ret* in neural crest stem cells in culture [43]. SIP1, a

transcriptional co-repressor, at least partially interacts with BMP signaling, and haploinsufficiency of *SIP1* causes Mowat-Wilson (M-W) syndrome, in which patients display mental retardation often associated with HSCR (~60% of the cases). Genetic ablation of *Sip1* gene leads to the absence of the ENS in the gut below stomach [77], a phenotype reminiscent of that of *Ret*-deficient mice. However, in M-W syndrome, there is no significant contribution of *RET* hypomorphic allele to HSCR expressivity, suggesting that *RET* and *SIP1* do not interact genetically. How the BMP signaling pathway interacts with the RET pathway in early ENS development remains unclear. BMP signaling does play a crucial role in specification and differentiation of the sympathetic neuronal lineage and is essential for *Ret* induction though whether it has the same function in ENS lineage development is unknown [78].

Retinoic acid (RA) is a strong inducer for *RET* expression in some neural crest-derived tumor cell lines [79]. Mice lacking Raldh2, an enzyme critical for RA synthesis during early development, die at E9-10.5 due to severe deficits in heart morphogenesis. This early embryonic lethality can be bypassed by all-tans-RA, and, in those rescued embryos, the ENS fails to develop normally [80]. Although retinoic acid influences diverse cell types including gut mesenchyme and neural crest cells, the phenotype observed in *Raldh2* mutants is at least partly explained by direct effects of retinoic acid on ENS precursors; a recent study, for example, clearly demonstrates that RA influences neuronal differentiation of purified ENS precursors in vitro [27].

Accumulating evidence suggests that RET activity also regulates *Ret* gene expression in vivo. In mice deficient for GDNF signaling, *Ret* expression is decreased in many RET-dependent neuronal populations [81, 82]. Moreover, when the *Ret* locus is knocked out by inserting GFP or tLacZ reporter under the *Ret* promoter, the levels of reporter expression in GDNF-dependent cells are significantly lower in homozygous than in heterozygous mice (Enomoto, H. personal data). Importantly, such decreases in reporter expression are not observed in mice carrying homozygous *Gfrα1* knock-in allele [83], suggesting that GDNF signaling regulates *Ret* specifically. Molecular constituents of the RET autoregulatory loop have yet to be identified.

Newly translated RET requires further glycosylation for its cell surface expression. In mice homozygous for the RETC620R mutation, RET is poorly glycosylated, and the ENS is absent in the gut below stomach [84], a phenotype identical to that of *Ret*-deficient mice. The RET maturation process may also depend on RET activity, as a decrease in RET expression leads to a decrease in the mature glycosylated form as compared to the immature form.

Unlike other receptor tyrosine kinases, which are degraded in the lysosome-dependent pathway, RET protein is degraded mainly by the proteasome. A recent study in sympathetic neurons shows that this process is regulated by the coordinated actions of CD2-associated protein, CD2AP, and the E3-ligase Cbl-3 [85]. It remains to be determined whether similar degradation machinery is at work in enteric neurons. Such knowledge will be vital to the understanding of post-translational regulation of RET expression and its influence on ENS development.

Other genes controlling ENS development and their relevance to RET signaling

In contrast to the regulation of RET in ENCDCs, much less is known about factors controlling GDNF in gut mesenchyme. Mice lacking Hlx, a homeobox transcription factor expressed in gut mesenchyme, display hypoganglionosis [86]. GDNF expression levels, however, are not altered in *Hlx*$^{-/-}$ gut. Disruption of several genes affects RET signaling, thereby causing ENS deficits. In mice deficient for both the *Sulf1* and *Sulf2* genes, neuronal innervation in the esophagus is reduced, and the deficit is assumed to arise from impaired desulfation of heparan sulfate [87], a known modulator of GDNF signaling. In *Sprouty2*-deficient mice, GDNF signaling is constitutively activated, and enteric neurons are increased in number [88]. In contrast, NPY deficiency impairs GDNF-induced proliferation and survival of ENCDCs in vitro and causes reduced numbers of nNOS-containing enteric neurons in mice [89].

The roles of Ret signaling in neural differentiation, neural guidance and postnatal maturation and function of the ENS remain unclear. Several genes have been shown to regulate these processes. For instance, the Notch pathway and Hand2 are involved in glial and terminal neuronal differentiation of ENCDCs [23, 34, 36, 37, 90]. BMP signaling also influences neuronal differentiation, presumably by promoting cell cycle exit of ENCDCs[14]. Neural guidance-related molecules, including Semaphorin3A, Netrin-DCC, ß1 integrin, and L1, have been shown to play a role in ENCDC migration and gangliogenesis[10, 21, 91, 92]. Finally, genetic inhibition of the PKA pathway in enteric neurons does not affect ENS formation at least at gross morphological levels but causes intestinal pseudo-obstruction [93].

While mouse studies have been invaluable in advancing our current knowledge of the genetic basis of ENS development and have lead to a much clearer understanding of the genetics HSCR a need has emerged for the use of other genetic model organisms to complement mouse studies so as to further our understanding of the genetics of ENS development. Furthermore the recent human genetic studies of HSCR that have led to the identification of additional HSCR susceptibility loci as well as the identification of several new loci and genes that have been implicated in other human diseases affecting the intestine and its motility such as inflammatory bowel diseases (IBDs) has created the need for the use of a genetic model organism in which investigators can comparatively quickly and cheaply validate the importance of potential genes in disease linked loci [32, 48, 94]. As a result several groups have begun to use the zebrafish model system to investigate the cellular and genetic basis of ENS development.

The Zebrafish Model System

In the past 15 years the zebrafish has emerged as a new model system to study vertebrate development [95]. The key reason for this emergence has been the ability to efficiently undertake forward genetic screens. To date most zebrafish screens have used the chemical mutagen ethylnitrosourea (ENU) to induce point mutations [96]. Classical positional cloning approaches are then used to identify affected genes in isolated mutants[97]. Whilst this approach has been very successful it is time consuming and does not always work. As a result, insertion mutagenesis approaches have been developed using retroviral and

transponson based methods that enable much more rapid identification of the mutated loci [98-100].

In addition to forward genetic approaches, reverse genetic approaches have also been developed to investigate specific gene function in zebrafish. Current and future reverse genetic methods in zebrafish have been recently reviewed so we will only discuss this approach briefly [101]. The most widely used method involves the transient knock down of a gene's expression using modified antisense oligonucleotides called morpholinos [102]. While morpholinos have proven to be extremely valuable, as with many antisense techniques, it has become apparent that there are off-site effects of using these reagents [103]. As a result other reverse genetic techniques have been developed, the most promising of which uses zinc finger nucleases to generate null and hypomorphic mutations [104, 105].

Anatomy of the zebrafish intestine and ENS and the development of gut motility

The zebrafish ENS like those of other vertebrates is derived from the vagal neural crest and, as with other vertebrates, coordinated gut motility is under direct intestinal neuron control [106-109]. The development and the final form of the zebrafish ENS and gut, however, are not identical to that of higher vertebrates. As in other vertebrates, zebrafish neural crest derived ENS precursors migrate from the vagal region to the anterior end of the gut primordium where they associate with it [110, 111]. Zebrafish ENCDCs then migrate as two parallel chains of cells along the length of the GI tract [110, 111]. This contrasts to the situation in mammals and avians where ENCDCs migrate in multiple chains that follow complex and unpredictable trajectories within a caudal direction [112, 113]. Furthermore, zebrafish lineage analysis experiments suggest that there is no sacral neural crest contribution to the ENS (Shepherd unpublished data). Other significant differences in the zebrafish ENS is that, ENCDCs do not form a submucosal plexus and the myenteric plexus is not ganglionated in the same manner as seen in amniotes [114, 115]. Potentially, the reduced intrinsic innervation in zebrafish may be a consequence of the less complex gut morphology. Zebrafish intestinal epithelium has a comparatively simple structure that is arranged in long folds rather than villi, and lacks a submucosal layer [114, 116]. Zebrafish, like amniotes, do have ICCs that are responsible for slow propagating waves [117].

Spontaneous, propagating gut contractions can be detected in zebrafish at 3.5 days post fertilization (dpf), before the onset of feeding (5-6 dpf). This correlates with the appearance of cells expressing neuronal markers that are first detected in the intestine at approximately 2 dpf stage [107, 115]. Neuronal number rapidly increases over the next 3 days and this correlates with the differentiation of the intestinal longitudinal and circular muscle layers [114, 115, 118, 119]. Studies from several labs indicate that zebrafish enteric neurons produce both excitatory and inhibitory neurotransmitters. Enteric neurons expressing adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), calcitonin gene-related polypeptide (CGRP), nitric oxide (NO) neurokinin-A (NKA), substance P, acetylcholine and serotonin have all been reported [108, 115, 118-122]. However the precise neurochemical coding of the embryonic larval and adult zebrafish ENS along with a detailed anatomical description of the morphology of the different types of enteric neurons in the zebrafish intestine does not currently exist.

Zebrafish exhibit complex patterns of intestinal motility. Anterograde waves of contraction spread from the middle intestine when fish start to feed. In addition, retrograde contractions also arise in the anterior intestine that probably compensate for the lack of a proper stomach in zebrafish and the necessity to mix food in the intestinal bulb. The posterior part of the zebrafish GI tract exhibits contractions with short propagation waves in both directions, with a frequency and pattern resembling mammalian colon. Regular and spontaneous gut motility is observed in larvae as well as in the adult fish in between feedings and these patterns of motility can be compared to the migrating motor complexes that occur between meals in adult mammals (109).

A series of elegant studies from the Holmberg lab has demonstrated that both an excitatory cholinergic tonus and an inhibiting nitergic tonus are present in zebrafish from just before or at the onset of feeding and that a co-functionality between these two pathways potentially balance the sweeping gut contractions [108, 122]. A subsequent study from this group demonstrated that the spontaneous gut contractions seen at 4 dpf are not initiated by intrinsic or extrinsic neuronal innervation [123]. Instead, the ENS appears to play a more important role in helping to regulate intestinal activity after the onset of feeding. This is similar to the situation in humans and mice where regular coordinated contractions can develop in the absence of enteric neurons [2, 124, 125]. The initiation of these spontaneous gut contractions at 4 dpf in zebrafish are potentially due to the ICCs.

Molecular basis of Zebrafish ENS development

While there are clear developmental and anatomical differences between the zebrafish ENS and the ENS of avians and mammals, in vivo studies show there has been a conservation of the molecular basis of ENS development between these species. Perturbation of the function of *sox10*, *gdnf*, *gfrα1*, *phox2b* and *sip1* in zebrafish, result in a loss of enteric neurons [110, 111, 126-128]. However perturbation of the *ednrb1* function does not result in an ENS phenotype in contrast to avians and mammals [129].

Significantly *ret* expression and function in ENS development is conserved in zebrafish. Zebrafish *ret* mRNA is expressed in ENS precursors and differentiating neurons in the developing gut[106, 109, 110]. Futhermore it has been recently reported that two *ret* isoforms (*ret9* and *ret51*) are also present in the zebrafish and that *ret51*, as in other vertebrates, is not required for the complete colonization of the gut by the ENS precursors [130]. RET's primary function in zebrafish ENS development appears to be transduction of the GDNF ligand signal through interaction with the two zebrafish GFRα1 orthologues. Perturbation of *ret*, *gdnf* and both *gfrα1* orthologues in zebrafish by morpholino knock-down results in a complete loss of ENS neurons and precursors in the intestine[110, 128]. The exact mechanism by which this loss occurs has not been determined. Whether RET signaling in zebrafish has the same wide range of biological activities in ENS development as shown in mice and humans is still to be determined. Furthermore, given there is no apparent affect on ENS development from the loss of *ednrb1* function it is not clear if there is any synergistic activity between the endothelin signaling pathway and the RET signaling pathways in zebrafish. Finally, while neurturin and artemin like immunoreactivity have been reported in zebrafish the function of these GFL ligands in zebrafish ENS development is

unknown [131, 132]. Morpholino knock-down of zebarfish *gfrα2* does not result in any apparent affect in ENS development but whether there this gene and these GFL family ligands have a later function in ENS development still needs to be addressed (Shepherd unpublished data).

In addition to the classically defined HSCR linked genes (*sox10, gdnf, ret, phox2b* and *sip1*) recent studies in zebrafish have provided novel insights into the pleiotropic Bardet-Biedl syndrome (BBS). HSCR is frequently associated as part of this disease's clinical phenotype. Perturbation of *bbs8* function, one of the genes associated with this syndrome, in zebrafish results in craniofacial and ENS defect [133]. Subsequently these defects were shown to be associated with a perturbation of the hedgehog signaling pathway [133]. Independently, hedgehog signaling has been shown to be essential for zebrafish ENS development with sonic hedgehog (SHH) acting as potent mitogen for ENS precursors[134]. The mitogenic effect of SHH on ENS has been previously demonstrated *in vitro* [16]. This study also reported that SHH inhibits the proneurogenic effects of GDNF and also inhibits GDNF's migration stimulating activity. Taken together, these studies provide a potential link between the HSCR phenotype seen in BBS patients, HH signaling and RET signaling.

Zebrafish Forward Genetic Screens

As mentioned previously, a key strength of the zebrafish model system is the ability to undertake forward genetic screens. While not specifically screening for ENS mutants, several ENS mutants were identified in the two large-scale genetic screens that were undertaken in Germany and the US in the mid 1990 [135, 136]. Some of the ENS mutants identified were in genes known to be essential for ENS development such as the mutant *colourless*, which is a *sox10* mutant [127]. However, other mutants were in genes that have not previously been associated with ENS development such as the DNAse polymerase delta 1 mutant *flathead*, the *elys* nucleopore assembly protein mutant *flotte lotte* and the *rpc2*, RNA polymerase III subunit mutant *slimjim* [114, 137-142]. All of these mutants have pleiotropic phenotypes and were not initially identified based on their ENS phenotypes, however these studies contribute to our knowledge about GI tract development and potentially may help in dissecting the genetic basis of human intestinal disorders.

Recently two smaller scale screens have been undertaken that have specifically focused on the identification of ENS mutations [121, 143]. Both of these screens used ENU mutagenesis. Mutants were identified based on a reduction in the number of enteric neurons determined immunocytochemically, using an antibody that specifically labels differentiated neurons. In addition, Kuhlman and Eisen developed a motility assay as a secondary screen for the mutants they identified. Both screens were successful. Pietsch and colleagues identified 6 ENS mutants while Kuhlman and Eisen described 13. In both screens the majority of the mutants have pleiotropic phenotypes however Kuhlman and Eisen identified 4 mutants whose phenotype consists of only a loss of enteric neurons. 17 out of 19 mutants identified in both screens have a diminished number of enteric neurons along the whole length of the intestine while the remaining 2 identified in the Kuhlman and Eisen screen have aganglionosis only in the posterior segment of the gut. Of specific note is the *gutwrencher* mutant, that has fewer enteric neurons along the complete length of the gut and has less

coordinated intestinal contraction waves [143]. The key next step for both of these screens has been the identification of the genes mutated in these mutants. The genetic basis of all of the Kuhlman and Eisen mutants is currently unknown.

In the second zebrafish ENS screen the genetic basis for 2 of the 6 mutants has been determined. One of the ENS mutants is an allele of the previously identified DNA polymerase delta 1 mutant *flathead* [139]. The second ENS mutant, *lessen*, is a null mutant in the *med24/trap100* gene [121].

Med24 is a component of the mediator co-transcriptional activation complex that has previously been shown to have an essential role in mouse embryogenesis, though no ENS phenotype has been reported due to the *med24* null mutant mouse being an early embryonic lethal caused by a placenta defect [144]. The *lessen* ENS phenotype results from a proliferation defect in the ENS precursors once they reach the intestine. Surprisingly Med24 is not expressed in ENS precursors but is expressed in the intestinal endoderm. Genetic chimera analysis revealed that wild type endoderm could rescue the *lessen* mutant phenotype when transplanted into *lessen* mutants, suggesting that an endoderm derived or endoderm regulated mitogen is reduced in the *lessen* mutant, thus causing the ENS phenotype [121].

Zebrafish Transgenics

A significant development in the zebrafish field has been the use of the transposons to rapidly generate transient and stable transgenic lines [145]. The major use of these transposon systems to date (principally the Tol2 transposon) has been the generation of a large number of GFP expressing transgenic lines in enhancer trap screens as well as the use of this system to Tol2 system to analyze regulatory regions of genes [145-149]. Of significant interest for scientists studying ENS development, the regulatory regions of *ret, phox2b* and *sox10* genes have been analyzed using this approach [146, 148, 149]. A key finding from the RET study was that the majority of human RET non-coding sequences are able to direct ret-specific GFP expression in zebrafish even though the regulatory regions had little to no sequence similarity [148]. This study demonstrated the ability to use the zebrafish to analyze human regulatory regions. As a result, zebrafish is model systems in which potential regulatory mutations associated with human disease loci, such as HSCR, may be rapidly and cheaply analyzed in vivo.

A further development in the use of transposons in zebrafish has been the demonstration that a Tol2 transposon based enhancer trap system can be used for insertional mutagenesis [99]. A similar genetrap/ mutagenesis approach has also been reported for a second transposon system the Sleeping Beauty transposon [98]. Application of this type of mutagenesis in a screen for intestinal/ENS mutants has great potential though no screens using this approach have been reported.

Microarrays

As an alternative approach to identify genes necessary for ENS development two groups have used a microarray-based approach to identify genes whose expression is modified in ENS mutant mouse intestines. RNA was isolated from mice deficient for RET and GFRα1

to identify differences in gene expression profiles between normal and aganglionic gut [150, 151]. In principle, genes found to have a higher abundance of expression in wild type gut compared to aganglionic intestine should mainly represent the distinct expression profile of enteric neurons. The list of differentially regulated genes between two studies had significant overlap. Both groups reported finding genes previously implicated in the ENS development like *ret*, *sox10*, *gfrα1* and *phox2b*, although not all of them were present in both arrays [150, 151]. Other differentially expressed genes fell into many different functional categories. Among well-represented classes were transcription factors (e.g. homeobox factors, Sox family factors, T-box factors or Ets family transcription factors), genes involved in cell adhesion like cadherins, protocadherins and CAMs (cell adhesion molecules), and microtubule-associated proteins (e.g. stathmin family). Mediators of many well described signaling pathways, including receptor ligands (e.g. FGF growth factors and chemokines), kinases (Map kinases) and G-protein subunits were also identified. Not surprisingly, numerous genes associated with synaptic functions (e.g. Synaptotagmins, SNARE complex proteins) were down regulated in both studies. In both studies antisense RNA probes were generated and *in situ* hybridizations were performed with selected genes to confirm that the selected genes had an ENS/ intestinal expression. All genes investigated in this manner were expressed in the embryonic mouse gut (E14 or E15.5), in the regions of ENS formation, thus validating the microarray results [150, 151].

In a subsequent study by Heanue and Pachnis [130] orthologues of selected genes identified in their mouse microarray study were demonstrated to have a comparable pattern of expression in zebrafish. This study provides further support that there is a conservation of expression and function of the ENS-related genes between zebrafish and other vertebrates.

Perspectives

Development of the ENS involves complex biological processes and has been attracting the interest of researchers in many distinct fields. With the expansion of the ENS research, the numbers of mouse and zebrafish mutants displaying ENS deficits will undoubtedly continue to increase. It is important to note that colonization of the gut by ENCDCs is the net result of cell proliferation, migration, survival and differentiation of those cells. Thus, it is crucial to examine each developmental process in a given mutant to more precisely understand how individual genes control ENS development. Such analysis will be facilitated by the recent advent of technologies that allow visualization of proliferation and individual cell behaviors in a population of cells by live imaging [152, 153]. Furthermore, new forward genetic screens in zebrafish utilizing both established transgenic lines that express GFP in ENS precursors or intestinal neurons, such as the *foxd3:egfp* and *huc:egfp* transgenic lines [154, 155], as well as newly developed screening methods for intestinal gut motility will only further enhance our ability to identify mutants and genes critical for ENS development and gut motility [156]. With regard to *Ret* biology, it is now crucial 1) to identify factors associating with human *Ret* enhancers, 2) to more precisely determine the actual RET dosage in ENCDCs in HSCR and 3) to explore the involvement of cell death in HSCR cases. The degree of contribution of cell death to the overall aganglionosis phenotype must also be assessed in HSCR models. Future identification of the entire set of HSCR susceptibility loci will help to give a more complete picture of gene networks crucial for ENS development. Currently existing and

newly developed mutants for these loci will provide vital information toward understanding the functional hierarchies and gene networks in the development and pathology of the ENS and intestinal motility. Studies using both the zebrafish and mouse model systems will be pivotal in achieving this understanding.

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