HHS Public Access

Author manuscript

Neurogastroenterol Motil. Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

Neurogastroenterol Motil. 2016 April ; 28(4): 498–512. doi:10.1111/nmo.12744.

Isogenic enteric neural progenitor cells can replace missing neurons and glia in mice with Hirschsprung disease

Ryo Hotta[1], **Lily Cheng**[1,2], **Hannah Graham**[1], **Weihua Pan**[1,3], **Nandor Nagy**[1,4], **Jaime Belkind-Gerson**[5], and **Allan M. Goldstein**[1,#]

1

2

3

4

5

Abstract

Background—Transplanting autologous patient-derived enteric neuronal stem/progenitor cells (ENSCs) is an innovative approach to replacing missing enteric neurons in patients with Hirschsprung disease (HSCR). Using autologous cells eliminates immunologic and ethical concerns raised by other cell sources. However, whether postnatal aganglionic bowel is permissive for transplanted ENSCs and whether ENSCs from HSCR patients can be successfully isolated, cultured, and transplanted *in vivo* remains unknown.

Methods—ENSCs isolated from the ganglionic intestine of Ednrb$^{-/-}$ mice (HSCR-ENSCs) were characterized immunohistochemically and evaluated for their capacity to proliferate and differentiate *in vitro*. Fluorescently-labeled ENSCs were co-cultured *ex vivo* with aganglionic Ednrb$^{-/-}$ colon. For *in vivo* transplantation, HSCR-ENSCs were labeled with lentivirus expressing GFP and implanted into aganglionic embryonic chick gut *in ovo* and postnatal aganglionic Ednrb$^{-/-}$ rectum *in vivo*.

Key Results—HSCR-ENSCs maintain normal capacity self-renewal and neuronal differentiation. Moreover, the Ednrb$^{-/-}$ aganglionic environment is permissive to engraftment by wild-type ENSCs *ex vivo* and supports migratrion and neuroglial differentiation of these cells following transplantation *in vivo*. Lentiviral-GFP-labeled HSCR-ENSCs populated embryonic chick hindgut and postnatal colon of Ednrb$^{-/-}$ HSCR, with cells populating the intermuscular layer and forming enteric neurons and glia.

[#]Corresponding Author: Allan M. Goldstein, Massachusetts General Hospital, Warren 1153, Boston, MA 02114, Tel: 617-726-0270, Fax: 617-726-2167, agoldstein@partners.org.

Contributions

RH, LSC, and WHP performed the research and analyzed the data. HKG provided technical assistance and maintained the mouse colonies. LSC, NN and RH performed immunohistochemistry. RH, JBG, and AMG designed the study. RH wrote the manuscript. All authors reviewed and approved the final manuscript.

Disclosure

The authors have no conflicts of interest.

Conclusions & Inferences—ENSCs can be isolated and cultured from mice with HSCR, and transplanted into the aganglionic bowel of HSCR littermates to generate enteric neuronal networks. These results in an isogenic model establish the potential of using autologous-derived stem cells to treat HSCR and other intestinal neuropathies.

Keywords

Enteric nervous system; Hirschsprung disease; cell therapy; endothelin receptor B; lentivirus

Introduction

The enteric nervous system (ENS) is an extensive network of neurons and glial cells within the wall of the gastrointestinal tract critically important in regulating gut motility and other fundamental aspects of gut function[1]. As a result, enteric neuropathies, in which enteric neurons are abnormal or congenitally absent, can cause significant morbidity. Hirschsprung disease (HSCR) is characterized by absent ganglion cells in the distal bowel due to failure of neural crest-derived precursors to colonize the entire gut. The aganglionic region produces a functional obstruction, and current treatment requires surgical resection of this segment. While surgery is life-saving, over 50% of children have persistent problems, including severe constipation, fecal incontinence, and enterocolitis[2,3]. Cell-based therapy offers the potential to introduce new neurons into the aganglionic region as a novel therapy for this disease[4–6].

Neuronal stem cells can be isolated from postnatal rodent and human intestine and are able to migrate and differentiate when transplanted into the embryonic gut[7–11]. More recently, it has been shown that enteric neuronal stem/progenitor cells (ENSCs) derived from neonatal rodent and human can be expanded in culture and transplanted into postnatal colon of mice *in vivo*, resulting in cell engraftment, migration and neuroglial differentiation[12–14]. These studies show that ENSC transplantation is achievable in postnatal recipient gut and offers the potential to replace missing neurons with neuronal stem cells for the treatment of enteric neuropathies. However, little research has been done to determine whether the postnatal aganglionic environment of HSCR is permissive to transplanted neuronal stem cells.

Several groups have demonstrated the isolation, expansion, and differentiation of ENSCs from the intestine of rodents and humans with HSCR[9,11,14,15], raising the possibility of establishing patient-derived ENSCs as donor cells for cell therapy. The use of patient-derived autologous ENSCs would have significant advantages over other cell sources by eliminating the risk of immunological rejection and minimizing ethical concerns[16]. However, whether ENSCs derived from HSCR patients have a similar capacity to form extensive neuroglial networks as cells derived from healthy individuals is unknown. Since nearly all patients with HSCR carry mutations that affect enteric neural crest-derived cells[17], patient-derived ENSCs may not be able to generate adequate enteric neurons and glial cells following transplantation[18].

In order to address these questions, we used Endothelin receptor type B (Ednrb)-null mice, which possess distal colorectal aganglionosis, as a model of HSCR in the current study. Our goal was to test whether the postnatal aganglionic colon is permissive for ENSCs and to

determine whether ENSCs isolated from Ednrb$^{-/-}$ mice could serve as a source of cells for isogenic transplantation into the aganglionic segment of recipient mice. Our results establish the feasibility of this approach and support the potential of autologous cell transplantation for neurointestinal diseases.

Materials and methods

Animals

Experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Ednrb^{tm1Ywa} mice on a hybrid C57BL/6J-129Sv background were purchased from Jackson Laboratory (JAX#003295). Homozygous pups (Ednrb$^{-/-}$) were identified by white coat color and exhibit distal aganglionosis[19]. Genotyping was performed using a polymerase chain reaction-based assay to distinguish wild type (Ednrb$^{+/+}$) from heterozygous (Ednrb$^{+/-}$) littermates[20]. We also used Actb-DsRed mice (strain Tg(CAG-DsRed*MST)1Nagy/J; Jackson Labs Stock #005441), in which all cells express red fluorescent protein[21]. Fertilized White Leghorn chicken (Gallus gallus) eggs were obtained from commercial breeders and maintained at 37°C in a humidified incubator. Embryos were staged by the number of embryonic days (E).

Tissue preparation and immunohistochemistry

Cells and tissues were fixed in 4% paraformaldehyde. For whole-mount preparations of mouse longitudinal muscle-myenteric plexus (LMMP), distal colon was opened along the mesenteric border, pinned on Sylgard-coated plates, and fixed. The mucosa and submucosa were removed. For cryosection, tissues were transferred to 15% sucrose overnight at 4°C. The medium was changed to 7.5% gelatin containing 15% sucrose at 37°C for 1 hour and rapidly frozen at −50°C in methylbutane (Sigma). Frozen sections were cut at 12 μm thickness.

Immunohistochemistry was performed as previously described [22]. Cells or tissues were fixed, washed, and permeablized with 0.1% Triton X-100 for 30 minutes. Primary antibodies included mouse anti-Tuj1 (1:100, Covance, Dedham, MA), mouse anti-HuC/D (1:100, Life Technologies, Carlsbad, CA) mouse anti-CN (kind gift from Dr. Tanaka [23]), rabbit anti-S-100 (1:100, NeoMarkers, Fremont, CA), rabbit anti-p75 (1:500, Promega, Madison, WI), rabbit anti-αSMA (1:100, Abcam, Cambridge, MA, USA), rabbit anti-nNOS (1:500, Santa Cruz Biotechnology), rabbit anti-calretinin (1:200, Life Technologies), rabbit anti-Nestin (1:200, Abcam), rabbit anti-synaptophysin (ab14692, 1:200, Abcam), goat anti-GFAP (1:500, Abcam), goat anti-ChAT (1:50, Millipore, Billerica, MA), goat anti-Ret (1:20 R&D Systems, Minneapolis, MN), goat anti-GFP (1:400, Rockland, Limerick, PA), and human antineuronal nuclear type 1 (Hu; 1:16,000 kind gift from Dr. Lennon). Secondary antibodies included goat anti-mouse IgG Alexa Fluor 546, goat anti-rabbit Alexa Fluor 488, donkey anti-goat Alexa Fluor 488, donkey anti-goat Alexa Fluor 546, donkey anti-mouse Alexa Fluor 546, and donkey anti-mouse Alexa Fluor 488 (Life Technologies; Carlsbad, CA). Cell nuclei were stained with DAPI (Vector Labs, Burlingame, CA). Images were captured with a Nikon 80i microscope or with a Nikon A1R laser-scanning confocal microscope

Enteric neuronal stem/progenitor cell (ENSC) cultures

DsRed mice were sacrificed on postnatal day 14–21 (P14–21) and the gastrointestinal tract from duodenum to anus removed. Small intestine of P10–14 Ednrb$^{-/-}$ mice or Ednrb$^{+/+}$ littermates were also used to obtain HSCR-ENSCs or non-HSCR-ENSCs. The LMMP was separated and dissociated with dispase (250 μg ml^{-1}; StemCell Technologies, Vancouver, Canada) and collagenase XI (1 mg ml^{-1}; Sigma Aldrich, St. Louis, MO) at 37°C for 1 hour with gentle pipetting. The cell suspension was passed through a 40 μm cell strainer and cultured at a density of 50,000 cells ml^{-1} in proliferation medium, consisting of Neurocult NSC Basal Medium (StemCell Technologies) supplemented with 20 ng ml^{-1} epidermal growth factor (EGF; StemCell Technologies) and 10 ng ml^{-1} basic fibroblast growth factor (bFGF; StemCell Technologies), 0.0002% Heparin (StemCell Technologies) and 100 U ml^{-1} Penicillin-Streptomycin (Life Technologies) for 7–10 days to form enteric neurospheres. Neurospheres were passaged every 7–10 days with gentle Accutase (StemCell Technologies) dissociation at 37°C for 20–30 minutes followed by re-plating.

To induce differentiation, neurospheres were dissociated with Accutase (StemCell Technologies) at 37°C for 30 minutes with gentle pipetting. The cell suspension was passed though a 40 μm cell strainer and plated at 50,000 cells ml^{-1} on glass-bottom chamber slides coated with 20 μg ml^{-1} fibronectin (Biomedical Technologies, Ward Hill, MA). Cells were cultured for 7 days in NeuroCult NSC Differentiation Medium (StemCell Technologies) and then processed for immunohistochemistry.

Differentiation assay

Neurospheres isolated from Ednrb$^{-/-}$ or Ednrb$^{+/+}$ littermates were dissociated with Accutase and replated as above. Following 7 days in differentiation conditions, cells were fixed and processed for immunohistochemistry. Following staining with the neuronal marker, Tuj1, random images were taken from each culture condition and neuronal density measured quantitatively using ImageJ software.

Proliferation assay

10 nM 5-ethynyl-2′-deoxyuridine (EdU) was added to the culture medium 24 hours prior to fixation. Neurospheres were dissociated with Accutase and centrifuged (800 g for 2 minutes; Shandon Cytospin 3) onto a poly-L-lysine slide. Neural crest cells were visualized using p75 immunoreactivity, with EdU incorporation detected using the ClickiT EdU Imaging Kit (Invitrogen). Ten random images were taken from each group and the number of p75+EdU− or p75+EdU+ cells counted using ImageJ software.

Preparation and transduction of lentiviral vector

The lentiviral plasmid (pLenti-GIII-CMV-GFP-2A-Puro) was purchased from Applied Biological Materials Inc and amplified using plasmid DNA Maxiprep Kit (Life Technologies) according to the manufacturer's protocol. Lentiviral vector stocks were produced by co-transfection of 293T cells with the transfer vector, packaging plasmid and a plasmid coding for the VSV-G envelope glycoprotein[24]. The viral titer was determined by qPCR using a probe against the WPRE element; stocks were stored at −80°C until use.

Enteric neurospheres from Ednrb$^{-/-}$ or Ednrb$^{+/+}$ mice were gently triturated to single cell suspensions using Accutase at 750 cells μl^{-1} and plated in proliferation medium. Following 2 hours at 37°C, lentivirus was added at multiplicity of infection (MOI) in the range of 3–6. After 48 hours, successful transduction was confirmed by green fluorescent protein (GFP) expression.

To determine transduction efficiency, enteric neurospheres were generated from lentiviral-transduced ENSCs and then dissociated and plated on fibronectin-coated cover slips. Presence of GFP fluorescence was quantitatively measured using ImageJ software to evaluate lentiviral transduction efficiency.

Co-culture of LMMP and neurospheres

LMMP was obtained from mouse colon and a 5×5 mm segment suspended as a catenary culture using a piece of filter paper with a rectangular-shaped window. DsRed expressing neurospheres were placed onto the LMMP and covered with 10 μL Matrigel (BD biosciences, CA, USA) to secure the neurospheres in position. Co-cultures were maintained for 4 or 7 days in medium containing 10% fetal bovine serum (Life Technologies) and 100 U ml^{-1} Penicillin-Streptomycin (Life Technologies), and then fixed overnight in 4% paraformaldehyde.

Measurement of ENSC migration on LMMP

Tile scans of wholemount preparations were taken with a Nikon AZ100 fluorescent microscope. Migration distance was measured by dividing the image into octants (Supp. Fig. 1) and measuring the distance from the edge of the neurosphere to the distalmost DsRed+ cell in each octant. The average distance was calculated to represent the migration distance in each preparation.

Neurosphere transplantation to aganglionic chick hindgut

Chick aganglionic hindgut was dissected from E5 chicken embryos. Three GFP+ neurospheres were implanted into the proximal hindgut mesenchyme using fine forceps under microscopic visualization. The recombinants were transplanted onto the chorioallantoic membrane (CAM) of an E10 chick host for 7 days as described previously[22,25].

In vivo transplantation of neurospheres to Ednrb$^{-/-}$ mice

Recipient 2-week-old Ednrb$^{-/-}$ or Ednrb$^{+/+}$ mice were anesthetized by isoflurane (Henry Schein Animal Health, Dublin, OH) inhalation. A circum-anal skin incision was made and the distal rectum exposed. One or 2 DsRed+ or GFP+ neurospheres, labeled with 0.1% methylene blue, were transplanted into a pocket created between the two muscle layers of the rectal wall. The pocket was closed with 8–0 nylon suture. At 1–2 weeks following surgery, recipient mice were sacrificed and the rectum removed.

In vivo proliferation studies

Intraperitoneal injection with 50 mg kg^{-1} EdU was performed 1 and 2 days following transplantation. The distal colon was removed, processed as above, and EdU incorporation

detected using the ClickiT EdU Imaging Kit (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR

Total mRNA was extracted from proliferating neurospheres using the RNeasy Mini kit (Qiagen, Santa Clarita, CA, USA) and cDNA synthesized with the Superscript III Reverse Transcription Kit (Invitrogen). Ednrb expression levels were measured using quantitative PCR (qPCR) with Gapdh as the internal standard. The primers used were: EdnrB forward, GAACTCCACGCTGCTAAGAATCATCTAC and EdnrB reverse, CAGCTTACACATCTCAGCTCCAAATGG. Relative expression was calculated by $2^{-\Delta Ct}$.

Statistical analyses

Data were expressed as means ± SD. Paired t-tests were used to evaluate the statistical significance between two groups. When more than two groups were compared, means were statistically compared by one-way ANOVA with Tukey's *post hoc* multiple-comparison tests of differences. Statistical significance was considered at $p<0.05$.

Results

DsRed+ enteric neurospheres engraft onto aganglionic colon *ex vivo*

Based on our previous isolation and propagation of postnatal mouse ENSCs[12,22,26], we generated DsRed-expressing enteric neurospheres to allow cell tracking following transplantation. The gastrointestinal tract was removed from two-week-old Actb-DsRed mice. Following mechanical and enzymatic dissociation, cells were cultured in medium containing EGF and bFGF for 7–10 days to form DsRed+ enteric neurospheres (Fig. 1A). When cultured on fibronectin-coated cover slips in medium devoid of EGF and bFGF, DsRed+ neurospheres differentiate into enteric neurons and glia expressing Tuj1 (Fig. 1B) and GFAP (Fig. 1C), respectively.

Previous reports suggest that aneural gut, including embryonic gut of HSCR mice[9], is more permissive for enteric neural crest cells than neural gut[27,28]. To test this in postnatal intestine, we co-cultured DsRed+ enteric neurospheres with LMMP colon explants isolated from postnatal day 7 (P7) $Ednrb^{-/-}$ mice or $Ednrb^{+/+}$ littermates. Immunostaining with Tuj1 shows extensive neuronal differentiation of cells migrating from the neurospheres (Fig. 1E). After 4 and 7 days of co-culturing, the extent of cell migration and fiber extension were quantitatively compared between neural and aneural environments (Fig. 1D,F) as described in Suppl. Fig. 1. Immunohistochemical analysis revealed that most of the DsRed-expressing cells migrate on the surface of the LMMP, with no difference in the location of the cells in the two different environments (data not shown). ENSCs migrate significantly further in an aganglionic environment compared to ganglionic recipient gut both at 4 days (394.3 ± 6.9 μm vs 107.7 ± 16.1 μm, Fig 1F) and 7 days (670.7 ± 71.3 μm vs 227.7 ± 26.9 μm, Fig. 1F). In normal ganglionic intestine, there was no significant difference in the migration distance between 4 and 7 days. In contrast, in aganglionic gut, there was a significant increase in migration distance between day 4 and day 7 (Fig. 1F).

Aganglionic HSCR colon is permissive to transplanted ENSCs *in vivo*

Based on our *ex vivo* experiments, we tested if DsRed+ ENSCs are able to engraft, migrate, and differentiate in the aganglionic colon of HSCR mice *in vivo*. Two-week-old Ednrb$^{-/-}$ mice were anesthetized and the aganglionic distal colon exposed through a perianal skin incision. DsRed+ enteric neurospheres were microsurgically implanted into a pocket created in the muscular wall of the colon (Fig. 2A). At 1–2 weeks following surgery, recipient mice were sacrificed and the distal colon removed. Of the 8 Ednrb$^{-/-}$ mice that underwent transplantation, DsRed+ cells were found in 7 recipients (5 at 1 week and 2 at 2 weeks following surgery). Wholemount preparation from recipient Ednrb$^{-/-}$ mice 2 weeks following surgery showed the presence of surviving transplanted ENSCs in the host gut, confirming their successful engraftment (Fig. 2B), along with projections of DsRed+ fibers emanating from the neurospheres (Fig. 2C, arrowheads). S-100 immunostaining demonstrates the presence of endogenous glial cells in the myenteric plexus of the proximal, ganglionated colon (Fig. 2D, arrowheads). An isolated glial fiber with no associated cell body can be seen in the aganglionic region, possibly representing an extrinsic fiber (Fig. 2D). Transplanted DsRed+ cells are located in the inter-myenteric layer of the aganglionic region (Fig. 2D,E; arrows), with fibers extending distally (Fig. 2E, arrowheads). The precise distance of longitudinal cell migration was difficult to determine in these preparations. DsRed+ ENSCs differentiate into neurons (Fig. 2F) and glia (Fig. 2G), suggesting that the aganglionic colon can support the survival, migration, and differentiation of transplanted ENSCs *in vivo*.

ENSCs can be isolated from ganglionic intestine of HSCR mice

While the successful isolation of ENSCs from postnatal intestine and their transplantation into aganglionic colon of HSCR mice represents an important step forward, clinical application of cell therapy will require autologous ENSCs transplantation. To determine if ENSCs can be isolated from mice with HSCR, we dissected the LMMP layer from the ganglionic small intestine of 1–2 week-old Ednrb$^{-/-}$ mice and formed neurospheres as described above (Fig. 3A–D). Immunohistochemistical characterization of sectioned neurospheres reveals the presence of neural crest-derived cells (Fig. 3A, p75), enteric neural crest cells (Fig. 3B, Ret), neurons (Fig. 3C, Tuj1), and glial cells (Fig. 3D, GFAP). The proportion of p75+ neural crest cells in HSCR-derived neurospheres was 46.9 ± 8.0 %, which is equivalent to that seen in control (non-HSCR) neurospheres (55.9 ± 13.8 %, n=3 in each group, p=NS). Upon differentiation, HSCR-derived ENSCs give rise to Tuj1+ neurons and S-100+ glial cells (Fig. 3E), as well as neuronal subtypes, including choline acetyltransferase (Fig. 3F, ChAT), neuronal nitric oxide synthase (Fig. 3G, nNOS), and calretinin (Fig. 3H).

Ednrb$^{-/-}$ ENSCs retain proliferation and neuronal differentiation potential

Although autologous stem cells have a number of advantages in regenerative medicine, the use of ENSCs from a patient with HSCR may be theoretically problematic[18]. We therefore tested whether HSCR-ENSCs from Ednrb$^{-/-}$ mice have an altered capacity for self-renewal or neuronal differentiation. Expression of Ednrb transcript in ENSCs isolated from Ednrb$^{-/-}$ mice and Ednrb$^{+/+}$ littermates was quantified by qPCR, confirming a lack of Ednrb

expression in HSCR-ENSCs (Fig. 3I). To examine their capacity for self-renewal, EdU was added to the culture medium while growing neurospheres and the proportion of proliferating neural crest cells was quantified immunohistochemically. Despite the loss of Ednrb expression in HSCR-ENSCs, no significant alteration was seen in the percentage of dividing neural crest cells within neurospheres from HSCR mice compared to those from non-HSCR mice (10.5 ± 5.5 % vs 8.8 ± 3.3 %, Fig. 3J). Dissociated neurospheres were also plated on fibronectin-coated cover slips to determine their ability of undergo neuronal differentiation, as determined by Tuj1 immunoreactivity. Enteric neuronal density was equivalent between ENSCs derived from Ednrb$^{-/-}$ and Ednrb$^{+/+}$ intestine (0.24 ± 0.07 mm^2 vs 0.28 ± 0.07 mm^2, Fig. 3K). These data suggest that the loss of Ednrb expression in HSCR-ENSCs does not alter their ability to proliferate or differentiate and supports their use for cell replacement therapy.

Lentiviral infection achieves long-term labeling of HSCR-ENSCs

In order to trace HSCR-ENSCs following transplantation, we generated a lentiviral vector expressing GFP, and transduced this construct into dissociated neurospheres. GFP fluorescence at 48 hours confirms a high efficiency of transduction, with 64.1 ± 3.8% of cells infected (Suppl. Fig 2A, Table 1). Lentivirus-mediated gene delivery results in stable and long-term transgene expression, even after neuronal or glial differentiation (Suppl. Fig. 2B, C). Immunohistochemical characterization of lenti-GFP-transduced cells demonstrated that 27.3 ± 2.1% and 35.9 ± 0.1% of GFP+ cells are neurons and glia, respectively (Suppl. Fig. 2B, C, and Table 1). Conversely, 53.2 ± 0.8% of Tuj1+ cells are labeled by GFP and 52.2 ± 5.9% of glial cells are GFP+.

We also transduced ENSCs isolated from Ednrb$^{+/+}$ littermates and found the overall transduction efficiency (61.6 ± 6.3%) and the proportion of neurons in the GFP+ transduced cells (27.4 ± 6.6%) to be similar to that seen with mutant ENSC. Interestingly, the loss of Ednrb expression was associated with a higher percentage of glial differentiation (43.2 ± 0.9%) as compared to Ednrb$^{+/+}$ ENSCs (27.6 ± 1.7%). Similarly, the proportion of GFP+ cells that underwent glial differentiation was greater in the HSCR-ENSC (35.9 ± 0.1% vs 24.3 ± 0.4%), suggesting that Ednrb$^{-/-}$-derived ENSCs preferentially differentiate into glia (Table 1).

HSCR-ENSCs survive, migrate, and differentiate in aganglionic chick gut

To determine the capacity of HSCR-ENSC to survive and generate neurons within the aneural gut environment, we implanted GFP+ HSCR-ENSC into the aganglionic hindgut of an embryonic day 5 (E5) chick (n=9), a stage at which enteric neural crest cells have not yet colonized the hindgut. Following neurosphere transplantation (Fig. 4A), hindguts were cultured on the CAM of an E10 host chick embryo for 7 days. GFP+ cells migrated all the way down the length of the chick embryonic gut, representing a distance of approximately 450 μm (Fig. 4B). Neuronal differentiation is shown by co-expression with Tuj1 (Fig. 4D) and Hu (Fig. 4E). The absence of staining with CN antibody, a chick-specific neuronal antibody[23], confirms that these are not endogenous chick-derived enteric neurons (Fig. 4C). Co-expression of GFP and GFAP confirms the presence of glial differentiation (Fig. 4F).

These findings demonstrate that HSCR-ENSCs survive, migrate, and differentiate in the embryonic aganglionic environment.

HSCR-derived ENSCs engraft in the postnatal aganglionic colon of HSCR mice *in vivo*

To test whether autologous HSCR-derived ENSCs can be transplanted into postnatal mouse colon *in vivo*, GFP+ HSCR-ENSCs were microsurgically implanted into the distal colorectal wall of 2 week-old Ednrb$^{-/-}$ or Ednrb$^{+/+}$ mice (n=9 Ednrb$^{-/-}$ and n=4 Ednrb$^{+/+}$; Fig. 2A). All recipient mice survived the procedure and GFP+ cells were found in 11 of 13 recipients (4 of 5 recipients at 1 week and 7 of 8 recipients at 2 weeks following transplantation). GFP + HSCR-ENSCs were present in the aganglionic colon with cells seen differentiating into neurons (Fig. 5A, arrows). A proportion of Tuj1+ cells are noted to be GFP-negative (Fig. 5A), likely representing neuronal differentiation of transplanted cells that lack lenti-GFP transduction, since the transduction efficiency is only about 60% (Table 1). Anti-αSMA staining of recipient colon shows HSCR-ENSCs in the submucosal area with few fibers projecting toward the muscular layer (Fig. 5B). Longitudinal cell migration was consistently observed and at 2 weeks following transplantation, HSCR-ENSCs migrated up to 940 μm in HSCR (Ednrb$^{-/-}$) mice. In comparison, HSCR-ENSCs migrated up to 1000 μm in non-HSCR (Ednrb$^{+/+}$) mice. With regard to differentiation of transplanted cells, co-localization of αSMA and GFP was infrequently seen in the aganglionic recipient colon (Fig. 5B,C).

To determine if transplanted cells proliferate and differentiate into neurons following transplantation, EdU was injected into recipient mice intraperitoneally following transplantation. Incorporation of EdU is observed in GFP+ HSCR-ENSCs-derived neurons (Fig. 5D–G, closed arrows), suggesting transplanted HSCR-ENSCs are able to proliferate and give rise to neurons *in vivo*. Tuj1-expressing neurons that co-express GFP, but do not incorporate EdU, are also seen (Fig. 5D–G, open arrows), representing either neurons that were already present within the neurosphere prior to transplantation or cells that differentiated following the EdU pulse. GFP+ HSCR-ENSCs also give rise to glial cells (Fig. 5H and H′) and enteric neuronal subtypes, including nNOS- (Fig. 5I and I′) and calretinin-expressing neurons (CalR; Fig. 5J). Finally, synaptophysin-immunoreactive vesicles are seen on GFP+ HSCR-ENSC-derived cells (SYP; Fig. 5K, arrows) in recipient aganglionic colon 1 week after transplant, suggesting synaptic formation. More abundant and robust synaptophysin-positive vesicles are seen in the recipient colon of wild-type Ednrb$^{+/+}$ mice 2 weeks following surgery (Fig. 5L and M).

Discussion

In the current study, we demonstrate that ENSCs can be isolated from the ganglionated intestine of mice with HSCR and have the capacity to generate neurons and glia both *ex vivo* and following transplantation into the aganglionic colon *in vivo*. These findings add to important previous work in this field and support the potential application of autologous cell-based therapy for treating enteric neuropathies.

ENSCs have been successfully isolated from embryonic[8–10,12,15] and postnatal[12,13,15,22,29–31] rodent gut and postnatal human intestine[8,10,11,14,32,33]. Identifying the optimal source of ENSCs for cell therapy applications is an important goal. We chose to

derive enteric neurospheres from unsorted cells dissociated from the gut wall. The cells in these spheres are heterogeneous, including both neural crest- and non-neural crest-derived cells[34]. We consistently find that approximately half of the cells within the neurosphere are neural crest-derived, and the absence of Ednrb expression did not alter this proportion. We find that our neurospheres gave rise to glia, neurons, and multiple neuronal subtypes *in vivo*, with proliferative potential, and little smooth muscle differentiation. It has been suggested that non-neural crest cells in the neurosphere may produce paracrine factors to support the neural crest cells, although this remains unknown[34]. Our observations support such a "bystander" effect and suggest that spontaneous (non-selected) neurospheres can be a therapeutic source for the treatment of enteric neuropathies.

To date, most studies have characterized ENSCs following transplantation into the preganglionated embryonic chick or mouse hindgut, i.e. prior to the arrival of migrating enteric neural crest-derived cells. The embryonic aganglionic colon of Ret- or Ednrb-deficient mice has also been successfully transplanted with enteric neuronal progenitors in co-culture experiments[7,9,28,35]. Transplantation into postnatal intestine *in vivo*, either normal or aganglionic, has been infrequently reported[12–14]. This represents a potentially significant hurdle as the postnatal gut microenvironment may not be sufficiently permissive to allow extensive colonization by enteric neural crest-derived cells, possibly due to the presence of a hostile extracellular matrix in the mature gut[28,36] or to the inhibitory effect of pre-existing neurons[27,28]. We found that transplanted cells are able to engraft in the postnatal ganglionic and aganglionic bowel. Interestingly, the extent of cell migration and neurofiber projection was significantly greater in the aganglionic environment in our *ex vivo* experiment. In *ex vivo* LMMP experiments, migration occurs almost exclusively on the surface of the LMMP rather than within the myenteric plexus, but this is due to the fact that cells are placed on top of the muscle surface *ex vivo*, rather than implanted into the gut wall as we do for *in vivo* transplantation. Surface migration on the LMMP is unlikely to contribute to functional integration of ENSCs. We use the LMMP explants to test the permissiveness of the environment and not as an indicator of how ENSCs will respond *in vivo* since the explanted LMMP offers a very different and artificial environment. Interestingly, our results suggest that the normal intestine may represent a less favorable milieu for ENSC migration, but the mechanisms underlying this are unknown. While transplanted cells were able to engraft effectively in the aganglionic colon *in vivo*, the area of engraftment and extent of neuronal differentiation were limited. Modifications of the transplanted cells or the recipient environment may be needed in order to enhance the success of ENSC transplantation.

The use of autologous ENSCs would be significantly advantageous for cell replacement therapy in order to minimize the risk of immune rejection, ethical concerns surrounding embryonic stem cells, and potential problems with clinical use of induced pluripotent stem cells[16,37]. The capacity of HSCR-derived ENSCs to proliferate, migrate, and differentiate following transplantation has not been previously explored. We found that ENSCs isolated from HSCR mice have an equivalent capacity for cell proliferation and neuronal differentiation in comparison to those isolated from wild-type littermates. This is consistent with previous work by Kruger et al[35], who demonstrated that ENSCs derived from Ednrb-deficient rats showed no alteration in their multipotency and proliferation. Furthermore, we

show that HSCR-ENSCs were able to replace missing enteric neurons and expressed synaptophysin-immunoreactive vesicles in the distal HSCR colon *in vivo*, suggesting functional connectivity. This is the first demonstration of successful engraftment and replacement of enteric neurons in HSCR colon using isogenic ENSCs. Anitha et al[38] injected an enteric neuronal cell line into the colon of Endrb$^{-/-}$ mice via laparotomy, with successful engraftment and differentiation into neurons and glial cells. However, this enteric neuronal cell line has a different genetic background from the Ednrb$^{-/-}$ recipient mice, raising the problem of immunological rejection, which plagues human solid organ and bone marrow allogeneic transplantation[39]. Successful isolation and culturing of ENSCs from postnatal Ednrb$^{-/-}$ mice enabled us to examine their *in vivo* behavior following transplantation into Ednrb-null mice from the same colony. Demonstrating the survival, proliferation, and appropriate differentiation of Ednrb$^{-/-}$ neuronal progenitors, and their transplantation into littermates, are important first steps toward achieving autologous cell transplantation.

Endothelin-3 (Et3) is produced by the mesenchyme of the developing gut[40–42] and acts through its transmembrane receptor, Ednrb, which is expressed by enteric neural crest cells[41,43]. Et3 has been proposed to enhance the proliferation of enteric neural crest cells[40,41] and to prevent the premature neuronal differentiation of multilineage progenitors, thereby maintaining enough progenitors to colonize the entire bowel[44–46]. Interestingly, we find that postnatal ENSCs can be cultured and expanded in the absence of Ednrb signaling and, furthermore, that these postnatal ENSCs can give rise to equivalent number of neurons despite the lack of Edrnb expression. These observations suggest that Ednrb is not required for postnatal ENSCs to proliferate and differentiate normally. During mouse embryogenesis, Ednrb signaling is temporally required specifically between E10 and E12.5[47,48], and its role postnatally is largely unknown. As inhibition of Et3 signaling failed to block colonization of the mouse hindgut by neural crest cells when applied after E13[48], it is clear that the role of Ednrb signaling is different in the early embryonic gut versus the late embryonic or postnatal gut, an area that merits further research.

We consistently observed that Ednrb-null ENSCs give rise to more glial cells in culture than wild-type ENSCs did. Although Kruger et al[35] demonstrated that Ednrb deficiency did not alter the potency of multilineage differentiation of embryonic gut NCSCs, including glial differentiation, a more recent study showed that Et3 decreased glial proliferation presumably by downregulating Sox10 expression, resulting in decreased glial cell numbers in cultured p75+ rat enteric neural crest cells[49]. Additionally, increased numbers and early appearance of S-100-expressing glial cells have been observed in developing peripheral nerves of Ednrb-null rats[50]. Our results are consistent with these observations and implicate a possible role for Ednrb signaling in balancing neuronal and glial cell numbers in the postnatal ENS.

A limitation of this study is that the recipient Ednrb$^{-/-}$ mice do not live longer than 4–5 weeks due to bowel obstruction and enterocolitis[51]. We previously showed that functioning enteric neurons can be generated from ENSCs 3–5 weeks following transplantation into wild-type mice *in vivo*[12]. Since it is not feasible to follow our recipient Ednrb$^{-/-}$ mice for this long after transplantation, functional analysis of recipient animals could not be performed. Although Anitha et al[38] showed improved colon motor function just one week

after transplantation of an enteric neuronal cell line into Ednrb$^{-/-}$ mice, further studies using other mouse models with longer survival, such as ENS injury caused by benzalkonium chloride (BAC)[14], are needed to demonstrate functional improvement following ENSC transplantation using HSCR-derived cells.

Lentiviral transduction proved to be a useful method to label ENSCs fluorescently in order to permit their long-term tracking. Previous studies have used retrovirus or adenovirus vectors to label enteric or CNS-derived neural stem cells transplanted into the gut[15,52,53]. However, the use of lentivirus has advantages over those previous approaches by infecting both dividing and non-dividing cells, providing long-term, stable transgene expression, and generating low immunogenicity[34,54,55]. Moreover, we achieved a higher transduction efficiency (>60%) than previously reported[52]. While GFP expression is retained after cell differentiation *in vivo*, a few GFP−/Tuj1+ cells were seen in the transplant site, showing the limitations associated with not achieving 100% cell labeling in our system. Nonetheless, gene delivery using lentiviral vectors may allow for genetic engineering of neuronal stem cells prior to transplantation in order to achieve expression of desired transgenes, such as sustained release of neurotrophic factors[52,53,56], or to optimize the host microenvironment. Similarly, lentiviral gene delivery could also be exploited to correct the genetic mutation present in patient-derived stem cells, such as *RET* or *EDNRB* mutations frequently found in human HSCR[57–59].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Vanda Lennon (Mayo Clinic) for human anti-Hu antibody and Dr. Hideaki Tanaka (Gunma University) for mouse anti-CN antibody. The lentiviral GFP virus was packaged at the MGH Vector Core Facility, Massachusetts General Hospital Neuroscience Center, Charlestown, MA funded by NIH/NINDS P30NS045776. RH is supported by grants from the Tosteson Fund for Medical Discovery at Massachusetts General Hospital and from the REACHirschsprung Foundation. LC is supported by an Ethicon Surgical Research Fellowship Award from the Society of University Surgeons. AMG is supported by the National Institutes of Health (R01DK103785).

References

1. Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol. 2012; 9:286–294. Epub 2012/03/07. [PubMed: 22392290]
2. Catto-Smith AG, Trajanovska M, Taylor RG. Long-term continence after surgery for Hirschsprung's disease. Journal of gastroenterology and hepatology. 2007; 22:2273–2282. [PubMed: 18031392]
3. Ieiri S, Nakatsuji T, Akiyoshi J, et al. Long-term outcomes and the quality of life of Hirschsprung disease in adolescents who have reached 18 years or older-a 47-year single-institute experience. Journal of pediatric surgery. 2010; 45:2398–2402. Epub 2010/12/07. [PubMed: 21129554]
4. Young HM. Neural stem cell therapy and gastrointestinal biology. Gastroenterology. 2005; 129:2092–2095. [PubMed: 16344074]
5. Hotta R, Natarajan D, Thapar N. Potential of cell therapy to treat pediatric motility disorders. Seminars in pediatric surgery. 2009; 18:263–273. [PubMed: 19782309]
6. Wilkinson DJ, Edgar DH, Kenny SE. Future therapies for Hirschsprung's disease. Seminars in pediatric surgery. 2012; 21:364–370. Epub 2012/09/19. [PubMed: 22985842]

7. Natarajan D, Grigoriou M, Marcos-Gutierrez CV, Atkins C, Pachnis V. Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture. Development (Cambridge, England). 1999; 126:157–168.
8. Lindley RM, Hawcutt DB, Connell MG, et al. Human and mouse enteric nervous system neurosphere transplants regulate the function of aganglionic embryonic distal colon. Gastroenterology. 2008; 135:205–216. [PubMed: 18515088]
9. Mosher JT, Yeager KJ, Kruger GM, et al. Intrinsic differences among spatially distinct neural crest stem cells in terms of migratory properties, fate determination, and ability to colonize the enteric nervous system. Developmental biology. 2007; 303:1–15. [PubMed: 17113577]
10. Almond S, Lindley RM, Kenny SE, Connell MG, Edgar DH. Characterisation and transplantation of enteric nervous system progenitor cells. Gut. 2007; 56:489–496. [PubMed: 16973717]
11. Metzger M, Caldwell C, Barlow AJ, Burns AJ, Thapar N. Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. Gastroenterology. 2009; 136:2214–2225. [PubMed: 19505425]
12. Hotta R, Stamp LA, Foong JP, et al. Transplanted progenitors generate functional enteric neurons in the postnatal colon. The Journal of clinical investigation. 2013; 123:1182–1191. Epub 2013/03/05. [PubMed: 23454768]
13. Dettmann HM, Zhang Y, Wronna N, et al. Isolation, expansion and transplantation of postnatal murine progenitor cells of the enteric nervous system. PloS one. 2014; 9:e97792. Epub 2014/05/30. [PubMed: 24871092]
14. Hetz S, Acikgoez A, Voss U, et al. In vivo transplantation of neurosphere-like bodies derived from the human postnatal and adult enteric nervous system: a pilot study. PloS one. 2014; 9:e93605. Epub 2014/04/05. [PubMed: 24699866]
15. Bondurand N, Natarajan D, Thapar N, Atkins C, Pachnis V. Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures. Development (Cambridge, England). 2003; 130:6387–6400.
16. Preynat-Seauve O, Krause KH. Stem cell sources for regenerative medicine: the immunological point of view. Seminars in immunopathology. 2011; 33:519–524. Epub 2011/05/21. [PubMed: 21598094]
17. Amiel J, Sproat-Emison E, Garcia-Barcelo M, et al. Hirschsprung disease, associated syndromes and genetics: a review. J Med Genet. 2008; 45:1–14. [PubMed: 17965226]
18. Iwashita T, Kruger GM, Pardal R, Kiel MJ, Morrison SJ. Hirschsprung disease is linked to defects in neural crest stem cell function. Science (New York, NY. 2003; 301:972–976.
19. Hosoda K, Hammer RE, Richardson JA, et al. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell. 1994; 79:1267–1276. [PubMed: 8001159]
20. Thiagarajah JR, Yildiz H, Carlson T, et al. Altered goblet cell differentiation and surface mucus properties in Hirschsprung disease. PloS one. 2014; 9:e99944. Epub 2014/06/20. [PubMed: 24945437]
21. Vintersten K, Monetti C, Gertsenstein M, et al. Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. Genesis. 2004; 40:241–246. Epub 2004/12/14. [PubMed: 15593332]
22. Belkind-Gerson J, Carreon-Rodriguez A, Benedict LA, et al. Nestin-expressing cells in the gut give rise to enteric neurons and glial cells. Neurogastroenterol Motil. 2013; 25:61–69. e67. Epub 2012/09/25. [PubMed: 22998406]
23. Tanaka H, Kinutani M, Agata A, Takashima Y, Obata K. Pathfinding during spinal tract formation in the chick-quail chimera analysed by species-specific monoclonal antibodies. Development (Cambridge, England). 1990; 110:565–571. Epub 1990/10/01.
24. Sena-Esteves M, Tebbets JC, Steffens S, Crombleholme T, Flake AW. Optimized large-scale production of high titer lentivirus vector pseudotypes. Journal of virological methods. 2004; 122:131–139. Epub 2004/11/16. [PubMed: 15542136]
25. Nagy N, Brewer KC, Mwizerwa O, Goldstein AM. Pelvic plexus contributes ganglion cells to the hindgut enteric nervous system. Dev Dyn. 2007; 236:73–83. [PubMed: 16937371]

26. Belkind-Gerson J, Hotta R, Nagy N, et al. Colitis Induces Enteric Neurogenesis Through a 5-HT4-dependent Mechanism. Inflamm Bowel Dis. 2015 Epub 2015/03/06.
27. Meijers JH, van der Sanden MP, Tibboel D, van der Kamp AW, Luider TM, Molenaar JC. Colonization characteristics of enteric neural crest cells: embryological aspects of Hirschsprung's disease. Journal of pediatric surgery. 1992; 27:811–814. [PubMed: 1379301]
28. Hotta R, Anderson RB, Kobayashi K, Newgreen DF, Young HM. Effects of tissue age, presence of neurones and endothelin-3 on the ability of enteric neurone precursors to colonize recipient gut: implications for cell-based therapies. Neurogastroenterol Motil. 2010; 22:331–e386. [PubMed: 19775251]
29. Kruger G, Mosher J, Bixby S, Joseph N, Iwashita T, Morrison S. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron. 2002; 35:657–669. [PubMed: 12194866]
30. Suarez-Rodriguez R, Belkind-Gerson J. Cultured nestin-positive cells from postnatal mouse small bowel differentiate ex vivo into neurons, glia, and smooth muscle. Stem cells (Dayton, Ohio). 2004; 22:1373–1385.
31. Becker L, Kulkarni S, Tiwari G, Micci MA, Pasricha PJ. Divergent fate and origin of neurosphere-like bodies from different layers of the gut. American journal of physiology. 2012; 302:G958–965. Epub 2012/03/01. [PubMed: 22361728]
32. Metzger M, Bareiss PM, Danker T, et al. Expansion and differentiation of neural progenitors derived from the human adult enteric nervous system. Gastroenterology. 2009; 137:2063–2073. e2064. Epub 2009/06/25. [PubMed: 19549531]
33. Hagl CI, Heumuller-Klug S, Wink E, Wessel L, Schafer KH. The human gastrointestinal tract, a potential autologous neural stem cell source. PloS one. 2013; 8:e72948. Epub 2013/09/12. [PubMed: 24023797]
34. Binder E, Natarajan D, Cooper J, et al. Enteric neurospheres are not specific to neural crest cultures: implications for neural stem cell therapies. PloS one. 2015; 10:e0119467. Epub 2015/03/25. [PubMed: 25799576]
35. Kruger GM, Mosher JT, Tsai YH, et al. Temporally distinct requirements for endothelin receptor B in the generation and migration of gut neural crest stem cells. Neuron. 2003; 40:917–929. [PubMed: 14659091]
36. Druckenbrod NR, Epstein ML. Age-dependent changes in the gut environment restrict the invasion of the hindgut by enteric neural progenitors. Development (Cambridge, England). 2009; 136:3195–3203.
37. Fairchild PJ. The challenge of immunogenicity in the quest for induced pluripotency. Nature reviews Immunology. 2010; 10:868–875. Epub 2010/11/26.
38. Anitha M, Joseph I, Ding X, et al. Characterization of fetal and postnatal enteric neuronal cell lines with improvement in intestinal neural function. Gastroenterology. 2008; 134:1424–1435. [PubMed: 18471518]
39. Loewendorf A, Csete M. Concise review: immunologic lessons from solid organ transplantation for stem cell-based therapies. Stem cells translational medicine. 2013; 2:136–142. Epub 2013/01/26. [PubMed: 23349327]
40. Nagy N, Goldstein AM. Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. Developmental biology. 2006; 293:203–217. [PubMed: 16519884]
41. Barlow A, de Graaff E, Pachnis V. Enteric Nervous System Progenitors Are Coordinately Controlled by the G Protein-Coupled Receptor EDNRB and the Receptor Tyrosine Kinase RET. Neuron. 2003; 40:905–916. [PubMed: 14659090]
42. Leibl MA, Ota T, Woodward MN, et al. Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum. Gut. 1999; 44:246–252. [PubMed: 9895385]
43. Lee HO, Levorse JM, Shin MK. The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. Developmental biology. 2003; 259:162–175. [PubMed: 12812796]

44. Wu JJ, Chen JX, Rothman TP, Gershon MD. Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. Development (Cambridge, England). 1999; 126:1161–1173.
45. Gershon MD. Endothelin and the development of the enteric nervous system. Clinical and experimental pharmacology & physiology. 1999; 26:985–988. [PubMed: 10626067]
46. Bondurand N, Natarajan D, Barlow A, Thapar N, Pachnis V. Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling. Development (Cambridge, England). 2006; 133:2075–2086.
47. Shin MK, Levorse JM, Ingram RS, Tilghman SM. The temporal requirement for endothelin receptor-B signalling during neural crest development. Nature. 1999; 402:496–501. [PubMed: 10591209]
48. Woodward MN, Kenny SE, Vaillant C, Lloyd DA, Edgar DH. Time-dependent effects of endothelin-3 on enteric nervous system development in an organ culture model of Hirschsprung's disease. Journal of pediatric surgery. 2000; 35:25–29. [PubMed: 10646768]
49. Gisser JM, Cohen AR, Yin H, Gariepy CE. A novel bidirectional interaction between endothelin-3 and retinoic acid in rat enteric nervous system precursors. PloS one. 2013; 8:e74311. Epub 2013/09/17. [PubMed: 24040226]
50. Brennan A, Dean CH, Zhang AL, Cass DT, Mirsky R, Jessen KR. Endothelins control the timing of Schwann cell generation in vitro and in vivo. Developmental biology. 2000; 227:545–557. [PubMed: 11071773]
51. Zhao L, Dhall D, Cheng Z, et al. Murine model of Hirschsprung-associated enterocolitis II: Surgical correction of aganglionosis does not eliminate enterocolitis. Journal of pediatric surgery. 2010; 45:206–211. discussion 211–202. Epub 2010/01/29. [PubMed: 20105605]
52. Sun NF, Zhong WY, Lu SA, et al. Coexpression of recombinant adenovirus carrying GDNF and EDNRB genes in neural stem cells in vitro. Cell biology international. 2013; 37:458–463. Epub 2013/03/19. [PubMed: 23504906]
53. Shu X, Meng Q, Jin H, et al. Treatment of aganglionic megacolon mice via neural stem cell transplantation. Mol Neurobiol. 2013; 48:429–437. Epub 2013/03/21. [PubMed: 23512482]
54. Sakuma T, Barry MA, Ikeda Y. Lentiviral vectors: basic to translational. Biochem J. 2012; 443:603–618. Epub 2012/04/18. [PubMed: 22507128]
55. Natarajan D, Cooper J, Choudhury S, et al. Lentiviral labeling of mouse and human enteric nervous system stem cells for regenerative medicine studies. Neurogastroenterol Motil. 2014; 26:1513–1518. Epub 2014/09/10. [PubMed: 25199909]
56. Georgievska B, Kirik D, Bjorklund A. Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. J Neurosci. 2004; 24:6437–6445. [PubMed: 15269253]
57. Heanue TA, Pachnis V. Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nature reviews. 2007; 8:466–479.
58. Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science (New York, NY. 2007; 318:1920–1923.
59. Raya A, Rodriguez-Piza I, Guenechea G, et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature. 2009; 460:53–59. [PubMed: 19483674]

Key Messages

- ENSCs can be isolated from wild-type (DsRed mouse) and HSCR (Ednrb−/−) mice with no significant difference in their capacity for cell proliferation and neuronal differentiation.
- *Ex vivo* co-cultures of wild-type ENSCs and aganglionic colon demonstrate that the aganglionic microenivronment is permissive for ENSC migration and proliferation.
- Wild-type ENSCs survive, migrate, and differentiate into neurons and glial cells following transplantation into the aganglionic colon of HSCR mice *in vivo*.
- HSCR-ENSCs can be transplanted *in vivo* into the aganglionic colon of mice with HSCR colon, where they are able to proliferate, migrate, and undergo neuroglial differentiation.

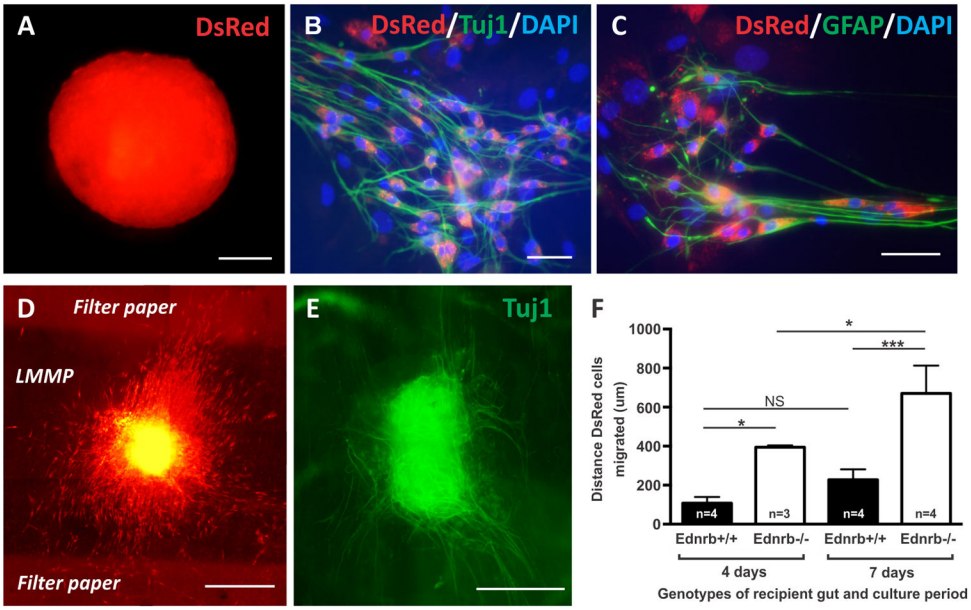

Figure 1. Isolation and transplantation of ENSCs into Ednrb$^{-/-}$ aganglionic colon ex vivo

Enteric neurospheres derived from Actb-DsRed mouse colon express red fluorescent protein (A). Enteric neuronal stem/progenitor cells (ENSCs) within those neurospheres differentiate into neurons (Tuj1, B) and glial cells (GFAP, C) that retain DsRed expression. DsRed+ enteric neurospheres co-cultured with LMMP isolated from an aganglionic colon of Ednrb$^{-/-}$ mice exhibit significant cell migration and fiber projection at 7 days (D), with extensive neuronal differentiation (E). ENSC migration is significantly better in aganglionic LMMP as compared to control (F) *: $p < 0.05$, ***: $p < 0.001$. Scale bars: 100 μm (A), 50 μm (B and C), 500 μm (D), and 200 μm (E).

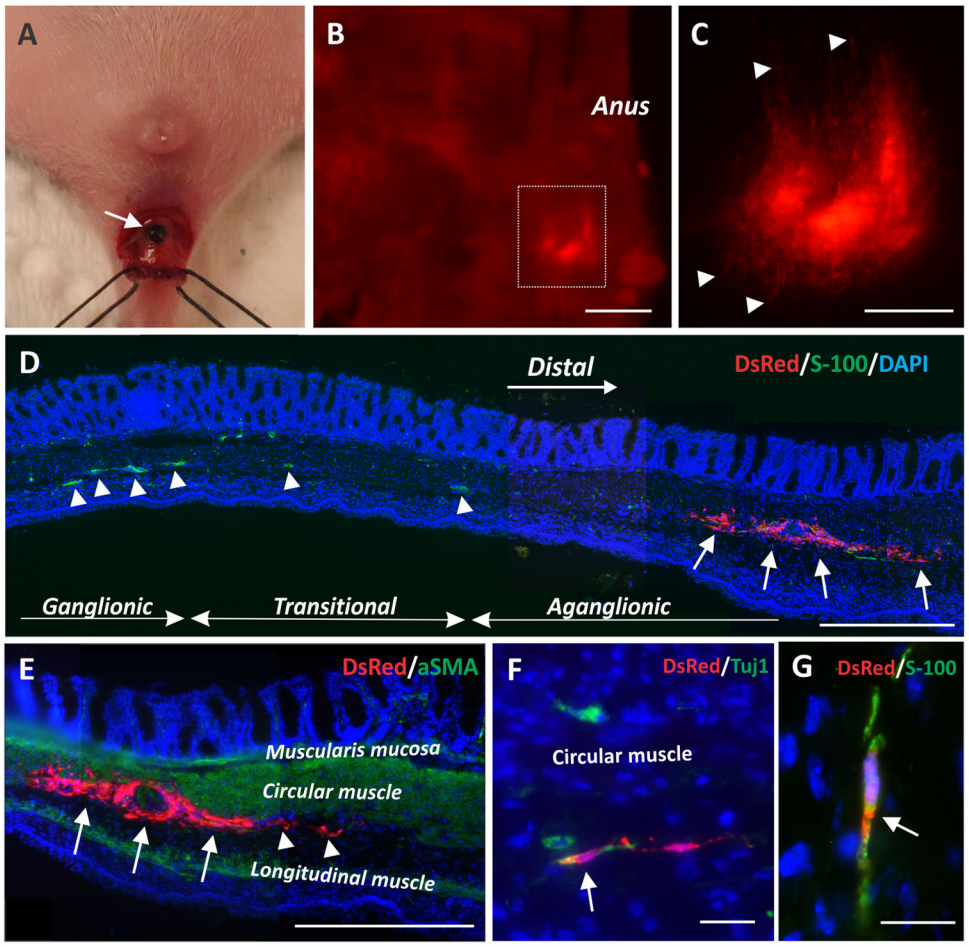

Figure 2. In vivo transplantation of ENSCs into aganglionic colon of Ednrb$^{-/-}$ mice

Methylene blue-labeled DsRed+ enteric neurospheres were microsurgically implanted into the rectal wall of Ednrb$^{-/-}$ mice via a trans-anal incision (A), and the colon fixed 1–2 weeks later. Wholemount immunostaining shows successful ENSCs engraftment (B) with migration of transplanted cells and prominent fiber projections (C, arrowheads). Recipient Ednrb$^{-/-}$ distal colon was sectioned longitudinally and examined immunohistochemically. Endogenous S100+ glial cells (D, arrowheads) are seen proximal to the transplanted DsRed + ENSCs (D, arrows), which are present in the inter-myenteric layer of the aganglionic region (E, arrows), with fibers extending distally (E, arrowheads). An isolated DsRed-nergative glial fiber, possibly extrinsically derived, is seen in the aganglionic region (D). Transplanted ENSCs differentiate into neurons (F) and glia (G). Scale bars: 1cm (B), 500 μm (C), 500 μm (D and E), and 50 μm (F and G).

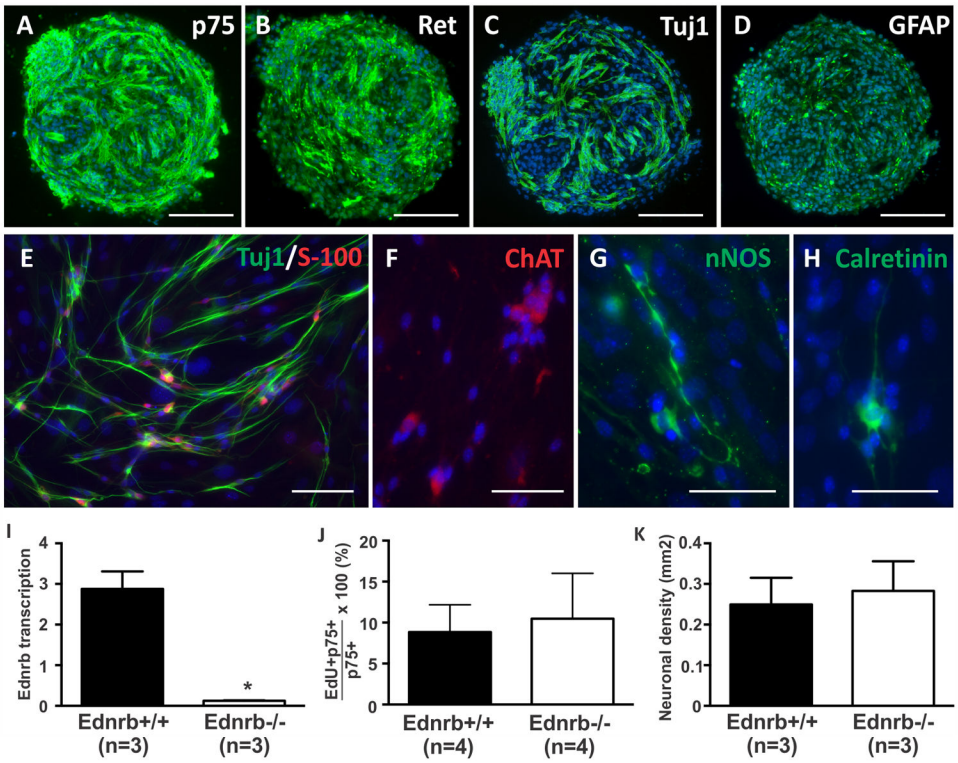

Figure 3. Isolation, characterization, proliferation and differentiation of ENSCs derived from Ednrb$^{-/-}$ mice

Immunofluorescent staining was performed on enteric neurospheres generated from dissociated small intestinal LMMP of Ednrb$^{-/-}$ mice (A–D). HSCR gut-derived ENSCs (HSCR-ENSCs) contain neural crest (A, p75; B, ret), neuronal (C, Tuj1), and glial (D, GFAP) cells. HSCR-ENSCs exhibit neuroglial differentiation following dissociation (E). A subset of neurons exhibits immunoreactivity to choline acetyltransferase (F, ChAT), nitric oxide synthase (G, NOS), or calretinin (H). qRT-PCR shows decreased Ednrb expression in HSCR-ENSCs (I), but no significant change in the rate of p75+ neural crest cell proliferation (J) or neuronal differentiation (K) compared to ENSCs from wild-type littermates. *$p <$ 0.05. Scale bars: 100 μm (A–E), 50 μm (F–H).

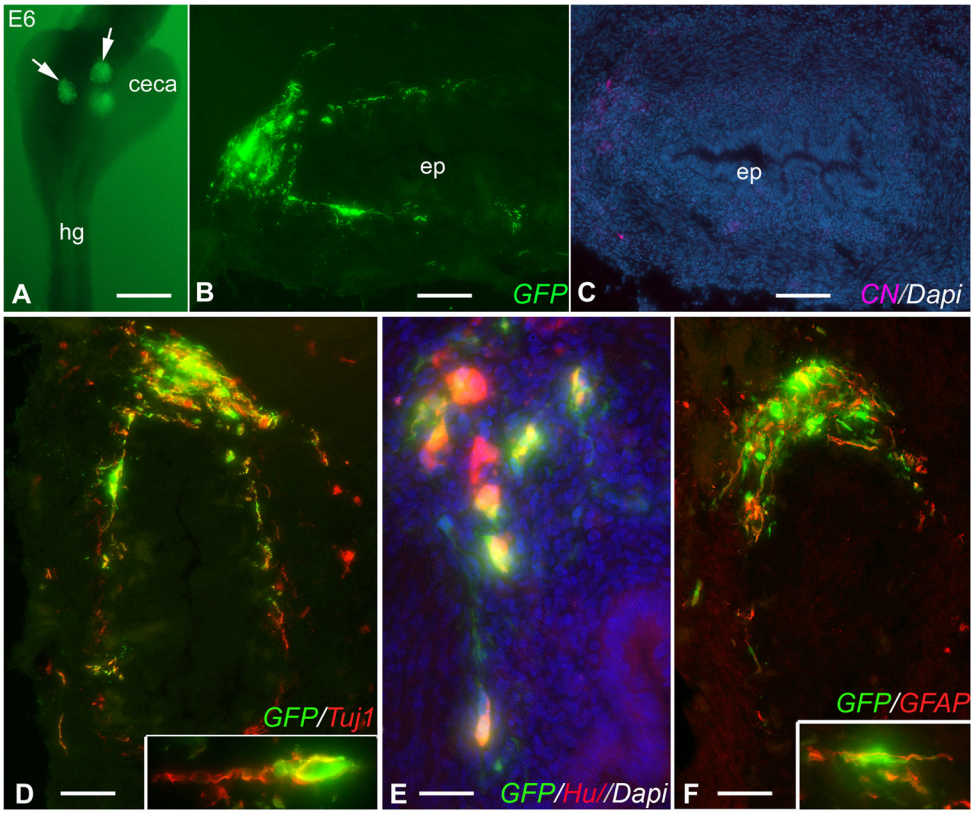

Figure 4. In ovo transplantation of ENSCs into embryonic aganglionic chicken hindgut

Aganglionic hindgut was removed from embryonic day 5 (E5) and GFP+ HSCR-ENSCs were transplanted into the cecal region (A, arrows). This transplanted tissue was grafted onto an E10 CAM. After 7 days, transplanted GFP+ HSCR-ENSCs migrate within the gut wall (B), project fibers (D) and differentiate into Tuj1+ (D) and Hu+ (E) neurons, and GFAP+ glial cells (F). GFP expressing cells show no CN staining (chick-specific neuron marker) in the gut (C), confirming they are not host-derived. Scale bars: 250 μm (A), 150 μm (B, C, D, and F), and 60 μm (E). ep, epithelium; hg, hindgut.

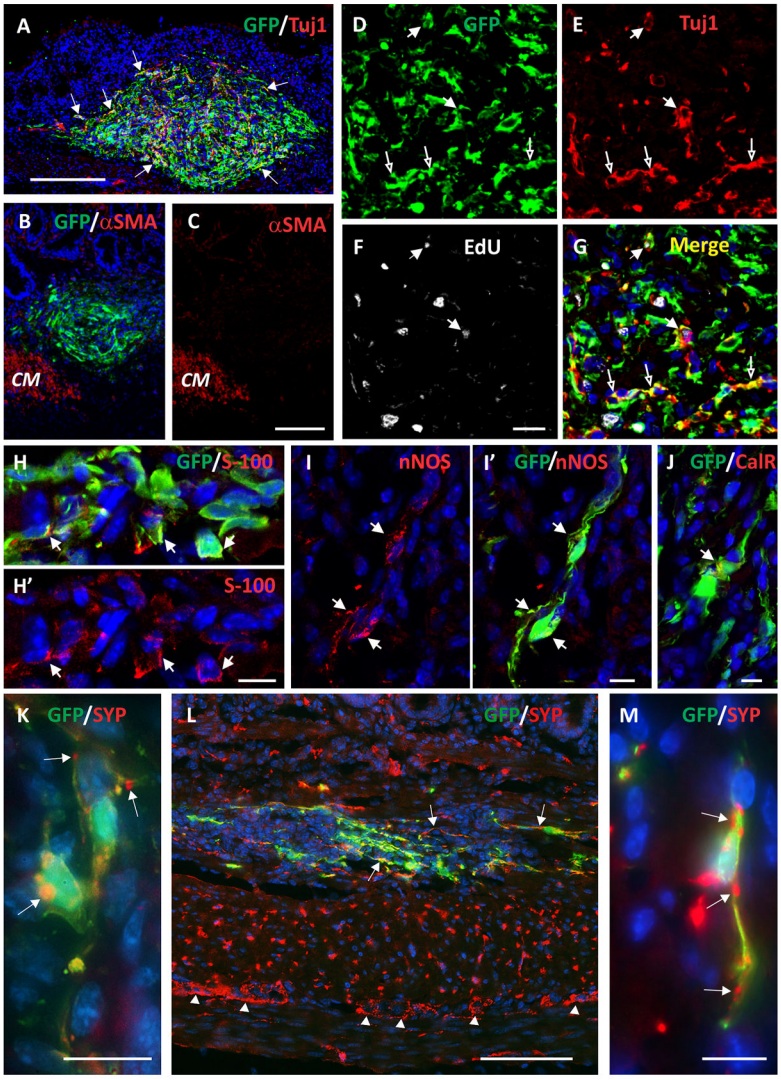

Figure 5. In vivo transplantation of Ednrb$^{-/-}$ ENSCs into aganglionic colon of Ednrb$^{-/-}$ mice

Lentiviral GFP-expressing HSCR-ENSCs were transplanted into aganglionic distal colon of Ednrb$^{-/-}$ mice and the colon fixed 1–2 weeks later. Transplanted cells engraft within the gut (A, arrows), mainly in the submucosal layer (B), and differentiate into Tuj1+ neurons (A, arrows), rarely giving rise to αSMA-expressing smooth muscle cells (B and C, CM; circular muscle). The presence of Tuj1+/GFP− cells likely represents neuronal differentiation of transplanted cells that lack lenti-GFP transduction. Neuronal proliferation following transplantation is demonstrated by incorporation of EdU and colocalization with Tuj1 and GFP (D–G, arrows). Non-dividing neurons are GFP+Tuj1+Edu− cells (D–G, open arrows). Transplanted ENSCs differentiate into glia (H and H′, arrows), nNOS-expressing neurons (I and I′, arrows), and calretinin (CalR) positive neurons (J, arrows). Synaptophysin (SYP) expressing vesicles are identified on transplanted ENSCs-derived cells in aganglionic colon at 1 week following surgery (K, arrows). Synaptophysin expression is seen more prominently in ganglionic recipient colon at 2 weeks following surgery (L and M, arrows) where the endogenous myenteric plexus also labels with synaptophysin (L, arrowheads).

Panels A–J were captured with confocal microscopy. Scale bars: 200 μm (A), 100 μm (B and C), 20 μm (D–J), 10 μm (K and M), and 100 μm (L).

Table 1

Characterization of lentiviral GFP (Lv-GFP) transduced cells

	Ednrb$^{-/-}$ ENSCs	**Ednrb$^{+/+}$ ENSCs**
Lv-GFP transduction efficiency (%)	64.1 ± 3.8	61.6 ± 6.3
GFP+Tuj+/GFP+ (%)	27.3 ± 2.1	27.4 ± 6.6
GFP+S-100+/GFP+ (%)	35.9 ± 0.1 **	24.3 ± 0.4
GFP+Tuj+/Tuj (%)	53.2 ± 0.8 *	41.0 ± 4.0
GFP+S-100+/S-100+ (%)	52.2 ± 5.9	59.0 ± 5.5
Tuj+/DAPI (%)	33.6 ±2.7	37.3 ±4.2
S-100+/DAPI (%)	43.2 ±0.9 ***	27.6 ±1.7

* P<0.05,

** p<0.01,

*** p<0.001