Genetic background impacts developmental potential of enteric neural crest-derived progenitors in the \textit{Sox10}^{Dom} model of Hirschsprung disease

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Abnormalities in the development of enteric neural crest-derived progenitors (ENPs) that generate the enteric nervous system (ENS) can lead to aganglionosis in a variable portion of the distal gastrointestinal tract. Cumulative evidence suggests that variation of aganglionosis is due to gene interactions that modulate the ability of ENPs to populate the intestine; however, the developmental processes underlying this effect are unknown. We hypothesized that differences in enteric ganglion deficits could be attributable to the effects of genetic background on early developmental processes, including migration, proliferation, or lineage divergence. Developmental processes were investigated in congenic \textit{Sox10}^{Dom} mice, an established Hirschsprung disease (HSCR) model, on distinct inbred backgrounds, C57BL/6J (B6) and C3HeB/FeJ (C3Fe). Immuno-staining on whole-mount fetal gut tissue and dissociated cell suspensions was used to assess migration and proliferation. Flow cytometry utilizing the cell surface markers p75 and HNK-1 was used to isolate live ENPs for analysis of developmental potential. Frequency of ENPs was reduced in \textit{Sox10}^{Dom} embryos relative to wild-type embryos, but was unaffected by genetic background. Both migration and developmental potential of ENPs in \textit{Sox10}^{Dom} embryos were altered by inbred strain background with the most highly significant differences seen for developmental potential between strains and genotypes. \textit{In vivo} imaging of fetal ENPs and postnatal ganglia demonstrates that altered lineage divergence impacts ganglia in the proximal intestine. Our analysis demonstrates that genetic background alters early ENS development and suggests that abnormalities in lineage diversification can shift the proportions of ENP populations and thus may contribute to ENS deficiencies \textit{in vivo}.

INTRODUCTION

Hirschsprung disease (HSCR) is defined by a lack of enteric ganglia, aganglionosis, in a variable portion of the distal intestine (OMIM No. 142623) (1,2). Due to the lack of ganglia, stool cannot be passed through the colon, and a dilation of the bowel wall, or megacolon, results (3). Treatment involves removal of the affected portion of the gut, pull-through of the unaffected portion, and re-attachment to the anus (4,5). This congenital disorder of the hindgut affects on average approximately 1/5000 live births, although significant differences exist in incidence between ethnic groups (6).

Mutations in \textit{RET} (7–9), \textit{EDNRB} (10–12), \textit{EDN3} (13,14), \textit{GDNF} (15–17), and \textit{SOX10} (18,19), genes that play important roles in the formation of the enteric nervous system (ENS), have been identified in HSCR patients. In addition, \textit{EDNRB} and \textit{SOX10} mutations are normally associated with Waardenburg–Shah Syndrome (WS4), which has additional neural

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crest (NC) defects beyond aganglionosis of the colon (19,20). Patients present with variable phenotypes depending in part on the primary gene defect. Patients with RET mutations typically exhibit greater extent of aganglionosis (11,21), whereas those with EDNRB mutations show lesser extent of aganglionosis (20). However, even in familial cases that share an identical gene alteration between individual family members, a large degree of variation in penetrance (whether the phenotype is present) and severity (the extent of gut length affected) can occur (3). This variation among familial cases is thought to be the consequence of other regions of the genome, ‘modifiers’, that interact with the primary mutation to influence the final disease phenotype.

Disease modifiers of HSCR have been explored in human populations, with the focus being placed on modifiers of RET (22,23). RET has been shown to act as a modifier itself in other syndromic diseases that display HSCR, including congenital central hypoventilation syndrome (24). However, these studies have mainly focused on SNP-based or linkage screens, with little attention to biological processes. Studies in mouse models of HSCR have allowed greater mechanistic insight into the origins of aganglionosis, but the majority of mouse models are recessive alleles that do not mimick the variability of aganglionosis seen in patient populations (25–28). As a result, insights as to which developmental processes are affected by modifier interactions and the impact of these interactions on aganglionosis have been lacking.

The Sox10Dom/+ model is the only dominant HSCR mouse model that exhibits the variable penetrance and severity of aganglionosis seen between HSCR patients. Sox10 encodes a transcription factor that is required to maintain the multipotency of enteric neural crest-derived progenitors (ENPs) and the differentiation of glial cells (29,30). The ‘Dom’ mouse mutation arose spontaneously on a C57BL/6 (B6) haplotype in a B6xC3HeB/FeJLe-a/a intercross (31). The mutation is a single base-pair insertion that results in the retention of the DNA-binding domain but the loss of the transcriptional activation domain from the Sox10 protein (32). The Dom protein has been shown to act in a dominant-negative fashion in vitro (33). Complete loss of Sox10 leads to a total absence of enteric ganglia in homozygotes (32). In addition to enteric deficits, Sox10Dom/+ mice also present pigmentation defects on the feet, ventrum and head. The Sox10Dom+ phenotype recapitulates features of WS4 in humans, which is characterized by intestinal aganglionosis and hypopigmentation as a consequence of mutations in the human SOX10 gene (19). When bred on a mixed genetic background, Sox10Dom+/+ mice exhibit variable aganglionosis. However, congenic lines of this allele maintained on B6 and C3Fe inbred genetic backgrounds differ significantly in phenotype. Sox10Dom+/+ mice on the B6 background more frequently exhibit aganglionosis (greater penetrance) and a larger extent of the distal gut is affected by aganglionosis (greater severity) than when the mutation is bred onto the C3Fe background (34).

While the basic elements of ENS development have been thoroughly studied, the effects of genetic background on discrete aspects of these processes are unknown. Normal ENS formation is a multi-step process that includes several migration phases, expansion of an initially small cell population, and creation of multiple cell lineages (35,36). ENPs originating from both the vagal and sacral levels of the neural tube contribute to the ENS. Vagal progenitors emigrate from the vagal neural tube at 9.5 days post-coitus (dpc) in the mouse and invade the proximal end of the developing gut. They then move caudally, colonizing the gut as it elongates and reaching the anus by 14.5 dpc (37). Sacral ENPs enter the hindgut and migrate in a reverse direction to vagal ENPs up to the level of the post-umbilicus (38). During colonization, both vagal and sacral ENPs proliferate and differentiate into neurons and glia (35). The multi-step complex nature of ENS ontogeny makes it highly susceptible to alterations in gene function or expression.

The impact of genetic background on development of ENPs has not previously been examined. Relative to wild-type progenitors, ENPs in Sox10Dom+/+ mice are delayed in their migration as they traverse the gut (39). Modifier genes in the genetic background that produce the distinct amounts of aganglionosis seen in the Sox10Dom+ congenic lines may be altering migration, enhancing or suppressing proliferation, or modulating developmental potential of ENPs. Developmental potential has been defined functionally as the ability of progenitors to form various lineages in response to developmental cues that regulate both the timing of differentiation and the proportions of cell types that are produced in vivo (40,41). Sox10 could impact any of these processes because the gene is expressed early during the initial migratory phases of NC and is maintained in ENPs and adult enteric glia (42). Determining the effects of genetic background on ENS development is critical to understanding the processes that lead to aganglionosis and the variation between HSCR patients.

It has been established that interplay between genes involved in ENS development can significantly affect the presentation of aganglionosis (34,43,44). The large disparity in aganglionosis severity and penetrance between the two congenic Sox10Dom+ strains must result from the effects of other genes in the distinct B6 and C3Fe genetic backgrounds that are modulating basic developmental processes in ENPs. The search for modifiers of aganglionosis between these two strains has identified multiple intervals in the mouse genome, but specific genes within these regions remain to be identified (45). Understanding the biological effect(s) of genetic background will aid in the identification of modifier genes. More importantly, these studies can illuminate previously unrecognized aspects that contribute to the complex etiology of aganglionosis.

To define the effect of genetic background on ENS development, we have undertaken the developmental study of ENPs in the B6,Sox10Dom and C3Fe,Sox10Dom congenic lines. We hypothesized that the differences in postnatal aganglionosis were the result of differences in ENS ontogeny during fetal development. ENPs were assayed for differences in migration, proliferation and developmental potential. Consistent with prior reports, we found that deficiency of Sox10 alters migration and frequency of ENPs. In addition, we observed that the developmental potential of ENPs in Sox10Dom mutants is substantially shifted, but no effect on ENP proliferation was seen. Most importantly, our studies demonstrate that genetic background substantially impacts both migration and developmental potential of ENPs. Developmental potential reflects the capacity of NC-derived progenitors to...
respond to environmental cues and generate distinct lineages in vivo. Our analysis suggests that genetic background can influence not only the migration of ENPs but, as a consequence of the effect on developmental potential, may also alter the composition of enteric ganglia that are formed in more proximal gut regions, thus contributing to the variation in HSCR-associated symptoms between patients.

RESULTS

Both the Sox10Dom mutation and strain background in the context of the Sox10Dom allele alter ENP migration

The initial migration of ENPs down the length of the fetal gut is an essential prerequisite for normal ENS development and function. Defective migration of ENPs has been documented in a variety of HSCR models, including EdnrB<sup>−/−</sup> mice (46), EdnrB<sup>−/−</sup> rats (47) and Edn3<sup>−/−</sup> mice (48–50). In addition, several genes implicated in the development of HSCR have also been studied in regards to ENP migration, including GDNF (51,52) and cell adhesion molecule L1 (53). It has been shown that mice with either the Sox10<sup>Dom</sup> mutation or a targeted deletion of Sox10 fail to completely colonize the distal bowel (39,54). The extent of aganglionosis in the Sox10<sup>Dom</sup> model is influenced by genetic background, as evidenced by the difference in phenotype seen between the B6 and C3Fe strains (34). However, the impact of strain background on the migration process during development has yet to be investigated.

To examine the effect of the Sox10<sup>Dom</sup> mutation and strain background on ENP migration, we used immunohistochemical detection of Phox2B, a known marker of all early ENPs (55), and Sox10 expression. Cells were classified into one of four categories: non-proliferating non-ENP cells (pH3<sup>−</sup>Sox10<sup>−</sup>); non-proliferating ENP cells (pH3<sup>−</sup>/Sox10<sup>+</sup>); proliferating non-ENP cells (pH3<sup>+</sup>/Sox10<sup>−</sup>); and proliferating ENP cells (pH3<sup>+</sup>/Sox10<sup>+</sup>). The percentage of proliferative progenitors in each gut region was determined by calculating the number of proliferating ENPs (pH3<sup>+</sup>/Sox10<sup>+</sup>) divided by the total number of ENPs (all Sox10<sup>+</sup> cells). Calculation of this percentage effectively normalized for any differences in total number of cells between wells so that the proliferative capacity of ENPs could be compared between genotype and strain classes for each gut region. This analysis found no significant differences in proliferation between wild-type and Sox10<sup>Dom</sup> ENPs or between Sox10<sup>Dom</sup>−/− ENPs of either strain background (Fig. 2).

Isolation of ENPs by flow cytometry in the mouse

Gut neural crest-derived stem cells (NCSCs) give rise to the ENS and comprise a few percent of fetal intestinal cells (40). NCSCs and progenitors can be identified in situ using probes for the transcription factors Sox10 or Phox2B. However, analysis of live cells requires use of cell surface markers. Surface antigens such as p75, α4-integrin or HNK-1 have been shown to reliably label ENPs in a variety of species, including quail, chick and rat (61–68). Combinations of these surface antigens have been used extensively in the rat to purify NCSC populations from the peripheral nervous system, including the ENS (40,41,47,51,69,70). However, HNK-1 has not previously been utilized in mouse models, as prior efforts to localize this antigen by immunohistochemistry in fixed cryo-sections were unsuccessful (66). The HNK-1 immunoreagent commonly used is an IgM monoclonal whose large size could stericly hinder its ability to cross-react with epitopes in fixed whole-mount tissues of other species compared with C3Fe,Sox10<sup>Dom</sup>−/− samples indicates that genetic background exacerbates the effect of the Sox10<sup>Dom</sup> mutation on ENP migration.

ENP proliferation is not affected by the Sox10<sup>Dom</sup> mutation or by strain background

ENPs are rapidly proliferating during colonization of the fetal intestine, and any loss of proliferative capacity could reduce the size of the progenitor pool needed to entirely populate the gut. Several NC genes are vital to the proliferation process, including EdnrB (56) and GDNF (57–59). We investigated the possibility that the altered migration evident in Sox10<sup>Dom</sup> mutants could be due to a proliferative effect that is further influenced by genetic background. To identify proliferating ENPs, we implemented immunocytochemical detection of Sox10, which labels all ENPs, and phospho-Histone H3 (pH3), a known marker of proliferating cells, and specifically quantified the fraction undergoing mitosis. Cells were dissociated from three regions of sub-dissected fetal intestine (mid-small intestine, cecum and hindgut) from 12.5 dpc Sox10<sup>Dom</sup>−/− and wild-type littermate embryos. Cell suspensions were allowed to settle onto wells coated with poly-D-lysine and fibronectin, then fixed and stained for pH3 and Sox10 expression. Cells were classified into one of four categories: non-proliferating non-ENP cells (pH3<sup>−</sup>/Sox10<sup>−</sup>); proliferating non-ENP cells (pH3<sup>+</sup>/Sox10<sup>−</sup>); non-proliferating ENP cells (pH3<sup>−</sup>/Sox10<sup>+</sup>) and proliferating ENP cells (pH3<sup>+</sup>/Sox10<sup>+</sup>).
Moreover, loss of antigenicity with fixation can be a complicating factor in immunohistochemical detection. To investigate the presence of the HNK-1 epitope on live, unfixed suspensions of dissociated mouse ENPs, we utilized C3Fe-Tg(Phox2B-H2BCFP) transgenic mice. This transgenic line expresses an H2BCerulean protein (CFP) under the control of the Phox2B promoter (72). As Phox2B is a widely recognized marker of ENPs, co-localization of HNK-1 with CFP + fetal gut cells from these transgenic embryos would demonstrate that murine ENPs do express a cross-reactive HNK-1 epitope.

Figure 1. Effect of strain background and Sox10Domm mutation on density and migration of ENPs. Density and migration of ENPs in Sox10Domm embryos and wild-type littermates can be seen in whole-mount 12.5 dpc fetal guts by immunohistochemical staining for Phox2B. Confocal images (×400 magnification) taken in the proximal foregut, before the cecum, and after the cecum are shown in relation to a schematic diagram of the developing gut. Image panels from representative single guts are shown for each genotypic class. Stippled lines outline the gut in the post-ecum image for Sox10Domm embryos to emphasize the gut wall border.
CFP^+ cells were isolated from fetal guts of 14.5 dpc C3Fe-Tg^Phox2B-H2BCFP^ embryos by flow cytometry. Cells were plated at a density of 150 cells per well and subjected to immunocytochemistry for p75 and HNK-1. Co-localization of the H2BCerulean marker and both p75 and HNK-1 cell surface markers was observed. In contrast, no HNK-1 immunoreactivity was noted in CFP^2 cells isolated from the same guts (Fig. 3A and B). Therefore, HNK-1 is expressed by ENPs in the developing murine gut.

To evaluate the potential for use of HNK-1 labeling to isolate ENPs, we examined the frequency of Sox10 and Phox2B expression among cells purified by flow cytometry for HNK-1 surface expression. Single cell suspensions from dissociated fetal gut of 12.5 dpc embryos were labeled for HNK-1 immunoreactivity. HNK-1^+ cells were sorted into plates at a density of 150 cells per well and immediately subjected to immunocytochemistry for Sox10 and Phox2B, commonly used markers of ENPs (73). We observed that approximately half of enteric HNK-1^+ cells are Phox2B^+, while fewer than 40% are Sox10^+ (Fig. 3F). Thus, expression of HNK-1 alone is not sufficient to purify a population of ENPs, which are known to express both Sox10 and Phox2B.

As p75 surface expression has been used frequently to enrich for ENPs using a variety of methods (74–76), we examined the frequency of Sox10 and Phox2B expression in p75^+ ENPs using flow cytometry to purify cells expressing this surface receptor. Interestingly, we found that less than half of total p75^+ enteric cells express Phox2B and less than a quarter express Sox10 (Fig. 3F). While these results could be due to the antibody source, purity, cross-reactivity, or the dilution of antibody applied, this analysis prompted us to attempt labeling with a combination of these two immunoreagents in an effort to obtain a population of ENPs that exhibited a high frequency of expression for Sox10 and Phox2B (72,73).

Double labeling of dissociated fetal gut cell suspensions with both p75 and HNK-1 led to isolation of a population that is highly enriched for ENPs. When single cell suspensions from 14.5 dpc fetal gut were exposed to both p75 and HNK-1 immunoreagents, plated, and immediately stained for Sox10 and Phox2B, we observed that ~95% of these cells express these known markers of enteric NC derivatives. Moreover, p75^+/HNK-1^+ ENPs also express Nestin, a marker expressed in all stem cells (77). Thus, the use of both p75 and HNK-1 in combination for flow cytometry increases the purity of the isolated ENP population.

NCSCs are also identified by their abilities to form neurospheres in non-adherent cultures and self renew. To establish that p75^+/HNK-1^+ fetal gut cells isolated by flow cytometry possess these capabilities, we plated cells from wild-type 14.5 dpc embryos at a density of 1000 cells per well in non-adherent plates. After 7 days in culture, neurospheres were counted, dissociated and replated at a density of 250 cells per well. The dissociated cells formed readily visible secondary neurospheres after 7 days of culture. (Supplementary

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**Table 1. Effect of strain background and Sox10Dom^+ mutation on ENP migration**

<table>
<thead>
<tr>
<th>Genotypic class</th>
<th>Density of progenitor cells along gut</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>B6.Sox10^+/+</td>
<td>3.9</td>
</tr>
<tr>
<td>Average</td>
<td>2.4</td>
</tr>
<tr>
<td>B6.Sox10^Dom+/+</td>
<td>4</td>
</tr>
<tr>
<td>Average</td>
<td>2.9</td>
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Density of Phox2B^+ cells was quantified on a four-point scale (1 = least dense, 4 = most dense) in eight equal-sized gut sections. Scores for individual guts and averages are shown (n ≥ 6). The following coloring system was applied: 0.0–0.4, white; 0.5–1.4, light gray; 1.5–2.4, medium gray; 2.5–3.4, dark gray; 3.5–4.0, black.
Material, Fig. S1). Thus, p75+/HNK-1+ cells in mouse fetal gut express markers of enteric NC-derived progenitors and exhibit both multipotency as well as self-renewal capacity.

**Effect of the Sox10Dom mutation and strain background on developmental potential of ENPs**

The ability to prospectively isolate ENPs provides the opportunity to assess the effect of genetic mutations and strain background on the multipotency, lineage potential and growth factor responsiveness of a defined population (47,51,78,79). Developmental potential can be assessed in vitro by prospectively isolating distinct populations of NCSCs and culturing these progenitors at low density. Under such conditions, individual cells form spatially distinct adherent colonies. The differentiation capacities of individual cells in vivo are reflected by the phenotypes of cells generated within each colony in vitro (40,41).

The effect of genetic background on the developmental potential of the progenitors that give rise to the ENS is unknown but is a crucial question as alterations in cell responsiveness and capacity to generate distinct lineages could alter the composition of enteric ganglia and potentially impact postnatal gastrointestinal function.

To investigate the effect of the Sox10Dom/+ mutant genotype on ENPs in the B6 and C3Fe congenic lines, we isolated ENPs from fetal guts of 12.5 dpc Sox10Dom/+ and Sox10+/+ embryos by flow cytometry. Representative flow profiles of dissociated fetal gut populations stained for p75 and HNK-1 are shown in Figure 4. For Sox10+/+ guts, approximately 1.5–2% of total cells stained brightly for p75/HNK-1, consistent with percentages of NCSCs reported by Bixby et al. (40). This fraction was collected for analysis of developmental potential in vitro. In contrast, Sox10Dom/+ littermate guts examined in parallel under identical gating conditions exhibited a consistent reduction in the percentage of ENPs present (0.4–0.8% of total cells), regardless of the strain background.

The fraction of fetal gut cells that are p75+/HNK-1+ differs significantly between wild-type and Sox10Dom/+ embryos (P < 0.005). This observation is consistent with the reduced numbers of Phox2B+ cells observed in Sox10Dom fetal guts by confocal microscopy.

To determine the effect of strain background and Sox10 genotype on the developmental potential of ENPs in vitro, we examined the phenotypes of colonies grown in adherent cultures at low density. We plated equal numbers of p75+/HNK-1+ progenitors for each genotype and strain background (500 cells per well, 52 cells/cm²). Colony phenotypes were examined by immunostaining for neuronal (peripherin), glial (glial fibrillary acidic protein (GFAP) and myofibroblast [smooth muscle actin (SMA)] lineage markers. ENP-derived colonies were either multipotent (containing all three cell types within a single colony; Fig. 5A and B) or restricted (containing only one or two cell types within a single colony; Fig. 5C and D). Multiple colony types were produced from both Sox10Dom/+ and Sox10+/+ ENPs, regardless of strain background (Table 2).

While all categories of ENPs were able to produce both restricted and multipotent colonies, we observed significant differences in the proportions of lineages produced by wild-type progenitors from the two strain backgrounds. B6 Sox10+/+ ENPs produced more glial derivatives, with 0.8% glial and 3.3% glial + myofibroblast colonies, when compared with C3Fe Sox10+/+ ENPs, which produced no glial colonies and 0.7% glial + myofibroblast colonies (Table 2). B6 Sox10+/+ ENPs produced significantly fewer neuronal derivatives, with 2.4% neuronal and 10.8% neuronal + myofibroblast colonies, compared with 10.5% neuronal and 24.8% neuronal + myofibroblast colonies from C3Fe Sox10+/+ ENPs. The frequencies of multipotent colonies, which comprised the largest proportion of total colonies in all cases, were also significantly different. The highest percentage of multipotent (N + G + M) colonies was observed in B6 Sox10+/+ cultures (63.7%), compared with 48.4% in C3Fe Sox10+/+ cultures. These results indicate that, even under ‘wild-type’ conditions, genetic background can substantially shift the developmental potential of ENPs in wild-type embryos.

The presence of the Sox10Dom mutation changed the developmental potential of ENPs and altered the ratios of colony types that were formed in vitro. This effect is more pronounced in progenitors isolated from B6 Sox10Dom/+ embryos. Four different categories of colony types were significantly shifted away from wild-type proportions in the B6 background: neuronal (5.3% Dom versus 2.4% wild-type), glial (0.3% Dom versus 0.8% wild-type), myofibroblast (8.1% Dom versus 8.8% wild-type) and glial + myofibroblast (0.7% Dom versus 3.3% wild-type) (Table 2). In contrast, only two classes of colonies from C3Fe Sox10Dom/+ ENPs were significantly shifted away from proportions produced by wild-type embryos: neuronal + glial (6.9% Dom versus 9% wild-type) and glial + myofibroblast (1.1% Dom versus 0.7% wild-type). These results indicate that the Sox10Dom allele alters the developmental potential of ENPs at the time that these cells are migrating within the gut. The greater number of colony classes shifted in the B6 strain reveals that developmental potential is a process impacted by the genetic background.
Effect of the Sox10Dom mutation and strain background on in vivo neurogenesis in the developing fetal gut

Our analysis of lineage potential in vitro demonstrated that the Sox10Dom allele has a marked impact on the proportions of cell types that derive from ENPs. In particular, greater numbers of neuronal-restricted progenitors occur in Sox10Dom/+ mutants, particularly those on the B6 genetic background. Previous studies have shown that the presence of immature neurons is increased in Sox10LacZ/+ mutants at 15.5 dpc, but this analysis did not take genetic background into account (54). An increase in neurons at this early developmental stage would have a patent effect on the functional ability of the fully-formed ENS.

To determine whether the proportion of neurons in the developing fetal gut was altered in Sox10Dom/+ mutants in vivo, we used immunohistochemical detection of Phox2B, a known marker of all early ENPs (55), and Hu, an early marker of differentiating neurons (80) in fetal gastrointestinal tracts from 13.5 dpc Sox10Dom/+ and Sox10+/+ embryos. The most distal 700 μm of the ENP-populated gut was identified, and the ratio of Phox2B+Hu+ cells to total Phox2B+ cells was calculated to determine the proportion of neurons in the developing fetal gut (Fig. 6A).

Consistent with our in vitro analyses, the proportion of Phox2B+Hu+ neurons was highest in the C3Fe,Sox10Dom/+ fetal gut. This percentage was approximately 1.6-fold higher than that found in C3Fe,Sox10+/+ gastrointestinal tracts (18.2 versus 11.6%), similar to the in vitro results (Fig. 6B). Although the analyses of developmental potential were highly significant in vitro between wild-type and B6,Sox10Dom/+ mutants, the proportion of neurons was more similar between the B6,Sox10+/+ and B6,Sox10Dom/+ fetal gut samples in vivo at this stage of development (14.0 and 16.9%, respectively; approximately 1.2-fold difference). None of the ratios of
Phox2B\(^+\)/Hu\(^+\) cells, either between mutants and wild-type littermates or between strains, proved statistically significant. The lack of significant differences could be due to the highly variable nature of the ENP defects combined with the relatively small number of animals examined in this study (6–12 in each class). Alternatively, significant differences may not have been seen due to the short time-frame between the harvest of p75\(^+\)/HNK-1\(^+\) ENPs at 12.5 dpc for the analysis of developmental potential and the double-label immunohistochemistry for Phox2B\(^+\)/Hu\(^+\) ENPs at 13.5 dpc. The 24 h developmental window \textit{in vivo} may not have been a sufficient time period for differentiation, whereas the \textit{in vitro} cultures for analysis of lineage divergence are scored for cell types after 2 weeks. Interestingly, the variation was greater in the Sox10Dom\(^+\) embryos, mirroring the wide spread of aganglionic severity seen in postnatal animals.

We postulated that the increase in neurons documented in B6.Sox10\(^{Dom/+}\) ENP colonies might be more visible in the foregut, which is completely populated at 13.5 dpc and further along in the differentiation process. In wild-type fetal gut from both the B6 and C3Fe strains, the density of cells in the proximal region was so great that quantitation was not practical (Fig. 6C and D). In contrast, the majority of gastrointestinal tracts from Sox10\(^{Dom/+}\) embryos from both strains had decreased numbers of progenitor cells in the foregut (Fig. 6E and I). Moreover, a subset of B6.Sox10\(^{Dom/+}\) and C3Fe.Sox10\(^{Dom/+}\) gastrointestinal tracts also exhibited abnormal patterning of the ganglion network (Fig. 6F–I). The patterning defects in the proximal foregut were not correlated with an increase in the proportion of neurons found in the distal gut; samples that had the greatest proportions of Phox2B\(^+\)/Hu\(^+\) ENPs at the migratory wave-front did not exhibit more apparent abnormalities in the proximal fetal gut. No defects of patterning in the fetal foregut were detected in any of the wild-type samples of either strain.

**Effect of the Sox10\(^{Dom}\) mutation and strain background on postnatal ganglion patterning**

The abnormal cell density, distribution and differentiation seen in 13.5 dpc Sox10\(^{Dom/+}\) foreguts and the possibility that deficient migration in the hindgut might lead to an overabundance of ganglia in the foregut led us to investigate patterning of proximal foregut ganglion in postnatal Sox10\(^{Dom/+}\) mutants. Previous studies of the Sox10\(^{Dom}\) allele have utilized acetylcholinesterase staining to visualize the ganglion network (34). However, this method only labels cholinergic neurons and does not visualize all neurons or ancillary support cells. Examination of the enteric plexi separate from the gut mucosa as gut muscle strips (GMS) allows better visualization of the ganglion network of the small intestine. Abnormal patterning of myenteric plexi in the proximal small intestine would be consistent with the concept that deficiencies in lineage divergence contribute to abnormalities of enteric ganglia development.

To examine the density and patterning of enteric ganglia in the proximal small intestine, GMS consisting of both the outer longitudinal and inner circular muscle layers were peeled...
away as an intact sheet from the proximal foregut of both postnatal (P15–P17) Sox10Dom/+ animals and wild-type littermates immediately below the gastric sphincter. These strips were divided into three sections to facilitate immunostaining with Phox2B antibody and imaged to view enteric ganglia (Fig. 7A). GMS from wild-type, Sox10+/+, animals exhibited large, round, brightly Phox2B+ nuclei of neuronal cells forming chain-like networks interspersed with smaller, spindle-shaped, faintly Phox2B+ enteric glia (Fig. 7A). Images from adjoining sections of representative animals from each strain and genotype class are provided in Supplementary Material, Figure S3. Regions exhibiting

Figure 5. Effect of strain background and Sox10Dom mutation on developmental potential of in vitro cultures of flow-sorted ENPs. Multiple colony types from cultured p75+/HNK-1+ ENPs from 12.5 dpc guts from Sox10Dom embryos and wild-type littermates are visualized by immuno-staining for GFAP (glia, yellow), SMA (myofibroblasts, green), peripherin (neurons, red) and DAPI (blue). (A) A multipotent colony displays staining for all three cell lineage markers within a single colony (×100 magnification). (B) Higher magnification (×200) images of cell types are shown from the regions indicated by the stipple boxes in (A). (C and D) Restricted colonies display only a subset of cell-type markers within a single colony (neuronal/gliaal and neuronal/myofibroblast, respectively) (×100 magnification).
normal latticework ganglion patterns were also present in
Sox10Dom/+ animals, and we did not identify any areas with
increased density of enteric ganglia in the Sox10Dom/+ mutants
(Fig. 7A–C). However, we did observe decreased density of
enteric ganglia in the Sox10Dom/+ mutants. In addition,
Phox2B staining identified large cells exhibiting cytoplasmic
Phox2B immunoreactivity and myofibroblast-like morphology
within and around the enteric ganglia. These myofibroblast-
like cells were present in all GMS of all genotypes, even the
Sox10Dom/+ mutants (Fig. 7A–C). However, we did observe
the increased density of enteric ganglia in the mutants
between HSCR patients are unknown. We have previously
shown to recapitulate Phox2B expression (72). Analysis
of Tg SoxB2+/− H2B-CFP mice that model multiple aspects of HSCR. The effects of the Sox10Dom mutation and genetic background on migration, proliferation and developmental potential were investigated. We found that genetic background had an effect on ENP migration in the presence of the Sox10Dom mutation. The Sox10Dom mutation also caused a decrease in the frequency of ENPs, regardless of the strain background. Our analysis further identified a previously unrecognized effect of the Sox10Dom mutation on developmental potential, the balance of lineages normally generated by ENPs. Genetic background exacerbated the shift in developmental potential produced by the Sox10Dom mutation. Moreover, we found that genetic background alone alters the proportions of cell types that derive from ENPs in wild-type animals, independent of the Sox10Dom genotype. In vivo analysis of enteric neural patterning in fetal and postnatal proximal intestine revealed that deficiency of Sox10 disrupts ENPs and enteric ganglia, respectively. Thus, our analysis has identified a novel pathological mechanism that likely contributes to abnormalities of enteric ganglia and may explain some of the variation in aganglionosis and post-surgical outcomes between HSCR patients.

Delayed migration of ENPs down the intestine is a consistent feature present in multiple HSCR mouse models, including Sox10Dom (19,39), Ednrb2−/− (46) and Edn3−/− (48,50). Kapur

Table 2. Effect of strain background and Sox10Dom+ mutation on developmental potential

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total colonies</th>
<th>Percentage of cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>N + G</td>
</tr>
<tr>
<td>B6.Sox10Dom/+</td>
<td>25.3 ± 7.2</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>B6.Sox10Dom+/−</td>
<td>23.9 ± 5.8</td>
<td>9.2 ± 7.3</td>
</tr>
<tr>
<td>C3Fe.Sox10Dom/+</td>
<td>24.1 ± 3.1</td>
<td>9.0 ± 7.8</td>
</tr>
<tr>
<td>C3Fe.Sox10Dom+</td>
<td>33.7 ± 13.9</td>
<td>6.9 ± 6.3</td>
</tr>
</tbody>
</table>

Percentages of colony types (N, neuronal; G, glial; M, myofibroblasts) relative to total number of colonies per well. Values shown are average ± SD (n ≥ 10). B6.Sox10Dom+ and C3Fe.Sox10Dom+ were compared with B6.Sox10Dom−/+ [∗ very significant (P < 0.05), † very significant (P < 0.01), ‡ very significant (P < 0.001)]. C3Fe.Sox10Dom+ was compared with C3Fe.Sox10Dom−/+ [very significant (P < 0.01), highly significant (P < 0.001)].

DISCUSSION

The mechanisms responsible for variation of aganglionosis between HSCR patients are unknown. We have previously reported that genetic background contributes to differences in postnatal aganglionosis (34). To identify developmental processes that underlie differences in aganglionosis between inbred strains, we utilized two congenic lines of Sox10Dom+/− mice that model multiple aspects of HSCR. The effects of the Sox10Dom mutation and genetic background on migration, proliferation and developmental potential were investigated. We found that genetic background had an effect on ENP migration in the presence of the Sox10Dom mutation. The Sox10Dom mutation also caused a decrease in the frequency of ENPs, regardless of the strain background. Our analysis further identified a previously unrecognized effect of the Sox10Dom mutation on developmental potential, the balance of lineages normally generated by ENPs. Genetic background exacerbated the shift in developmental potential produced by the Sox10Dom mutation. Moreover, we found that genetic background alone alters the proportions of cell types that derive from ENPs in wild-type animals, independent of the Sox10Dom genotype. In vivo analysis of enteric neural patterning in fetal and postnatal proximal intestine revealed that deficiency of Sox10 disrupts ENPs and enteric ganglia, respectively. Thus, our analysis has identified a novel pathological mechanism that likely contributes to abnormalities of enteric ganglia and may explain some of the variation in aganglionosis and post-surgical outcomes between HSCR patients.
et al. (39) detailed the lack of complete bowel colonization in *Sox10<sup>Dom</sup>/+* mutants utilizing the DBH-LacZ transgenic allele, assaying both fetal and postnatal time points. However, this study used mice on a mixed B6–C3Fe background and was not able to assess the impact of genetic background on migration. We observed a marked difference in numbers and extent of migration of ENPs in *Sox10<sup>Dom</sup>/+* embryos on two different strain backgrounds. B6.<sup>Sox10<sup>Dom</sup>/+</sup> embryos had a more pronounced deficit than those on the C3Fe background. Thus, fetal ENP migration correlates closely with the significant differences in aganglionosis seen postnatally in these two congenic strains. Wild-type embryos were comparable between the two strains; therefore, strain background alone had no effect on ENP migration. It is only in the presence

Figure 6. In vivo neurogenesis in *Sox10<sup>Dom</sup>* embryos. (A) Gastrointestinal tracts from 13.5 dpc *Sox10<sup>+/+</sup>* and *Sox10<sup>Dom</sup>/+* embryos were immunostained for Phox2B (green) and Hu (red) to visualize developing neurons at the distal wave-front of migration (×400 magnification). (B) The proportion of double-positive (Phox2B<sup>+</sup> Hu<sup>+</sup>/Phox2B<sup>+</sup>) cells was determined in the most distal 700 µm portion of the gut. The foregut region in these samples was also examined for cell staining and distribution (×400 magnification). *Sox10<sup>+/+</sup>* guts from the B6 (C) and C3Fe (D) backgrounds display normal dense population of the foregut. *Sox10<sup>Dom</sup>/+* guts presented with decreased ENP density alone (E) or decreased density accompanied by abnormal patterning (F–H). (I) The phenotype of foregut ENP staining was characterized in *Sox10<sup>Dom</sup>/+* guts.
of the Sox10\textsuperscript{Dom} mutation that genetic modifiers in the B6 genetic background exert an effect on ENP migration; these are likely one or more of the Sox10\textsuperscript{Dom} modifier loci that have previously been mapped between these strains (45).

Multiple theories could be posited for the defect in migration seen in Sox10\textsuperscript{Dom}/+ guts. The lack of complete population could be attributed to cell death in the progenitor population. While apoptosis has been demonstrated in early migrating NC derivatives just outside the neural tube in Sox10\textsuperscript{Dom} mutants (32), efforts investigating cell death within the intestinal wall of Sox10\textsuperscript{Dom}/+ mutants have definitively shown no increase in apoptosis when compared with wild-type littermates (54). Another possibility is a ‘blockade’ against migration that holds progenitors in the foregut and prevents further progression down the developing gut. However, we did not observe increased density of progenitors in the foregut of Sox10\textsuperscript{Dom}/+ mutants, regardless of age (12.5 dpc, Fig. 1; 13.5 dpc, Fig. 6E–H; P15–P17, Fig. 7B–G).

Current models of ENS development posit that ENPs at the migratory wave-front possess an increased capacity for proliferation and drive the extension of the cell population down the gut (84). However, our results show equal rates of proliferation across different regions of the gut. Previous work by Okamura and Saga (85) suggested that wild-type ENPs in the stomach and intestine had equal proliferative capacity. Young et al. (86) assayed proliferation in BALB/c

![Ganglion patterning in Sox10\textsuperscript{Dom} mutants. (A) Gut muscle layers were stripped intact from proximal foreguts, divided into three sections and immunostained for Phox2B. Sox10\textsuperscript{10+} ganglia present as chain-like arrangements of neurons with interspersed glia in all sections (×200 magnification). (B) Similar ganglia can be found in Sox10\textsuperscript{Dom/+} GMS (×200 and ×400 magnifications designated by stippled outline; neurons identified by arrow, glia identified by open arrowhead). Myofibroblast-like cells (identified by closed arrowhead) labeled by Phox2B cytoplasmic staining were also visible in both wild-type and mutant GMS (C). The presence of myofibroblast-like cells is increased in B6.Sox10\textsuperscript{Dom/+} animals, usually in conjunction with neurons at the expense of glia (D and E). Severely affected B6.Sox10\textsuperscript{Dom/+} animals have additional patterning defects including abnormal morphology of neurons (F) and the presence of only myofibroblast-like cells (G).](https://academic.oup.com/hmg/article-abstract/19/22/4353/2527055)
embryonic guts and also detected no regional differences in proliferation in a wild-type situation. Our studies examined multiple regions throughout the fetal intestine including the foregut and found no regional or strain-specific difference in ENP proliferation in wild-type animals, nor did we detect any significant differences in ENP proliferative capacity between $Sox10^{+/+}$ and $Sox10^{Dom/+}$ embryos at 12.5 dpc. Other HSCR mouse models have similarly documented a deficit in migration without any deficits in proliferation of ENPs (87). Even when we specifically assayed proliferation of wave-front ENPs in the distal hindgut, no effect of the $Sox10^{Dom}$ allele on proliferation was detected. While proliferation is obviously essential to ENS ontogeny and may contribute to HSCR pathophysiology in some cases (57,85,88), it did not differ between $Sox10^{Dom}$ mutant and wild-type embryos and thus does not contribute to the etiology of aganglionosis in this particular HSCR model.

The ability to isolate viable ENPs for further study is vital to truly understanding the processes involved in ENS ontogeny. Surface p75 expression has been used routinely for enrichment of ENPs in mouse and rat (74–76). HNK-1, a marker of migrating NC, has also been used to this end in the chick and the rat (60,89–92). Isolation of pure enteric NCSCs in the rat has been achieved by dual-labeling of p75 with other markers including P0 and α4-integrin (40,41,47,69,70). This process has been hindered in the mouse due to a perceived lack of cell surface markers that could be utilized for flow cytometry. Use of HNK-1 immunoreactivity to capture mouse enteric NCSCs has not been attempted because sectional analysis on fixed tissue was not successful (61). To eliminate any complications due to fixation or the physical size of the HNK-1 IgM monoclonal, we sought to immunostain freshly dissociated live cells, thus circumventing problems of permeability. Using this methodology, we demonstrated that murine ENPs do in fact express HNK-1, thus allowing isolation by flow cytometric methods used in other species. Moreover, we demonstrate that double-labeling of mouse ENPs with p75 and HNK-1 identifies a population of highly enriched enteric stem cells. To conclusively demonstrate the progenitor characteristics of p75/HNK-1+ enteric cells, we confirmed that these cells could form neurospheres, were able to self-renew and expressed NC markers. Ready growth of primary and secondary neurospheres from cells isolated by p75/HNK-1 staining confirms that this cell population is made up of progenitors. Moreover, the significant enrichment of cells expressing NC markers isolated by flow cytometry for p75/HNK-1 staining, especially compared with populations isolated using a single marker, indicates that this cell population contains specifically NC-derived progenitors. These qualities demonstrate the purity and utility of the p75+/HNK-1+ population for subsequent studies of enteric development.

Several studies have examined the frequency of enteric NC-derived progenitors in murine HSCR models. Decreased numbers of ENPs, ranging up to 60% of the total progenitor population, have been described in NC mutants (47,54,85). Our flow cytometric studies similarly identified a 65% reduction, on average, in numbers of p75+/HNK-1+ cells in $Sox10^{Dom/+}$ fetal gut. This decrease in progenitors was not influenced by genetic background as the reduction in ENPs did not differ between B6 and C3Fe strains. Despite the reduction in frequency of p75+/HNK-1+ cells in $Sox10^{Dom/+}$ mutants, we did not see any difference in the proportion of multipotent colonies between $Sox10^{Dom/+}$ and $Sox10^{+/+}$ ENPs. The proportions of multipotent colonies were also maintained in EdnrBSL mutant progenitors (47). Thus, while there is a substantial reduction in the number of p75+/HNK-1+ cells in $Sox10^{Dom/+}$ mutant guts, the proportion of total progenitors that are multipotent remains the same. This raises an interesting question: what happens to the rest of the progenitor population in vivo? An obvious explanation for the decrease in p75+/HNK-1+ progenitors and the final fates of the missing progenitors in $Sox10^{Dom}$ mutants is not apparent. Future studies using Cre-driven lineage tracing methods are needed to determine the fate of these cells in the gut.

Lineage divergence as a pathological mechanism has not been previously investigated in HSCR models. The majority of studies investigating ENS defects have focused strictly on hindgut aganglionosis and evaluated cell survival (93,94), or migration and proliferation, as detailed above. Those that have looked at lineage segregation have focused on strictly neuronal cell fates (54,85). The concurrent study of multiple lineages provides a complete picture of the cell fate capacity exhibited by ENPs. Our in vitro studies of developmental potential demonstrate that the responsiveness of the p75+/HNK-1+ populations differs between strains and genotypes and, as a result, their ability to give rise to various lineages within the culture environment must also differ. Moreover, our in vivo analysis of enteric neurogenesis and postnatal ganglia is consistent with our in vitro developmental potential studies.

Our analysis of early phases of lineage segregation revealed marked differences in developmental potential, the proportions of differentiated lineages that arose in culture, between wild-type and $Sox10^{Dom/+}$ ENPs. Specifically, B6,$Sox10^{Dom/+}$ ENPs increase the proportion of N-only colonies at the expense of other cell types. Paratore et al. observed an analogous increase in neuronal derivatives within dorsal root ganglia cultures from $Sox10^{LacZ/+}$ mutants (30) and noted an increase in neuronal precursors in mid-gut sections from $Sox10^{LacZ/+}$ embryos (54). Our in vitro analysis of neurogenesis, performed earlier in development, identifies the onset of differential neuronal development between wild-type and $Sox10^{Dom/+}$ ENPs. The consistency between our findings and those of Paratore et al. (30,54) strengthen the concept that differences in lineage potential detected in vitro reflect differences of lineage segregation in vivo.

An increase in neurons in vivo could account for the decrease in cell density and migration seen in $Sox10^{Dom}$ embryos by whole-mount immuno-staining. Premature differentiation of neurons could contribute to a decrease in migration (95,96). Studies by Hao et al. (95) demonstrated that immature TH+ neurons migrate at approximately 30–40% of the speed at which ENPs migrate. Wu et al. (96) also concluded that differentiating neurons have impaired migration in their studies on the effect of endothelin 3 on ENPs. The absence of proliferation among differentiated neurons (97) further limits the ability of ENPs to completely populate the intestine because a minimum density of enteric...
cells is required to provide the necessary cues for chain-based migration (84,98,99). Thus, the decreased rate of migration and lack of proliferation in committed neuroblasts may both contribute to aganglionosis. It is possible that the Sox10<sup>Dom</sup> allele has a distinct effect on migration alone, separate from the migration defect caused by the primary effect on lineage divergence. However, it is not possible to uncouple these effects at this time. A recently reported conditional allele of Sox10 will likely facilitate such studies in the future (100).

Most notably, our studies demonstrate a distinct and novel effect of genetic background on developmental potential independent of any mutation. We observed highly significant differences in enteric lineages produced by wild-type ENPs of the B6 and C3Fe strains, specifically an increase in neuronal lineages in the C3Fe strain. Because wild-type B6 and C3Fe mice have functional ENSs, the differences in developmental capacity and responsiveness between these two strains were unexpected. Our studies suggest that the ENS, similar to other biological systems, has a certain degree of plasticity in its composition. While neurons and glia must be present in the ganglia that comprise the ENS, the relative proportions of the two cell types appear to be flexible, at least at this developmental stage. It is only in the presence of a disease-causing mutation, such as the Sox10<sup>Dom</sup> allele, that these inherent differences modulate the threshold at which disease pathogenesis occurs. The fact that fewer lineage shifts occur in C3Fe,Sox10<sup>Dom</sup> ENPs demonstrates that this genetic background provides a greater buffer to absorb disruptions in lineage divergence caused by mutations. This effect is consistent with the protective effect of the C3Fe genetic background (34). The molecular mechanisms behind this substantial difference in lineage segregation have not yet been identified. This overall effect of genetic background is most likely the cumulative result of multiple genes that participate in enteric NC development, as suggested by the identification of multiple aganglionosis modifiers between the B6 and C3Fe inbred strains (45).

In vivo imaging of neurogenesis based on detection of Phox2B<sup>+</sup>/Hu<sup>+</sup> ENPs identified abnormal patterning in the proximal foregut of Sox10<sup>Dom/+</sup> fetal intestine. This atypical patterning ranged from depletion of neuroblasts, seen in C3Fe,Sox10<sup>Dom</sup> samples, to reduced ENP numbers and aberrant distributions in the gut wall, including both punctate, disconnected cell bodies and sparse, chain-like ganglion arrangements in B6,Sox10<sup>Dom/+</sup> mutants. The reduction of progenitor cells in the proximal fetal gut of both strains demonstrates that the Sox10<sup>Dom</sup> allele does not simply delay migration in a manner that causes accumulation of ENPs in the small intestine. Rather, our findings implicate the process of lineage divergence, both at the wave-front of migration and in patterning the distribution of ENPs in the proximal foregut.

We extended our in vivo analysis to examine the distribution of enteric ganglia in the proximal small intestine of postnatal Sox10<sup>Dom/+</sup> mutants. We did not observe any increase in the density of enteric ganglia in the mutants when compared with wild-type littermates. However, we did observe decreased density of enteric ganglia, as well as aberrant ganglia composition in the Sox10<sup>Dom</sup> heterozygotes. We have previously reported reduced density and altered patterning of enteric ganglia in the proximal foregut of EdnrB<sup>+</sup> samples, to reduced ENP numbers and aberrant distributions in the gut wall, including both punctate, disconnected cell bodies and sparse, chain-like ganglion arrangements in B6,Sox10<sup>Dom/+</sup> mutants. The reduction of progenitor cells in the proximal fetal gut of both strains demonstrates that the Sox10<sup>Dom</sup> allele does not simply delay migration in a manner that causes accumulation of ENPs in the small intestine. Rather, our findings implicate the process of lineage divergence, both at the wave-front of migration and in patterning the distribution of ENPs in the proximal foregut.

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suggest abnormalities that impact initial development of ENPs may also affect the function of enteric ganglia that are formed in more proximal gut regions. Our studies in congenic mouse strains reveal that a large amount of natural variation exists in the population of ENPs that will eventually form the ganglia of the ENS. We hypothesize that these different progenitors may be able to give rise to functional ganglia in a stress-free environment, but the addition of a disease-causing mutation renders them less able to generate a normal repertoire. The level at which they are still able to function obviously covers a wide spectrum, hence the variation in post-operative outcomes. Our findings suggest that new methods to examine the functionality of residual proximal enteric ganglia are needed to improve patient outcomes after surgery.

In conclusion, we have shown that genetic background plays a key role in determining the final impact of disease-causing mutations, such as Sox10DelDom/+. The Sox10DelDom allele itself impacts migration, frequency and developmental potential of the ENPs that go on to form the ENS. However, only migration and developmental potential are substantially modulated by genetic background. We also document significant natural variation in developmental potential between ‘wild-type’ strains of mice that appear to account for differences in penetrance and severity of aganglionosis when disease-causing mutations are introduced. The disruption of lineage divergence is a novel pathogenic mechanism for the development of aganglionosis and may explain the differential outcomes of HSCR patients post-resection. Studies such as these that explore both disease-causing mutations and the contribution of genetic background are vital to understanding complex diseases such as HSCR.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. B6.Sox10DelDom and C3Fe.Sox10DelDom congenic lines were established and maintained by backcrosses to C57BL/6J and C3HeB/FeJ stocks, respectively. Both strains have been bred onto their respective inbred strains for >40 generations.

Fetal mouse dissections

Timed matings of congenic Sox10DelDom males and wild-type females were set up to obtain staged mouse fetuses, designating the morning of plug formation as 0.5 dpc. Fetal gastrointestinal tracts (stomach to anus) were sub-dissected for analysis.

Genotyping

Genomic DNA was isolated from limb buds by proteinase K digestion as previously described (34). Genotype at the Sox10 locus was determined using PCR-based methodology by established methods (32). For rapid genotyping, limb buds were digested in Fast Lysis Buffer (0.45% Tween, 2.5 mM MgCl2, 0.1 M KCl, 10 mM Tris pH 8.3) with proteinase K at 90°C for 12 min. Genotype at the Sox10 locus was determined using Real-Time Quantitative PCR (Applied Biosystems). In brief, each reaction composed of 1X TaqMan Fast Universal PCR Master Mix (No AmpErase UNG), 1X TaqMan Sox10Del SNP Genotyping Assay Mix, 10% 1X Betaine and 20% fast-prepped DNA was run in triplicate on the Fast Amplification Protocol (95°C for 20 s; 95°C for 1 s then 60°C for 20 s, 40 cycles) on a 7900HT Fast Real-Time PCR System (ABI). The Sox10Del SNP Genotyping assay was designed with the following specifications: Sox10Del F Primer, TCGGCGCGGAAGAAC; Sox10Del R Primer, GCAGACGCCCTCTTCTT; Sox10Del VIC (Sox10+/+) Probe, TCGGCTTCCCCCGC; and Sox10Del FAM (Sox10Del/+) Probe, CTGGCTTCCCCGCCC.

Analysis of migration

Fetal guts at 12.5 dpc were dissected, and immunohistochemistry was performed as previously described (107). ENPs were stained using polyclonal rabbit anti-mouse Phox2B antibody (108) at 1:750 and detected by donkey anti-rabbit Cy3-conjugated secondary antibody, while the cecum and hindgut, with the cecum designated as section 4. The density of Phox2B+ cells was qualitatively scored on a whole-number four-point scale, with zero being a lack of Phox2B+ cells and four being numerous Phox2B+ cells, for each individual section. Scoring was completed in a strain- and genotype-blinded fashion (109). Scores for each individual section of the gut were averaged across strain- and genotype-matched embryos, with seven to nine embryos from two separate litters per genotypic class.

Analysis of proliferation

Fetal guts at 12.5 dpc were dissected and then partitioned into segments comprised of the foregut (mid-small intestine, approximately equal in size to the cecum), cecal and hindgut (entire) regions. Individual gut segments were dissociated into a single cell suspension as previously described (40). Dissociated cells were plated in Opti-MEM medium (Gibco) at a density of ~1/4 gut segment per well in a 24-well tissue culture plate coated with poly-d-lysine and fibronectin as previously described (40,88). Plates were incubated at 37°C for 1 h to allow cells to adhere to the plate surface and then fixed on ice in neutral-buffered formalin (NBF) with Triton X-100. Cells were immunostained with antibodies to polyclonal rabbit anti-mouse Sox10 (110) (1:100) to identify all ENPs and monoclonal mouse anti-mouse phospho-Histone H3 (phH3, Abcam, 1:2000) to identify proliferating cells. Primary immunoreagents were detected with donkey anti-mouse Cy2- and donkey anti-rabbit Cy3-conjugated secondary antibodies (Jackson Immuno Research, 1:1000). Fetal guts were flat-mounted in Aqua Poly/Mount (PolySciences, Inc.) and cover-slipped for imaging. Confocal microscopy was performed on a Zeiss Scanning Microscope LSM510 at ×200 and ×400 magnifications to visualize ENPs expressing endogenous Phox2B in fetal guts. Tiled confocal images of whole-mount fetal guts were divided into eight equal sections encompassing the mid-small intestine, cecum and hindgut, with the cecum designated as section 4. The density of Phox2B+ cells was qualitatively scored on a whole-number four-point scale, with zero being a lack of Phox2B+ cells and four being numerous Phox2B+ cells, for each individual section. Scoring was completed in a strain- and genotype-blinded fashion (109). Scores for each individual section of the gut were averaged across strain- and genotype-matched embryos, with seven to nine embryos from two separate litters per genotypic class.
double-positive (Sox10+/pH3+) cells relative to the total of all Sox10+ cells was calculated to determine the proliferative frequency of ENPs in each gut region. Ratios were averaged across strain- and genotype-matched embryos, with 6–10 embryos per class. Averages and standard deviations were compared by Welch’s t-test and are shown in Figure 2.

Identification of HNK-1 immunoreactivity on mouse ENPs

Fetal guts from 14.5 dpc C3Fe-Tgphox2B−/422BCFP mice were dissected, and transgenic embryos were identified by CFP fluorescence. Transgenic (CFP+) and non-transgenic (CFP−) fetal guts were dissected, pooled and dissociated to generate single cell suspensions. Viability dye (7-AAD, Molecular Probes, 1:1000) was added to cell suspensions prior to FACS isolation to exclude dead cells. Cell sorts and analyses were performed on a BD FACSAria flow cytometer using a 100 micron nozzle. CFP− and CFP+ cells were directly sorted into self-renewal medium within 24-well tissue culture plates coated with poly-d-lysine and fibronectin as described at a density of 150 cells per well (40). After incubating briefly at 37°C to allow cells to adhere to plate well surfaces, cells were fixed on ice in NBF with Triton X-100. Cells were immunostained with antibodies to polyclonal rabbit anti-mouse p75 (Chemicon, 1:2000) or monoclonal mouse anti-human HNK-1 (BD Pharmingen, 1:400; cross-reactive with murine protein). Primary immunoreagents were detected with donkey anti-rabbit Cy3- or donkey anti-mouse Cy3-conjugated secondary antibodies (Jackson Immuno Research, 1:1000) and DAPI. Cells were imaged using an Olympus IX71 inverted microscope.

Flow-cytometric isolation of p75+/HNK-1+ ENPs

Fetal guts at 12.5 and 14.5 dpc were dissected and dissociated. ENPs were labeled in solution using polyclonal rabbit anti-mouse p75 (Chemicon, 1:2000) and/or monoclonal mouse anti-human HNK-1 primary antibodies (BD Pharmingen, 1:400; cross-reactive with murine protein). Secondary fluororescent antibodies (goat anti-rabbit FITC, Jackson Immuno Research, 1:400; goat anti-mouse PE, Jackson Immuno Research, 1:400) were used to detect primary immunoreagents. Viability dye (7-AAD, Molecular Probes, 1:1000) was added to cell suspensions prior to FACS isolation to exclude dead cells. Cell sorts and analyses were performed on a BD FACSARia flow cytometer using a 100 micron nozzle. p75+/HNK-1+ cell measurements were determined by employing a series of gates to ensure this population only included single viable cells with high levels of marker staining (Supplemental Material, Fig. S3).

Immunohistochemical labeling of p75+/HNK-1+ progenitors

p75+/HNK-1+ cells were directly flow-sorted into self-renewal medium within 24-well tissue culture plates coated with poly-d-lysine and fibronectin as described at a density of 150 cells per well (40). Equivalent numbers of unsorted, p75+, HNK-1+ and p75+/HNK-1− cells were distributed into similar plates to serve as controls. After incubating briefly as above at 37°C to allow cells to adhere to the plate well surface, cells were fixed on ice in NBF with Triton X-100. Immunostaining with antibodies for progenitor markers including polyclonal rabbit anti-mouse Sox10 (Generously provided by R. Hakami and W. Pavan), polyclonal rabbit anti-mouse Phox2B (108) (1:750), and monoclonal mouse anti-mouse Nestin (Chemicon, 1:200) was performed. Primary immunoreagents were detected with either donkey anti-rabbit Cy3- or donkey anti-mouse Cy3-conjugated secondary antibodies (Jackson Immuno Research, 1:1000) and DAPI. Cells were visualized and counted using a DMi6000B microscope (Leica). The total number of DAPI+ cells and the number of DAPI+ cells positive for the individual progenitor marker being examined were determined for each individual well. The ratio of double-positive (DAPI+, Progenitor Marker+) relative to the total count of DAPI+ cells was calculated to determine the percentage of cells positive for each marker. Percentages were averaged across wells for each antibody, with eight replicates performed for each antibody in all three cell populations. Averages and standard deviations were calculated between unsorted and p75+/HNK-1− cells, unsorted and p75+/HNK-1+ cells, p75+/HNK-1− and p75+/HNK-1+ cells, p75+ and p75+/HNK-1− cells, and HNK-1+ and p75+/HNK-1− cells using Welch’s t-test and are listed in Figure 3.

Neurosphere culture of p75+/HNK-1+ progenitors

p75+/HNK-1+ cells were directly flow-sorted into self-renewal medium within 6-well non-adherent tissue culture plates at a density of 1000 cells per well. After 7 days, neurospheres were counted, pooled and dissociated using standard procedures (111). The concentration of cells (cells/ml) was determined, and dissociated cells were replated into self-renewal medium in 6-well non-adherent tissue culture plates at a density of 250 cells per well. After 7 days, neurospheres were again counted. This analysis was performed on two biological replicates. The number of isolated neurospheres can be found in Supplemental Material, Figure S2.

Analysis of developmental potential

Fetal guts at 12.5 dpc were dissected, pooled according to strain and Sox10 genotype, and dissociated. ENPs were isolated by flow cytometry as detailed above. p75+/HNK-1+ cells were directly sorted at low density (500 cells per well, 52 cells/cm²) into self-renewal medium within 6-well tissue culture plates coated with poly-d-lysine and fibronectin under conditions established for the analysis of in vitro developmental potential (40). Identical gating conditions were used for Sox10+/+ and Sox10+/dom+/+ cells. After 7 days, the medium was changed to a differentiation medium (40). Following 7 days in differentiation medium, immuno-staining was performed to determine the composition of resulting colonies. Cultures were washed and then fixed on ice in NBF with Triton X-100. After fixation, cultures were immunostained with antibodies to detect distinct lineages within individual colonies. Antibodies used included polyclonal rabbit anti-mouse Peripherin (Chemicon, 1:1000), monoclonal mouse anti-human GFAP-Cy3 (Sigma, 1:800), monoclonal mouse anti-human αSMA-FITC (Sigma, 1:800) and donkey anti-rabbit Cy3.
anti-rabbit Cy5-conjugated secondary antibody (Jackson Immuno Research, 1:1000). Cultures were post-fixed in NBF and counter-stained with DAPI to label nuclei. The number of total colonies per well was counted. Colonies were classified based on detection of distinct lineage IHC markers within individual colonies. Peripherin detected neurons, GFAP detected glia and SMA detected myofibroblasts. Colonies were categorized as either multipotent (neuronal + glial + myofibroblast, N + G + M) or uni- or bi-potent based on the observation of IHC staining within the colony: neuronal only (N), glial only (G), myofibroblast only (M), neuronal + glial (N + G), neuronal + myofibroblast (N + M) or glial + myofibroblast (G + M). Each well comprised one replicate. Colony-type percentages were determined by dividing the number of colonies displaying a particular phenotype by the total number of colonies in a single well. Percentages were averaged across replicates. Non-parametric statistical analyses were applied because standard deviations were not equivalent. Average percentages were compared by the Wilcoxon rank-sum test.

**Analysis of in vivo neurogenesis**

Embryonic guts at 13.5 dpc were dissected, and immunohistochemistry was performed as previously described (107). ENPs were stained using polyclonal rabbit anti-mouse Phox2B antibody (108) at 1:750 and detected by donkey anti-rabbit Cy2-conjugated secondary antibody (Jackson Immuno Research; 1:750) and detected by donkey anti-human Texas Red-conjugated secondary antibody (Jackson Immuno Research, 1:100). Embryonic guts were flat-mounted in Aqua Poly/Mount (Polysciences, Inc.) and cover-slipped for imaging. Confocal microscopy was performed at 400 magnifications on a Zeiss Scanning Microscope LSM510 to visualize the ganglion network in proximal small intestine from P15 to P17. Confocal microscopy at 400 magnifications was performed on a Zeiss Scanning Microscope (Olympus) at ×200 and ×400 magnifications.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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