Enteric Nervous System Stem Cells Derived From Human Gut Mucosa for the Treatment of Aganglionic Gut Disorders

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BACKGROUND & AIMS: Enteric nervous system stem cells (ENSSCs) provide potential therapeutic tools to replenish absent ganglia in Hirschsprung's disease. Although full-thickness human postnatal gut tissue can be used to generate ENSSCs, reliance on its harvesting from surgical resection poses significant practical limitations. This study aimed to explore whether gut tissue obtained utilizing minimally invasive routine endoscopy techniques could be used to generate ENSSCs and whether such cells retain the potential to generate an ENS upon transplantation into aganglionic gut.

METHODS: Postnatal human gut mucosal tissue obtained from children undergoing gastrointestinal endoscopy was used to generate cell cultures in which ENSSCs were contained within neurosphere-like bodies (NLBs). These NLBs were characterized by immunostaining, and their potential to generate components of the ENS, in vitro and upon transplantation into models of aganglionic gut, was examined.

RESULTS: Gut mucosal biopsy specimens were obtained from 75 children (age, 9 months–17 years). The biopsy specimens contained neural cells and ENSSCs and, on culturing, generated characteristic NLBs at all ages examined. Postnatal mucosa-derived NLBs contained cells that, akin to their embryonic counterparts, were proliferating, expressed ENSSC markers, were bipotent, and capable of generating large colonies in clonogenic cultures and multiple ENS neuronal subtypes. Upon transplantation, cells from NLBs colonized cultured recipient aganglionic chick and human hindgut to generate ganglia-like structures and enteric neurons and glia.

CONCLUSIONS: The results represent a significant practical advance toward the development of definitive cell replenishment therapies for ENS disorders such as Hirschsprung's disease.

Hirschsprung's disease (HSCR), with an incidence of 1:5000 live births, is the commonest identifiable developmental disorder of the enteric nervous system (ENS), characterized by a failure of its complete formation, leaving a distinctive absence of enteric ganglia (aganglionosis) in a variable segment of distal bowel.1,2 This leads to peristaltic misregulation and tonic contraction within the affected gut causing intestinal obstruction. Current treatment for HSCR is limited to surgical resection of aganglionic bowel, but, despite tremendous expertise, often spanning the lifetimes of individual surgeons, the long-term outcomes postsurgery remain poor, irrespective of the degree of gut aganglionosis.3–7 Alternative forms of treatment are required if a satisfactory outcome is to be achieved for this common disorder.

Advances in molecular and stem cell biology have provided new avenues for therapy for ENS disorders and have led to the development of the ENS stem cell field.1,8,9 In this respect, the persistence in fetal and postnatal life of the original neural crest-derived precursors of the ENS, variably referred to as ENS progenitor cells10,11 or neural crest stem cells,12,13 has led to the possibility of these cells being used as therapeutic tools for ENS replenishment in disorders characterized by a dysfunctional or absent ENS.10–12,14,15 These studies demonstrated that such multipotent “stem” cells retain characteristics of early ENS progenitors and could be harvested either as single cells or within cellular aggregates termed neurospheres or neurosphere-like bodies (NLBs). These could then be transplanted into models of aganglionic gut, which they colonize to ultimately form components of the ENS. Such studies underpin the drive to translate this research in animal models to humans, with the hypothesis that stem cells, capable of generating a sufficient ENS to effect functional recovery when transplanted into the affected guts of patients with HSCR or other ENS disorders, could eliminate the need for less efficient surgical treatments.1,16

Significant progress has been made in the identification and harvesting of ENS stem cells from human gut. We and other groups have reported the isolation of such cells, contained within neurospheres from postnatal human gut including from the ganglionic segment of HSCR.
These studies have relied on obtaining full-thickness gut tissue from resection specimens obtained at surgery or postmortem, which poses significant practical limitations on the very likely requirement for repeated harvesting of fresh tissue.

Here, we describe the generation of ENS stem cell-containing NLBs from human postnatal gut mucosal tissue obtained using conventional, minimally invasive intestinal endoscopic techniques. We show that cells derived from such NLBs, upon transplantation into models of aganglionic gut, are capable of colonizing such tissue and differentiating appropriately into ENS neuronal and glial cells. This study provides a significant and necessary advance for the development of practical enteric neuronal transplantation treatments for ENS disorders such as HSCR.

**Materials and Methods**

**Human Embryonic and Postnatal Gut Material**

Human embryonic material (8–12 weeks gestational age) was obtained from the joint Medical Research Council and the Wellcome Trust-funded Human Developmental Biology Resource under ethical approval from the Joint UCL/UCLH committees on the ethics of human research. Staging of embryos was carried out using the Carnegie system and the gastrointestinal tract dissected out and prepared for tissue culture.

Human postnatal tissue was obtained from patients of Great Ormond Street Hospital (GOSH), London, UK, under ethical approval from the ICH/GOSH REC-O4S626 and with fully informed consent. Tissue was either (1) ganglionic and/or aganglionic full-thickness gut samples or (2) endoscopic gut mucosal biopsy specimens obtained from children undergoing gut resection surgery or endoscopic investigation, respectively, for gastrointestinal disorders including HSCR.

**Generation of Human NLBs**

To generate human ENSSC-containing NLBs from both human embryonic and postnatal gut tissue, we used a modification of the method that has been successfully employed by members of the group in work with murine gut to 7 days before processing for immunohistochemistry.

**In Vitro Clonogenic Assays**

A near single cell suspension was obtained from 7-day-old postnatal gut mucosal NLB cultures by treatment with Accutase enzyme solution (PAA, Yeovil, UK) for 10 minutes at 37°C and passage through a 50-µm cell filter. For the clonogenic culture assay, pure single cells were identified on clone sorting via flow cytometer MoFlo XDP (Beckman Coulter, Luton, UK), and each cell was plated into individual wells of fibronectin-coated, 96-well plates (4 plates/patient) before culturing for 14 days in NLB standard medium 50%, supplemented with conditioned medium (from 7-day-old NLB cultures). Any colonies subsequently generated from each single cell were microscopically identified by phase contrast and subsequently stained via immunocytochemistry using antibodies for TuJ1 and S100 as described.

**Neurosphere Differentiation Cultures and BrdU Incorporation**

To assess the range of neuronal subtypes generated, in vitro NLBs were collected, dissociated, and seeded onto fibronectin-coated 12-well culture dishes under differentiation conditions. Differentiation of dividing cells within NLBs was examined using a 5-bromo-2-deoxyuridine (BrdU) proliferation assay and immunostaining (see Supplementary Materials and Methods).

**Immunohistochemistry**

Immunohistochemistry was performed on cryosections (12 µm) of frozen human gut samples and on NLBs. Gut mucosal biopsy specimens and full-thickness gut segments were prepared as previously described. 17 NLBs were incubated in blocking solution (phosphate-buffered saline [PBS] containing 10% sheep serum, 0.1% Triton X-100) for 30 minutes at room temperature then primary and secondary antibodies were applied (see Supplementary Materials and Methods).

**Preparation of Recipient Gut Cultures**

For cell transplantation experiments, human embryonic or postnatal NLBs were injected into aganglionic recipient guts of embryonic day (E) 5 chick embryos placed on E8 chick chorioallantoic membrane (CAM). Following preparation of the CAM (see Supplementary Materials and Methods), 2-mm segments of aganglionic E5 chick hindgut (nerve of Remak removed) were placed on it and NLBs transplanted adjacent to the rostral end of recipient guts. Prior to injection, some NLBs were incubated with the nuclear stain Hoechst 33342 (10 µg/mL) for 30 minutes to enable cell labelling and facilitate observation with a fluorescence stereomicroscope. In later studies, the Hoechst stain was not used because of potential interference with cell differentiation. Eggs were incubated up to a further 12 days before injected guts were processed for immunohistochemistry.

For the experiments utilizing human aganglionic gut as recipient tissue, small segments of aganglionic gut tissue from HSCR patients were prepared by removing the mucosal and submucosal layers (to leave intact smooth muscle layers) and cut into 1- to 2-mm² pieces for use in organotypic tissue cultures as previously described for murine gut18 (see Supplementary Materials and Methods). For transplantation experiments, the human NLBs were washed with PBS and, using a glass micropipette, injected into the center of the cultured recipient guts. The cultures were then maintained for up to 7 days before processing for immunohistochemistry.
Results

**Human Endoscopic Gut Mucosal Biopsy Specimens Contain Both Neural and Stem Cells of the ENS**

Gut biopsy specimens (Figure 1A) from 75 children were collected and prepared for cell culture and immunostaining. The children were aged between 9 months and 17.3 years (mean age, 9.2 years) and consisted of 43 males and 32 females. Six children had HSCR (biopsy specimens from proximal ganglionic bowel). For comparison, full-thickness gut tissue samples were collected from 8 children aged between 3 months and 17.9 years (mean age, 5.9 years), comprising 4 males and 4 females, and including 2 children with HSCR (tissue from proximal ganglionic bowel).

To characterize the endoscopic biopsy specimens, samples from each of 12 patients were also fixed and sectioned for immunohistochemistry. Of these, 9 comprised gut mucosa alone and 3 contained fragments of adherent submucosa in addition (Figure 1B). Immunostaining of all biopsy specimens with the neuronal marker TuJ1 revealed a rich neuronal network within the mucosa, which consisted of both nerve fibers and neuronal cell bodies (Figure 1C). Although the majority of neurons was present as single cells, they did occasionally appear in small clusters reminiscent of enteric ganglia. To determine whether any ENSSCs were present within the mucosa, sections were immunostained using a panel of markers including the low-affinity neurotrophin receptor p75 and the transcription factor Sox10 (double stained with glial fibrillary acidic protein [GFAP] to exclude cells committed to a glial lineage). These immunostains showed that there were both p75 (data not shown) and Sox10+GFAP--cells (Figure 1D) within the mucosa consistent with the presence of ENSSCs within this location.

**NLBs Can Be Generated From Human Embryonic and Postnatal Gut**

Both embryonic and postnatal gut tissues (mucosal biopsy specimens and full thickness) were dissociated and plated into cell culture maintained under conditions designed to promote the generation of ENSSC-containing NLBs. For the mucosal biopsy specimens, NLBs were generated in 52 out of the 75 sets of cultures established. Of the remaining 23 (patients aged 1.5–17.3 years; mean, 10.7 years; and 14:9 male/female), 10 showed evidence of bacterial contamination and were discarded, and, in the rest, the cell cultures either failed to establish at all or no NLBs were evident by day 14. In the 52 patients whose cultures generated NLBs (age, 0.8 –16.5 years; average, 8.6 years; 30:22 male/female), 10 showed evidence of bacterial contamination and were discarded, and, in the rest, the cell cultures either failed to establish at all or no NLBs were evident by day 14. In the 52 patients whose cultures generated NLBs (age, 0.8 –16.5 years; average, 8.6 years; 30:22 male/female), small cell aggregates were visible by day 5, which went on to form characteristic NLBs by days 10 –14 (Figure 2C and D). NLBs were generated from all HSCR biopsy specimens and appeared equivalent to those from non-HSCR gut. There were no apparent differences between the numbers and size of NLBs generated from embryonic gut mucosal samples (Figure 2C and D; average diameter at postnatal age 1–12 years, 55.7 μm; and postnatal age 11–18 years, 58 μm) and those generated from the full-thickness samples (Figure 2E and F; average diameter, 60 μm). Although NLBs generated from postnatal gut were of a similar size, their efficiency of generation appeared to decrease with in-
creasing postnatal age. NLBs generated from embryonic cultures formed slightly earlier (days 7–10; Figure 2A and B) and in larger numbers and were larger compared to their postnatal counterparts (Figure 2A–D; average diameter, 79 μm).

**Human Embryonic and Postnatal Mucosal NLBs Contain Differentiated Neurons and Glia as Well as Cells That Are Proliferative and Express Markers of ENSSCs**

To characterize the NLBs generated in both human embryonic gut and postnatal gut mucosal primary cultures, they were immunostained using a panel of markers to determine the presence of cells that were dividing (Ki67), expressed markers of neural crest or neural stem cell status (p75, Nestin, and Sox10), or were differentiated neurons and glia (Figures 3 and 4). Sox10 immunoreactivity was assessed using double immunostaining with the glial marker GFAP to exclude Sox10-positive cells that had committed to the glial lineage. Both embryonic and postnatal NLBs contained cells that were Ki67 positive (Figures 3A and 4A), although the number of immunopositive cells was far greater in the former, consistent with their larger size (Figure 2). Similarly, both embryonic and postnatal mucosal NLBs contained p75 (Figures 3B and 4B), Nestin (Figures 3C and 4C), and Sox10 (Figures 3D and 4D) positive cells consistent with the idea that they contain ENSSCs. Again, the proportion of cells positive for each marker was visibly greater in the embryonic compared with the postnatal NLBs. Differentiated neurons and glia (evident by their immunoreactivity to TuJ1 and GFAP, respectively) were present in large numbers in both embryonic and postnatal NLBs (Figures 3E and 4E; and 4F). Taken together, these immunohistochemical findings are consistent with the NLBs being of neural crest origin.
Human Postnatal Mucosal NLBs Contain ENSSCs Capable of Generating Colonies Comprising Neurons and Glia in Clonogenic Cultures

To confirm further the presence of ENSSCs within NLBs, the latter were harvested from human postnatal gut mucosal primary cultures; dissociated into near single cell suspension; and, using flow cytometry, single cells plated into individual wells of 96-well plates and cultured for 14 days. Any colonies generated were identified microscopically and subsequently immunostained using antibodies for TuJ1 and S100. Such clonogenic assays were carried out on NLBs generated in cultures from 4 patients (age, 7–16.5 years; average age, 12.9 years; 2:2 male/female). Using phase contrast microscopy, single cells were visible within the wells in the first 24 hours after plating (Figure 5A, inset). By day 10, although no colonies were generated from the 16.5-year-old male, an average of 3.9% of wells (range, 1%–10.7%) from the remaining 3 experiments (average age, 11.6 years) contained large colonies of cells (Figure 5A, low power; 5B, high power), which by 14 days into culture consisted of an average of 1300 cells/colony. Cells within colonies showed characteristic neural morphology under phase contrast (Figure 5B), and immunostaining revealed cells immunoreactive for both the neuronal marker TuJ1 (Figure 5C) and glial marker S-100 (Figure 5D). These experiments confirmed the clonogenic ability and bipotentiality of cells contained within NLBs and, given their isolation from primary NLBs, also suggested that they were self-renewing.

Human Embryonic and Postnatal NLBs Can Generate a Variety of ENS Appropriate Mature Neuronal Subtypes

To assess the therapeutic potential of NLBs prior to in vivo transplantation, they were examined for their ability to generate ENS appropriate neuronal phenotypes
in vitro. Embryonic and postnatal mucosal NLBs were dissociated into cell suspension and plated into culture under conditions that favor differentiation. After 7 days, the cultures were immunostained with a panel of neuronal markers. Both embryonic and postnatal NLBs were able to generate cells representative of neuronal (TuJ1, Supplementary Figure 1A and G) and ENS neuronal phenotypes (CGRP, NOS, Ser, VIP, and ChAT; Supplementary Figure 1B–F and 1H–K).

**Human Postnatal Mucosal NLBs Contain Dividing Cells That Express Neuronal and Glial Lineage Markers**

To determine whether neodifferentiation was present in dividing cells seen within NLBs, BrdU was added to the culture medium of proliferating NLBs prior to dissociation and culture. At 14 days, cells within the cultures of dissociated NLBs expressed markers for neurons (PGP9.5, Supplementary Figure 2A) and glia (S100, Supplementary Figure 2B). BrdU incorporation was detected in cells immunopositive for the pan-neuronal marker PGP9.5 (Supplementary Figure 2C) and the glial marker GFAP (Supplementary Figure 2D). These results suggest that NLBs generated from postnatal mucosal biopsy specimens contained dividing cells that differentiate into neurons and glia, consistent either with neodifferentiation within uncommitted ENS progenitors or within progenitor cells committed to neuronal or glial lineages.

**Cells From Human Embryonic and Postnatal Gut Mucosal NLBs Can Colonize Aganglionic Gut Maintained in Culture and Show ENS Appropriate Localization and Differentiation**

To assess best the therapeutic potential of embryonic and postnatal mucosal NLBs, these were grafted onto aganglionic embryonic (E5) chick hindgut explants maintained on the CAM (Figures 6A–F and 7A and C–F) for up to 12 days before harvesting and analysis. Although ungrafted aganglionic chick hindgut explants
showed equivalent growth and anatomical maturation to
grafted explants, they never contained cells immunopo-
sitive for neuronal or glial markers (Figure 7B). Both
embryonic and postnatal NLBs engrafted onto the recip-
ient tissue irrespective of gestational or postnatal age. By
day 3, cells originating from the NLBs were evident in
deeper gut layers (data not shown) and, by day 7 onward,
were present in large numbers within submucosal and
myenteric regions of gut (Figures 6B–F and 7C–F).

In the embryonic NLB grafting experiments, NLB-derived
cells (labeled with a fluorescent Hoechst 33342 nuclear
stain) appeared to organize into presumptive ganglia within
the submucosal and myenteric regions by day 7 (Figure 6B,
and data not shown). By day 10, differentiated neuronal
(Figure 6C) and glial cells (Figure 6D) were present within
the deeper layers, and, by day 12, there was evidence of
differentiation into more mature neuronal subtypes (sero-
tonin and choline acetyltransferase-containing neurons;
Figure 6E and F, respectively).

In the postnatal NLB experiments, by days 7–10, large
clusters of neuronal cells were evident within the myenteric
and submucosal regions presumably close to the NLB graft
(Figure 7C). By days 10–12, numerous neuronal cells were
evident radially throughout the myenteric and submucosal
regions (Figure 7D) and organized into presumptive ganglia
(Figure 7D, inset). By day 12, transplanted cells showed
organization into ganglia as well as differentiation
into mature neuronal subtypes (vasoactive intestinal
polypeptide and nitric oxide synthase-containing neu-
rons; Figure 7E and F, respectively). In both embryonic
and postnatal experiments, the transplanted cells were
also capable of longitudinal migration. In 3 of 12
sequential postnatal grafting experiments, transplanted
cells had colonized the entire length of recipient guts by
culture end (up to 3600 μm, data not shown).

Cells From Postnatal NLBs Can Colonize
Segments of Aganglionic Human HSCR Gut

In an attempt to recapitulate the recipient gut envi-
ronment of HSCR gut, postnatal NLBs were grafted onto
segments of HSCR aganglionic gut maintained in organo-
typic cultures. Some of the segments were left ungrafted,
and, 7 days later, immunostaining showed parallel neuro-
fibers coursing through the tissue, representative of
the presumed extrinsically originating nerve fiber bundles
characteristically seen in aganglionic HSCR tis-
sue (Figure 8A). Human postnatal and embryonic NLBs
were grafted upon this donor tissue, and, within a few
days, neuronal processes and cell bodies extended away
from the NLB (Figure 8B and C). By day 7, the NLBs had
formed robust connections across the aganglionic tissue
reminiscent of nerve plexi (Figure 8C and D). Cross sec-
tions through the grafted segments showed the NLBs
had integrated within the recipient tissue in aggregates
(Figure 8F) compared with the ungrafted segments (Fig-
ure 8E). These studies showed that, at least in the cases of
human HSCR tissues tested here, the gut is receptive of
and supportive of human postnatal NLBs.

Discussion

We have shown that ENSSC-containing NLBs can
be generated from postnatal human gut mucosal biopsy
specimens obtained using conventional endoscopic tech-
niques. Such NLBs appear to be generated with the same efficiency as those from full-thickness gut tissue. Importantly, they exhibit characteristics similar to NLBs generated from embryonic human gut tissue taken at developmental stages appropriate for ENS formation, including the ability to generate a range of neuronal phenotypes normally encountered in a mature, functional ENS. Upon transplantation into recipient aganglionic gut, cells derived from NLBs are capable of colonizing this tissue in appropriate locations and differentiating into neurons and glia.

Not only do the results of our study concur with previous work from us and others showing that ENSSCs, contained within neurospheres or NLBs, can be derived from postnatal human gut,11,14,15 but, most significantly, they represent novel data that shows that ENSSCs that are self-renewing, bipotential (capable of generating neurons and glia), and have clonogenic ability can be generated from gut mucosal biopsy specimens beyond the neonatal period through into late childhood. This is significant given that the initial diagnosis and subsequent management of almost all congenital (including HSCR) and indeed many acquired ENS disorders occurs within the childhood period. Thus, if we consider any cell replenishment therapy for ENS disorders, especially autologous strategies, then both the harvesting of therapeutic ENSSCs and their transplantation needs to occur within this time frame. Our data demonstrate the feasibility of this process.

The ability to harvest ENSSC-containing gut tissue, using conventional endoscopic techniques to obtain gut mucosal biopsy specimens, represents a major practical step forward in the development of definitive ENS stem cell therapies. To date, including our current data, we and others have reported the isolation of ENSSCs, contained within neurospheres from postnatal human gut utilizing full-thickness gut tissue obtained at postmortem or resection from surgery.11,14,15 In establishing therapeutic protocols in humans, however, the need for gut resection and often major surgery to harvest tissue provides practical and ethical obstacles. Although such surgery may be
inevitable in HSCR patients (eg, for bowel decompression), it is unlikely, given the limited migration capacity of transplanted ENSSCs,¹⁰,¹¹ that a single procedure would provide sufficient ENSSCs to replenish the large amount of aganglionic or dysfunctional gut seen in HSCR. Perhaps the repeated requirement for obtaining ENSSCs over time could be overcome by their continued expansion and propagation in culture for long periods.¹¹,¹⁵ Repeated harvesting of fresh gut tissue, however, as opposed to prolonged culture, is likely to be preferable given that the latter may further impair the already restricted developmental potential of postnatal cells¹² and reduce levels of engraftment.¹³ Endoscopy would provide a widely accepted, minimally invasive, and reliable means of obtaining such tissue and, moreover, utilizes a regenerating source of tissue that is repeatedly accessible. Pediatric endoscopy is increasingly being done as a short, day case procedure.

Although from a therapeutic prospect, the precise source for the ENSSCs generated from the gut mucosal specimens is somewhat immaterial, it does raise some interesting questions about the structure of the ENS and niche of ENSSCs within the gut. There appear to be 3 possibilities to explain why endoscopic biopsy specimens are capable of generating ENSSC containing NLBs. The first is that ENSSCs are derived from adherent fragments of submucosal enteric ganglia where they formed discrete ganglia-like clusters between the muscle layers. Evidence of differentiation into vasoactive intestinal polypeptide (E) and nitric oxide synthase (F) neuronal subtypes was also evident by day 12. No such neuronal cells were present within control non-transplanted aganglionic chick hindgut explant cultured on CAM for 12 days (B, green, TuJ1 immunoreactivity). Scale bar in B represents 200 μm for B, C, and D and scale bar in E represents 50 μm in E and F.
Anatomically distinct ENS populations within the gut wall may not only serve different functions, but ENSSCs at each niche may possess varied developmental potentials, which may underlie potential differences in ENS plasticity and repair in response to damage/inflammation. In establishing their therapeutic potential, our in vitro data not only show that postnatal gut mucosal NLBs contain ENSSCs but, akin to the developmental potential of embryonic NLBs, they are also capable of generating a range of ENS neuronal phenotypes. It is likely that such neurons are the consequence of neodifferentiation given confirmation of the clonogenic and bipotent capability of presumptive ENSSCs contained within NLBs, incorporation of BrdU into NLB-derived cells differentiating into neurons and glia, and the experience of us and others that previously differentiated neurons are not present at establishment of primary cultures of dissociated intestine. Both embryonic and postnatal NLBs are moreover able to colonize aganglionic recipient gut and give rise to an anatomically appropriate ENS, including the generation of mature neuronal subtypes. The range of these subtypes, although limited, are reflective of a mature ENS and consist of both Mash-1-dependent and -independent lineages. Given that the in vitro data are not supportive of a restricted potential of ENSSCs, these findings either suggest that the limited time course of the CAM gut culture system (12 days posttransplantation) is insufficient to enable visualization of, or that the gut environment is restrictive to, terminal neuronal differentiation. The latter is unlikely, however, given numerous studies showing that embryonic aganglionic gut, including that of known animal models of aganglionosis (Ret, EDNRB mutants), is receptive to wild-type ENSSCs and can support mature neuronal subtypes. Further long-term differentiation studies are required but may only be possible following in vivo transplantation of ENSSCs into postnatal recipient gut. There are data to suggest that such postnatal gut is receptive to transplanted neural stem cells, although these studies used embryonic nongut-derived cells, which may not possess the same enteric potential as gut-derived stem cells.

Figure 8. Human postnatal NLBs are capable of colonizing segments of HSCR aganglionic gut maintained in tissue culture. (A) Nontransplanted aganglionic HSCR gut (patient aged 8 months) after 7 days organotypic culture showing presence of parallel TuJ1+ve neuronal fibers (arrowheads) within the smooth muscle. (B) Human embryonic NLB 5 days after placement on aganglionic gut segment from same patient showing extensive neuronal processes (arrowheads). (C) After 7 days, transplanted postnatal NLBs (arrows) from 14-year-old patient have interconnected with each other across the surface of the recipient gut forming a plexus-like arrangement (D, high power). (E and F) Cross sections through 7-day cultured aganglionic gut either nontransplanted (E) or transplanted (F) showing absence of neuronal clusters in the former but discrete clusters upon the surface and within the deeper layers (F, inset) of transplanted gut (F). Scale bar in A represents 200 μm in A, C, E, and F and 50 μm in D. Scale bar, 50 μm in B.
We have also shown that ENSSCs can be harvested from, and transplanted into, the ganglionic and aganglionic segments of HSCR gut, respectively, confirming the feasibility of autologous transplantation. It is very likely, however, that ENSSCs isolated from HSCR patients, where the proportion of RET mutations are high, will be inherently dysfunctional and will require further biologic or genetic manipulation prior to transplantation.

In conclusion, by realizing the potential of the gut mucosa as a source of ENSSCs, this study provides a significant and necessary advance for the development of practical enteric neural transplantation treatments for ENS disorders such as HSCR and might have a wider applicability for the establishment of neural components within the many facets of regenerative medicine.

**Supplementary Data**

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2009.02.048.

**References**


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Conflicts of interest
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Supplementary Materials and Methods

Generation of Human Neurosphere-Like Bodies

Human embryonic gut was incubated in 1 mg/mL Collagenase/Dispase (Roche, Mannheim, Germany) in phosphate-buffered saline (PBS) for up to 15 minutes at 37°C. Digested tissue was triturated and washed, and the cells were seeded onto 35-mm Petri dishes coated with fibronectin (2 μg/cm², Sigma Chemical Co, St. Louis, MO) and maintained in Dulbecco’s modified Eagle medium (DMEM)/F-12 medium supplemented with penicillin (100 U/mL; Sigma Chemical Co), streptomycin (100 μg/mL; Sigma Chemical Co), L-glutamine (2 mmol/L; Sigma Chemical Co), N2 (1:100, Invitrogen, Karlsruhe, Germany), basic fibroblast growth factor (20 ng/mL; Peprotech, London, UK), and epidermal growth factor (20 ng/mL; Peprotech). Culture medium was replaced every 3–4 days, and cultures were monitored for the generation of neurosphere-like bodies (NLBs).

Postnatal gut mucosal biopsy samples were obtained using endoscopic techniques. From each patient, up to 6 individual biopsy specimens (2–4 mm) were obtained for culturing and, where possible, also processed for immunohistochemistry. To generate NLBs, the postnatal gut biopsy specimens (both mucosal and full thickness) were mechanically then enzymatically dissociated (collagenase XI 750 U/mL; Sigma Chemical Co, and dispase II 250 μg/mL, Roche, Mannheim, Germany) for 15–30 minutes at 37°C to generate a cell suspension, which was plated in culture to generate NLBs as described for embryonic cultures above.

Neurosphere Differentiation Cultures

To assess the range of neuronal subtypes generated, in vitro NLBs were collected, dissociated, and seeded onto fibronectin-coated, 12-well culture dishes in DMEM/F-12 medium without growth factors and supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mmol/L), N2 (1:100), and 2% fetal calf serum (Invitrogen). Cell cultures were maintained for 14 days then processed for immunohistochemistry.

Immunohistochemistry

Immunostaining was performed on cryosections (12 μm) of frozen human gut samples, NLBs, and recipient gut samples. Gut mucosal biopsy specimens and recipient gut segments were chemically fixed in 4% paraformaldehyde (PFA) for 4 hours at 4°C, rinsed repeatedly in PBS (3–5 minutes), left overnight in 15% sucrose solution in PBS at 4°C, and then transferred to a solution containing 15% sucrose, 5% gelatin in PBS at 37°C for 1 hour. Tissues were finally placed in blocks, oriented appropriately in cooling gelatin solution, and frozen in isopentane, precooled in liquid nitrogen to −60°C. NLBs were centrifuged at 180g and then frozen in OCT embedding compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Cryosections of NLBs were fixed in 4% PFA for 10 minutes at room temperature and rinsed in PBS before application of blocking solution (PBS containing 10% sheep serum, 0.1% Triton X-100) for 30 minutes at room temperature.

Primary antibodies (Supplementary Table 1) were applied and visualized with the respective fluorochrome-conjugated secondary antibodies Alexa 488 or Alexa 568 (Invitrogen, Paisley, UK). Stained sections were cover-slipped with Vectashield containing Dapi (Vector Labs, Peterborough, UK) and images captured on a Zeiss Axioskop microscope (Zeiss, Jena, Germany) equipped with a Leica DC-500 digital camera and Leica Firecam (v1.20) software, or on a Leica Confocal microscope (Leica Microsystems, Bucks, UK). All figures were assembled and annotated using Adobe Photoshop (v9.2 software; Adobe Inc, San Jose, CA).

Preparation of Recipient Gut Cultures

To prepare chorioallantoic membrane (CAM) for culturing of recipient aganglionic gut, fertilized brown Gold Line eggs (Gallus gallus domesticus) were obtained from commercial sources and incubated at 37.5°C in a temperature-controlled brooder and humidified atmosphere. Access to the CAM was gained by cutting a window in the eggshell after 3 days incubation. Three milliliters albumen was withdrawn, and eggs were incubated for a further 5 days. For transplantation experiments, 2-mm segments of aganglionic E5 chick hindgut were prepared by carefully removing the nerve of Remak and placing the segments onto the CAM for 1 day prior to NLB transplantation.

For the experiments utilizing human aganglionic gut as recipient tissue, small segments of aganglionic gut tissue (from HSCR patients) were prepared by removing the mucosal and submucosal layers to leave intact smooth muscle layers. The residual gut was then cut into 1-to 2-mm² pieces for use in organotypic tissue cultures as previously described for murine gut. Briefly, the pieces were transferred onto uncoated membrane inserts (Millipore CM. 0.4-μm pore size; Millipore, Watford, UK) positioned within wells of a 6-well plate, each containing 1 mL culture medium, prior to NLB transplantation.

BrdU Labeling

Cell proliferation was visualized by 5-bromo-2-deoxyuridine (BrdU) proliferation assay. BrdU (10 mmol/L) was added for 1 hour to the culture medium of proliferating spheres and further cultured for another 14 days under differentiation conditions. Detection of ethanol-fixed, BrdU-positive cells was carried out with the BrdU Detection Kit I (Roche).

Reference

Supplementary Figure 1. Generation of diverse ENS appropriate neuronal phenotypes from human embryonic and postnatal NLBs. NLBs were dissociated and plated into cell culture under differentiation conditions. At 7 days, cultures from embryonic (panels A–F) and postnatal (panels G–L) NLBs showed immunoreactivity for the pan-neuronal marker TuJ1 (A and G) along with the neuronal subtypes calcitonin gene-related peptide (B and H), nitric oxide synthase (C and I), serotonin (D and J), vasoactive intestinal polypeptide (E and K), and choline acetyltransferase (F and L). Scale bars, 20 μm for A–L.
Postnatal mucosal NLBs contain dividing cells that express neuronal and glial lineage markers. To determine whether neodifferentiation was present in dividing cells seen within NLBs, BrdU was added to the culture medium of proliferating NLBs prior to dissociation and culture for another 14 days under differentiation conditions. At 14 days, cells within the cultures of dissociated postnatal mucosal NLBs expressed markers for neurons (A, PGP9.5) and glia (B, S100). BrdU incorporation (green staining) was detected in cells immunopositive for the pan neuronal marker PGP9.5 (C, arrows) and the glial marker GFAP (D, arrow). Scale bar, 50 μm for A–D.

**Supplementary Table 1.** Primary Antibodies Used in Immunostaining

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>Rabbit</td>
<td>1:600</td>
<td>Dako, Ely, UK</td>
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<tr>
<td>β-tubulin III: TuJ1</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Covance, Princeton, USA</td>
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<td>Tyrosine hydroxylase: TH</td>
<td>Rabbit</td>
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<td>BD, Heidelberg, Germany</td>
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<td>Smooth muscle actin: SMA</td>
<td>Mouse</td>
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<td>Nitric oxide synthase: NOS</td>
<td>Rabbit</td>
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<td>AbD Serotec, Oxford, UK</td>
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<td>Choline acetyl transferase: ChAT</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Sigma, Gillingham, UK</td>
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<tr>
<td>Vasoactive intestinal peptide: VIP</td>
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<td>AbD Serotec, Oxford, UK</td>
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<td>Serotonin</td>
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<td>DiaSorin, Dietzenbach, Germany</td>
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<tr>
<td>Calcitonin gene related peptide: CGRP</td>
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<td>Ki-67</td>
<td>Rabbit</td>
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<td>Novocastra, Newcastle, UK</td>
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<td>1:250</td>
<td>Promega, Southampton, UK</td>
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<tr>
<td>Sox10</td>
<td>Mouse</td>
<td>1:5</td>
<td>Dr. David J. Anderson; Caltech, Pasadena, CA</td>
</tr>
<tr>
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<td>Chemicon, Schwalbach, Germany</td>
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<tr>
<td>S100</td>
<td>Rabbit</td>
<td>1:600</td>
<td>Dako, Ely, UK</td>
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