cmgh
CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Enteric Neuron Imbalance and Proximal Dysmotility in Ganglionated Intestine of the *Sox10*$^{Dom/+}$ Hirschsprung Mouse Model

Melissa A. Musser,[1] Hernan Correa,[2] and E. Michelle Southard-Smith[1]

[1]Division of Genetic Medicine, Department of Medicine, and [2]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee

SUMMARY

The *Sox10*$^{Dom/+}$ Hirschsprung disease model exhibits imbalance of neuron subtypes throughout the intestine. These alterations suggest a novel role for *Sox10* in neuron specification and, in light of negligible inflammation, likely contribute to deficits in gastric emptying and small intestine motility.

BACKGROUND & AIMS: In Hirschsprung disease (HSCR), neural crest-derived progenitors (NCPs) fail to completely colonize the intestine so that the enteric nervous system is absent from distal bowel. Despite removal of the aganglionic region, many HSCR patients suffer from residual intestinal dysmotility. To test the hypothesis that inappropriate lineage segregation of NCPs in proximal ganglionated regions of the bowel could contribute to such postoperative disease, we investigated neural crest (NC)-derived lineages and motility in ganglionated, postnatal intestine of the *Sox10*$^{Dom/+}$ HSCR mouse model.

METHODS: Cre-mediated fate-mapping was applied to evaluate relative proportions of NC-derived cell types. Motility assays were performed to assess gastric emptying and small intestine motility while colonic inflammation was assessed by histopathology for *Sox10*$^{Dom/+}$ mutants relative to wild-type controls.

RESULTS: *Sox10*$^{Dom/+}$ mice showed regional alterations in neuron and glia proportions as well as calretinin+ and neuronal nitric oxide synthase (nNOS)+ neuronal subtypes. In the colon, imbalance of enteric NC derivatives correlated with the extent of aganglionosis. All *Sox10*$^{Dom/+}$ mice exhibited reduced small intestinal transit at 4 weeks of age; at 6 weeks of age, *Sox10*$^{Dom/+}$ males had increased gastric emptying rates. *Sox10*$^{Dom/+}$ mice surviving to 6 weeks of age had little or no colonic inflammation when compared with wild-type littermates, suggesting that these changes in gastrointestinal motility are neurally mediated.

CONCLUSIONS: The *Sox10*^{Dom} mutation disrupts the balance of NC-derived lineages and affects gastrointestinal motility in the proximal, ganglionated intestine of adult animals. This is the first report identifying alterations in enteric neuronal classes in *Sox10*$^{Dom/+}$ mutants, which suggests a previously unrecognized role for Sox10 in neuronal subtype specification. *(Cell Mol Gastroenterol Hepatol 2015;1:87–101; http://dx.doi.org/10.1016/j.jcmgh.2014.08.002)*

Keywords: Aganglionosis; Enteric Nervous System; Neural Crest.

The enteric nervous system (ENS) regulates multiple gastrointestinal (GI) functions, including motility, secretion, and inflammatory processes.[1] The ENS originates from neural crest-derived progenitors (NCPs) that migrate from the neural tube to colonize the entire intestine.[1] The normal function of the ENS relies upon complete colonization of the bowel as well as appropriate lineage segregation of NCPs to generate a balanced repertoire of distinct neuron classes and glial cell proportions.

In Hirschsprung disease (HSCR), migrating NCPs fail to populate the distal intestine, leading to a variable length of aganglionic bowel.[2] Mutations in *RET*, *EDNRB*, *EDN3*, or *SOX10* cause HSCR in patients, although other genetic variants influence disease penetrance and the extent of aganglionosis.[2–6] Despite surgical resection of the aganglionic segment, many HSCR patients suffer from residual chronic constipation (5% to 33% of patients) and decreased bowel function.[7] In addition, a substantial number of patients suffer from Hirschsprung associated-enterocolitis.[8] Differences in surgical procedures and recovery explain some adverse outcomes, yet many patients suffer from residual symptoms where no iatrogenic cause is found. An understanding of the processes that contribute to residual symptoms in HSCR patients would serve to better predict which patients will suffer from HSCR-related sequelae and to guide treatment options.

Prior evidence from mouse models with mutations that affect the ENS yet exhibit no overt aganglionosis suggests that deficits in enteric NCP lineage segregation contribute to GI dysmotility.[9] Chronic GI dysfunction in HSCR patients after surgery suggests that HSCR susceptibility genes (eg, *SOX10*) not only contribute to aganglionosis but may also affect ganglionated regions of the bowel. It has been suggested that *Sox10* affects multipotency of neural crest (NC)-derived cells and neuronal and glial specification. However, these

Abbreviations used in this paper: **BAC, bacterial artificial chromosome; ENS, enteric nervous system; GI, gastrointestinal; HSCR, Hirschsprung disease; IHC, immunohistochemistry; NC, neural crest; NCP, neural crest-derived progenitor; nNOS, neuronal nitric oxide synthase; P, postnatal; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.**

2352-345X
http://dx.doi.org/10.1016/j.jcmgh.2014.08.002

implications are derived from in vitro experiments or from other NC-derived structures such as dorsal root ganglia.[10–12] Although *Sox10* is essential for enteric NCP migration and colonization of the bowel, studies to elucidate the role of *Sox10* in NCP fate specification in the ENS in vivo have not been undertaken. Given established roles for *Sox10* outside the ENS and the presence of residual symptoms in HSCR patients, we hypothesized that perturbations in *Sox10* disrupt NCP lineage segregation and alter the function of ganglionated bowel in the $Sox10^{Dom/+}$ HSCR mouse model. To test this hypothesis, we fate-mapped NCPs using a Cre-LoxP system. Fate-mapping and immunohistochemical labeling of cell types in the myenteric plexus revealed that the normal complement of NC-derived lineages is disrupted in the enteric ganglia of $Sox10^{Dom/+}$ mutants. These changes are region specific, and disturbances in specific cell types in the colon correlate with extent of aganglionosis. Alterations seen in neuronal subtype proportions in $Sox10^{Dom/+}$ animals suggest a novel role for Sox10 in neuronal class specification.

Because changes in neuron ratios within enteric ganglia can alter GI motility, we investigated the potential for aberrant intestinal transit in the proximal small intestine of this HSCR model. GI motility assays exposed alterations in gastric emptying and small intestine transit that were age and sex dependent. Our results show that the $Sox10^{Dom}$ HSCR mutation alters NC lineage segregation and GI motility despite the presence and normal density of ENS ganglia in the proximal small intestine. Such changes could partially explain adverse outcomes in surgically treated HSCR patients and help clinicians better identify and treat patients at high risk for experiencing postsurgical GI dysfunction.

Materials and Methods

Animals

$Sox10^{Dom/+}$ and homozygous *B6.Cg-Gt(ROSA)-26Sor*$^{tm9(CAG\text{-}tdTomato/Hze/J}$, hereafter $R26R^{tdTom}$, were maintained on a C57BL/6J background. A *Sox10*-Cre bacterial artificial chromosome (BAC) construct was generated from the regulatory elements of *Sox10* within a well-characterized *Sox10* BAC[13] to obtain high levels of Cre in NC derivatives. The construct includes a nuclear localized Cre sequence connected to a human growth hormone mRNA stabilization sequence.[14,15] LoxP sites in the BAC flanking arms were removed as described by Boyle et al (2008) prior to microinjection of the BAC into fertilized mouse eggs by standard procedures.[16] The resulting $Tg^{(Sox10\text{-}CrehGH)1Sout}$ line, hereafter *Sox10-Cre*, was made congenic on the C3HeB/FeJ background. Transgene-driven reporter expression mirrors known *Sox10* expression in NC-derived lineages (Rosebrock J, Buehler DP, DeKeyser JL, et al., in preparation). Experimental animals for NC-derived lineage quantification were from crosses between $Sox10^{Dom/+}$; *Sox10*-Cre or $Sox10^{Dom/+}$; *Sox10*-Cre/Cre mice to $R26R^{tdTom}$ mice. $Sox10^{Dom/+}$ mice were identified by the presence of white feet and belly spotting as well as discernible hypoganglionosis and/or aganglionosis revealed by the absence of tdTomato fluorescent ganglia in the distal intestine. The $Sox10^{Dom/+}$ recapitulates HSCR in humans with animals exhibiting varying lengths of aganglionosis within the bowel despite harboring the same HSCR-causing mutation. The presence of the *Sox10*-Cre transgene was verified by polymerase chain reaction (PCR) genotyping with primers specific for fragment amplification of the Sp6 arm (Forward: Reverse: GGCACTTTCATGTTATCTGAGG), T7 arm (Forward: AAGAG CAAGCCTTGGAACTG; Reverse: TCGAGCTTGACATTGT AGGAC), and Cre-Recombinase (Forward: GCGGCATG GTGCAAGTTGAAT; Reverse: CGTTCACCGGCATCAACGTTT). Thermocycler conditions for all primers sets listed were as follows: 94°C for 5 minutes [(94°C for 30 seconds, 55°C for 30 seconds, 0.5-second ramp up to 72°C, 72°C for 30 seconds, 0.5-second ramp up to 94°C) 35 times], 72°C for 10 minutes, 4°C indefinitely. The Institutional Animal Care and Use Committee at Vanderbilt University approved all experimental protocols.

Immunohistochemistry

Regions of the duodenum, ileum, and midcolon were collected from postnatal (P) 15–19 day $Sox10^{Dom/+}$ and $Sox10^{+/+}$ littermates. Laminar muscle preparations containing myenteric plexus were isolated and subjected to immunohistochemical (IHC) analysis using the reagents described in Tables 1 and 2.[17] After incubation in primary antibodies, all tissues were rinsed in 1X phosphate-buffered saline (PBS)/0.1%Triton X-100 solution followed by incubation in secondary antibody dilution in block for 1 to 1.5 hours at room temperature. Rinses and incubation in a second secondary antibody dilution was repeated as previously described for double labeling. After secondary antibody incubation, tissues were rinsed in 1X PBS/0.1%Triton X-100 followed by rinses in 1X PBS. Tissue samples were stored in the dark in 1X PBS at 4°C before being mounted onto slides with Aqua-Poly/Mount mounting medium

Table 1. Primary Antibodies Used in Immunohistochemical Analysis

Primary antibody antigen	Host	Supplier	Catalog	Dilution	Tissue fix times
HuC/D	Human	Gift of V. Lennon	NA	1:10,000	20–25 min at RT or O/N at 4°C
FoxD3	Rabbit (polyclonal)	Gift of T. Labosky	NA	1:400	O/N at 4°C
Calretinin	Goat (polyclonal)	Millipore	AB1550	1:2500	20–25 min at RT
NOS1 (K-20)	Rabbit (polyclonal)	Santa Cruz Biotechnology	sc-1025	1:600	O/N at 4°C
s100A1	Sheep (polyclonal)	QED Biosciences	56201	1:3000	O/N at 4°C

Abbreviations: NA, not applicable; O/N, overnight; RT, room temperature.