ORIGINAL ARTICLE

Human and Murine Tissue-Engineered Colon Exhibit Diverse Neuronal Subtypes and Can Be Populated by Enteric Nervous System Progenitor Cells When Donor Colon Is Aganglionic

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Purpose: Tissue-engineered colon (TEC) might potentially replace absent or injured large intestine, but the enteric nervous system (ENS), a key component, has not been investigated. In various enteric neuropathic diseases in which the TEC is derived from aganglionic donor colon, the resulting construct might also be aganglionic, limiting tissue engineering applications in conditions such as Hirschsprung disease (HD). We hypothesized that TEC might contain a diverse population of enteric neuronal subtypes, and that aganglionic TEC can be populated by neurons and glia when supplemented with ENS progenitor cells in the form of neurospheres.

Materials and Methods: Human and murine organoid units (OU) and multicellular clusters containing epithelium and mesenchyme were isolated from both mouse and human donor tissues, including from normally innervated and aganglionic colon. The OU were seeded onto a biodegradable scaffold and implanted within a host mouse, resulting in the growth of TEC. Aganglionic murine and human OU were supplemented with cultured neurospheres to populate the absent ENS not provided by the OU to rescue the HD phenotype.

Results: TEC demonstrated abundant smooth muscle and clusters of neurons and glia beneath the epithelium and deeper within the mesenchyme. Motor and afferent neuronal subtypes were identified in TEC. Aganglionic OU formed TEC with absent neural elements, but neurons and glia were abundant when aganglionic OU were supplemented with ENS progenitor cells.

Conclusion: Murine and human TEC contain key components of the ENS that were not previously identified, including glia, neurons, and fundamental neuronal subtypes. TEC derived from aganglionic colon can be populated with neurons and glia when supplemented with neurospheres. Combining tissue engineering and cellular replacement therapies represents a new strategy for treating enteric neuropathies, particularly HD.

Introduction

CONGENITAL OR ACQUIRED defects in the enteric nervous system (ENS) can affect any region of the gastrointestinal tract, resulting in several distinct enteric neuropathies that are incompletely understood and inadequately treated such as gastroparesis and esophageal achalasia.^{1–6} Among congenital enteric neuropathies, Hirschsprung disease (HD) is the most common, with an incidence of 1 in 5000 live births.^{7,8} The etiology of HD is incomplete cephalic to caudal migration of the ENS precursor vagal neural crest cells within the gut during fetal development, resulting in absent neurons and glia in the distal colon, termed aganglionosis. The obstruction caused by

the distal aganglionic segment of intestine is fatal without surgical resection.⁹ Despite resection, many patients with HD still do not have complete resolution of their symptoms.^{10,11} In a subset of HD patients, aganglionosis affects the entire intestine, which cannot be resected, leading to devastating consequences.

The ENS consists of diverse neuronal subtypes and enteric glia arranged within a complex network of ganglia predominately located in the submucosa (submucosal plexus) and between the circular and longitudinal muscle layers (myenteric plexus) of the gastrointestinal tract.¹² Crucial roles of the ENS, many of which occur autonomously of the central nervous system (CNS), include control and modulation of motility, secretion, vasomotor function, and afferent sensing of stretch

and luminal contents.¹³ Although the development of the ENS has been investigated, little is known about postfetal homeostatic maintenance and repair. Early evidence suggests very limited *in vivo* neurogenesis and only in certain injury models,^{14,15} thus mandating the need for regenerative strategies.

The limited options for enteric neuropathies have driven the development of ENS progenitor cell therapeutics and efforts to populate aganglionic colon with an ENS. Originally developed in the CNS,^{16,17} neurosphere culture is an established method to isolate and expand ENS progenitor cells, which can be obtained from mucosal biopsy with relatively noninvasive endoscopy for a readily available autologous cell source.¹⁸ ENS progenitor cells have been transplanted in various *in vitro* and *in vivo* models and shown to differentiate into neurons and glia.^{18–29} Early evidence indicates that these cells are functional and able to electrophysiologically integrate within the host's ENS.^{28,29}

In parallel, tissue-engineered intestine is a promising therapy for organ replacement from autologous cells. Our laboratory has developed tissue-engineered colon (TEC) from innervated human and rat colonic tissue digested into multicellular clusters termed organoid units (OU) and shown that it contains differentiated epithelium, smooth muscle, and glial cells,^{30,31} which are all donor derived. The tissue-engineered intestine contains approximately three times the number of initially implanted cells, making autologously sourced donor tissue a viable option.³² However, patients with enteric neuropathies will require not only replacement tissue, but correctly innervated intestine, otherwise, functionally obstructed and phenotypically HD-like intestine will result. The presence of key neuronal subtypes and other critical ENS elements has not been confirmed in TEC and must precede progress toward human therapy.

We hypothesized that the addition of neuronal and glial progenitors to aganglionic donor colonic tissue would form all of the key components of TEC, including the ENS. Therefore, we sought to first identify the ENS elements in TEC derived from normal, ganglionated colon. We then aimed to replicate these elements in TEC derived from aganglionic murine and human tissue by supplementing aganglionic OU with ENS progenitor cells delivered as neurospheres.

Materials and Methods

Mouse strains

β -Actin/eGFP transgenic mice (gift from Dr. Wagers, Harvard Stem Cell Institute) and CAG::mRFP1 (B6.Cg-Tg(ACTB-mRFP1)1F1Hadj/J JAX Stock No. 005884) mice were maintained on a C57BL/6 background. *Ednr^b^{-/-}* mice were also maintained on a C57BL/6 background (B6.129S7-*Ednr^b^{-/-}*tm1Ywa>/FrykJ JAX Stock No. 021933).³³

Hosts for the *Ednr^b^{-/-}* TEC alone were β -actin/eGFP mice. *Ednr^b^{-/-}* TEC supplemented with neurospheres were implanted into both immunocompetent CAG::mRFP1 mice and immunosuppressed NOD/SCID gamma mice (Jackson Laboratories) irradiated with 350 cGY before implantation. All human TEC, both with and without supplemental neurospheres, were implanted into irradiated adult NOD/SCID gamma mice.

Preparation of organoid units

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Chil-

dren's Hospital Los Angeles (CHLA). Ganglionated mouse colon was obtained from 2- to 3-week old β -actin/eGFP mice. Aganglionic mouse colons were resected from 2- to 3-week old *Ednr^b^{-/-}* mice distal to the transition point and immunofluorescence staining was performed to verify aganglionosis.

Human colon samples were obtained with the approval of the Institutional Review Board of CHLA from surgical specimens that would have otherwise been discarded. The normal ganglionated human rectosigmoid colon samples were obtained from a 9-month-old to a 2-year-old male patient. OU were derived from the latter sample to identify ENS elements in TEC generated from normal human colon. Aganglionic rectosigmoid colon was obtained from a 7-week-old boy with HD and aganglionosis was verified by our own sections and the official pathology report. Both the native aganglionic control tissue and aganglionic OU were derived from this sample.

To create either murine or human multicellular OU containing epithelial and mesenchymal tissue, colonic tissue was emptied of its luminal contents, cut open longitudinally, and gently washed in 4°C Hank's balanced salt solution (HBSS; Gibco) containing 100 U/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B (Anti-Anti; Invitrogen) in serial washes until the supernatant was clear. The tissue was then cut into ~ 2 mm³ pieces and enzymatically digested with 800 U/mL collagenase type 4 (Worthington) and 0.12 mg/mL of dispase (Invitrogen) in HBSS at 37°C for 20 min. Digestion was halted with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), followed by trituration with a 10-mL serological pipette. The tissue was centrifuged at 190 g for 10 min to pellet the OU. The supernatant was then removed and the OU pellet was washed by adding DMEM plus 10% FBS and gently inverting the tube several times. This was repeated for a total of three washes.

Scaffold preparation and seeding

Two millimeters thick nonwoven polyglycolic acid scaffold (>95% porosity; Concordia Fibers) was wrapped around glass capillaries with an outer diameter of 1.5 mm and sealed with poly-L-lactic acid (DURECT Corporation) in chloroform (Sigma-Aldrich). This was sterilized with 80% ethanol, coated with 0.4 mg/mL type 1 collagen (Sigma-Aldrich) for 20 min at 4°C, rinsed with phosphate buffered saline (PBS, phosphate buffer 10 mM, KCl 2.7 mM, NaCl 137 mM, pH 7.4), and dried at room temperature within a dessicator. These were stored in the dessicator to prevent hydrolysis and degradation, and used within 12 weeks. Scaffolds were cut into 3 mm segments and OU consisting of ~ 3 million cells were directly pipetted into the lumen and onto the entire outer surface to seed the scaffold.³⁴

Preparation of enteric neurospheres

Enteric neurospheres were derived from the colons of β -actin/eGFP mice aged 3–4 weeks. After resection, luminal contents were removed and the colon was cut open longitudinally. The tissue was then rinsed with HBSS plus 100 U/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B (Anti-Anti; Invitrogen) in serial washes until the supernatant was clear. To remove the epithelium, the tissue was then treated with 20 mL of 5 mM of EDTA (Sigma) in 1 $\times$ PBS at 4°C for 30 min

with gentle rocking. The reaction was halted by adding 20 mL of DMEM plus 10% FBS, and the supernatant was discarded. Finely minced <1 mm pieces were then digested enzymatically with 800 U/mL type IV collagenase and 0.12 mg/mL dispase at 37°C for 20 min. Digestion was halted with DMEM plus 10% FBS, followed by trituration with a 10-mL serological pipette. The cells were then centrifuged at 190 g for 10 min and the pellet was washed with DMEM plus 10% FBS for a total of three washes. The cells were resuspended in 10 mL of FBS and passed through a 40- μ m cell strainer to isolate a single-cell suspension.

Following centrifugation and removal of the supernatant, cells were resuspended in 12 mL of the culture medium consisting of DMEM with 100 U/mL penicillin, 100 μ g/mL streptomycin, 250 ng/mL amphotericin B, 1% N2 supplement (Gibco), 1% FBS, 40 ng/mL epidermal growth factor (EGF; PeproTech), and 20 ng/mL basic fibroblast growth factor (FGF; Sigma). Within 1–2 days, neurospheres began to form. The culture was purified every 3–4 days by aspirating the culture medium containing the free-floating neurospheres, centrifuging the solution, removing the supernatant, and then resuspending the cells in fresh culture medium and applying this to a new culture plate. Neurosphere cultures were purified three times for a total of 9–12 days before adding them to OU.

Characterization of enteric neurospheres

Brightfield and fluorescent microscopy verified live neurospheres that were positive for green fluorescent protein (GFP). A sample of neurospheres was placed in agarose, fixed in formalin, blocked in paraffin, and sectioned for immunofluorescence staining. p75 identified the presence of ENS progenitor cells and glial fibrillary acidic protein (GFAP)-labeled enteric glial cells (Table 1). Neuronal class III β tubulin (Tuj-1) and alpha smooth muscle actin (α -SMA) identified differentiated neurons and mesenchyme, respectively. Cells from two neurosphere cultures were counted by a trained observer to determine the distribution of neuronal, glial, and mesenchymal cells.

Another sample was placed into a chamber slide with a prodifferentiation culture medium containing a higher concentration of FBS (10%) and no EGF or FGF for 7 days followed by wholemount fixation. This was stained with Tuj-1 and GFAP antibodies to assess for differentiated neurons and glia (Table 1). They were also stained with Ki67 antibody to identify proliferating cells.

Creation of TEC supplemented with neurospheres

Neurospheres were removed from culture and washed in DMEM. The solution was then centrifuged and the supernatant was removed. The neurospheres were then mixed with the aganglionic OU by gentle pipetting so that each implant contained 40–50 neurospheres. This mixture was seeded onto the scaffold as described above.

Implantation of seeded scaffolds and harvest of murine TEC

Each seeded scaffold was surgically implanted in a host mouse by wrapping it with omentum, securing the omental layers and scaffold with a suture, returning it to the peritoneal cavity, and closing the original incision. Postprocedure, host mice were given 1 mg/mL of ibuprofen (Major Pharmaceuticals) in the drinking water *ad libitum* for 3 days and trimethoprim/sulfamethoxazole (Hi-Tech Pharmaceuticals) 1 mg/mL *ad libitum* for 4 weeks.

At 4 weeks, the host mice were euthanized with carbon dioxide followed by cervical dislocation. The implants, now in the form of TEC, were removed, fixed in formalin, and embedded into paraffin blocks for histological analysis.

Histology and immunofluorescence

Five micrometers thick sections were stained with Hematoxylin and eosin (H&E) by standard protocol. For immunofluorescence, slides underwent heat-induced antigen retrieval

TABLE 1. ANTIBODY LIST

	Species	Company	Catalog No.	Dilution
Primary antibody				
E-cadherin (E-cad)	Mouse	BD Transduction Laboratories	610181	1:200
Villin	Mouse	Santa Cruz Biotechnology	Sc-66022	1:100
Mucin 2 (Muc2)	Rabbit	Santa Cruz Biotechnology	Sc-15334	1:100
Chromogranin A (ChgA)	Rabbit	Abcam	Ab15160	1:100
Neuronal class II beta-tubulin (Tuj-1)	Mouse	Covance	MMS435P-250	1:500
Glial fibrillary acidic protein (GFAP)	Mouse	Cell Signaling Technology	3670	
Alpha-smooth muscle actin (α -SMA)	Rabbit	Abcam	ab5694	1:100
Choline acetyltransferase (ChAT)	Rabbit	Bioss	Bs-0042R	1:100
Neuronal nitric oxide synthase-1 (nNOS)	Rabbit	Santa Cruz Biotechnology	sc-648	1:200
Calbindin	Rabbit	Millipore	AB1778	1:100
S-100 beta (s100b)	Rabbit	Abcam	Ab52642	1:100
Vasoactive intestinal peptide (VIP)	Rabbit	Bioss	Bs-0077R	1:100
Ki67	Rabbit	Thermo Scientific	RM-9106-S1	1:100
Nerve growth factor receptor, p75	Rabbit	Millipore	AB1554	1:200
Green fluorescent protein (GFP)	Chicken	Abcam	ab 13970	1:200
Beta-2 microglobulin (B2mcg)	Rabbit	Abcam	ab15976	1:100
Secondary antibody				
AlexaFluor anti-mouse 488	Goat	Life Technologies	A11029	1:200
AlexaFluor anti-rabbit 555	Goat	Invitrogen	A21429	1:200
AlexaFluor anti-chicken 488	Goat	Abcam	ab150169	1:200

in sodium citrate (H-3300 Unmasking Solution; Vector Laboratories) boiled in a microwave for 12 min. Slides were incubated at 4°C overnight with the following primary antibodies (Table 1): villin, mucin-2 (Muc2), chromogranin A (ChgA), Tuj-1, GFAP, α -SMA, choline acetyltransferase (ChAT), neuronal nitric oxide synthase-1 (nNOS), vasoactive intestinal polypeptide (VIP), Ki67, P75, and GFP. Cell nuclei were marked with 4',6 diamidino-2-phenylindole dihydrochloride (DAPI; Life Technologies) and coverslips were mounted with ProLong Gold (Life Technologies). Images were obtained with fluorescent microscopy (Leica DMI 6000B) and analyzed with Fiji³⁵ and/or by a trained observer. For quantification of GFP/Tuj-1 and GFP/s100b colocalization, images from three different TEC were counted.

Results

TEC derived from WT mouse colon and normal human colon

After 4 weeks, murine TEC demonstrated key components of the large intestine. On H&E staining, murine TEC demonstrated crypt architecture and epithelium similar to

native murine colon, but a comparatively more rudimentary mesenchyme was observed (Fig. 1A, F, K). In murine TEC generated from β -actin/eGFP OU, lineage tracing with GFP immunofluorescence demonstrated the donor origin of the epithelium (Fig. 1G), replicating the GFP⁺ epithelium of native β -actin/eGFP colon (Fig. 1B). Staining for cell type specific markers villin (enterocytes), Muc-2 (goblet cells), and ChGA (enteroendocrine cells) revealed that murine TEC developed a villin-lined epithelium, mucin-filled goblet cells, and occasional enteroendocrine cells (Fig. 1H, I), thus replicating native murine colon (Fig. 1C, D). In TEC, the lumens beside the epithelia were mucin filled as well. Proliferation of the epithelium was evaluated with Ki67 staining and demonstrated proliferative crypt-based epithelial cells in murine TEC (Fig. 1J), similar to native colon (Fig. 1E). Unlike native colon, proliferation was also evident in the mesenchyme. Costaining for GFP with mesenchymal marker α -SMA (Fig. 1L, higher magnification 1M) or neuronal marker Tuj-1 (Fig. 1N, higher magnification 1O) indicated a mixed host/donor origin of the mesenchyme (GFP = 29% $\pm$ 13%) and neurons (GFP = 48% $\pm$ 11%).

FIG. 1. Murine TEC derived from β -actin/eGFP OU originates from donor OU and contains a differentiated epithelium and mesenchyme. On Hematoxylin and eosin staining, TEC exhibits crypt architecture and epithelium (F, K) similar to native murine colon (A). Donor colon was taken from β -actin/eGFP mice, which stains ubiquitously for GFP (B). The epithelium of murine TEC derived from β -actin/eGFP OU and much of the mesenchyme are GFP⁺, indicating donor origin of these cells (G). Differentiated epithelial subtypes are found in TEC, including villin-lined colonocytes, mucin-filled goblet cells, and enteroendocrine cells (H, I), similar to native murine colon (C, D). Mucin is present within the lumen of TEC (H). Virtually all observed proliferation in native murine colon localized within the epithelial crypt bases (E). In contrast, murine TEC demonstrated significant proliferation in the mesenchyme as well as in the crypt bases (J). Smooth muscle beneath the epithelium (L) only partially colocalizes with GFP (M, higher magnification), indicating mixed host/donor origin of mesenchyme. Subepithelial nerve cells are also of mixed host/donor origin (N, O higher magnification). Scale bars 25 μ m in (M, O), all other scale bars 100 μ m. GFP, green fluorescent protein; OU, organoid units; TEC, tissue-engineered colon. Color images available online at www.liebertpub.com/tea

Murine TEC (Fig. 2C, D) also demonstrated a large amount of smooth muscle located subjacent to the epithelium, often in multiple layers, although it was not consistently in the discrete muscularis mucosa, inner circular, and outer longitudinal layers as in native murine colon (Fig. 2A,

FIG. 2. The mesenchyme of human and murine TEC consists of smooth muscle, neurons, and glia. Native murine colon demonstrates typical laminated layers of smooth muscle with Tuj-1⁺neurons and GFAP⁺ glia in the submucosal and myenteric plexi (A, B). Human native colon contains thicker muscle layers, and larger neuronal structures as seen in a Tuj-1-stained myenteric ganglion (E) and S100β⁺ glial staining of a submucosal ganglion (F). In murine and human TEC, smooth muscle is abundant, but not strictly organized into the circular and longitudinal layers (C, G). Neurons and glia in murine and human TEC are loosely clustered within the smooth muscle and subjacent to the epithelium (C, D, G, H). All scale bars 100 μm. GFAP, glial fibrillary acidic protein. Color images available online at www.liebertpub.com/tea

B). Clusters of Tuj-1⁺neuronal and S100β⁺ glial staining in murine TEC were found mostly within tracts of the smooth muscle, but also in areas just below the epithelium in a rudimentary replication of native enteric ganglia localization to the submucosal space and area between the circular and longitudinal muscle layers.

Compared to native murine colon, native human colon had relatively thicker smooth muscle layers, a larger submucosal space, and large ganglia in the myenteric plexus and submucosal space (not shown) (Fig. 2E, F). In human TEC, large amounts of smooth muscle were present in close association with the epithelium (Fig. 2G), although it was not organized consistently into distinct layers. Neural and glial staining with Tuj-1 and S100β, respectively, revealed neurons and glia in close proximity to the epithelium or deeper within the smooth muscle (Fig. 2G, H), although typical ganglion-like structures were not achieved.

The extent of neuronal differentiation was evaluated with antibodies to ChAT, nNOS, VIP, and calbindin to clarify the functional identity of these neurons. In native murine and human colon, the myenteric ganglia displayed a large proportion of ChAT and nNOS neurons (Fig. 3A, B, I, J), while VIP and calbindin were found in fewer numbers and most prominently in the submucosal space (Fig. 3C, D, K, L). Similarly, ChAT and nNOS staining in murine and human TEC were observed in a majority of neurons (Fig. 3E, F, M, N), and VIP and calbindin expressing neurons were present, but in smaller proportion and typically in close proximity to the epithelium (Fig. 3G, H, O, P).

Neurospheres derived from β-actin/eGFP mice

Two neurosphere cultures were created, each from the colons of four β-actin/eGFP mice. On brightfield imaging, neurospheres with typical spherical morphology and green fluorescence floated in the supernatant of the culture medium (Fig. 4A, B). Immunofluorescent staining of sectioned neurospheres revealed that many cells expressed the ENS progenitor cell marker p75 alone (45–60%) as well as glial cells coexpressing p75 and GFAP (17–32%) (Fig. 4C). Many cells also expressed Tuj-1 (25–45%) (Fig. 4D), but few expressed α-SMA (1–5%) (Fig. 4E). Under prodifferentiation culture conditions, neurospheres gave rise to neurons and glia, many of which were positive for Ki67, indicating these were actively cycling cells, likely differentiated from neurosphere progenitors, rather than surviving terminally differentiated neurons and glia (Fig. 4F, G).

TEC derived from aganglionic mouse and human colon, with and without supplemental neurospheres

Aganglionic colon was obtained from Ednrb^{-/-} mice (Fig. 5A) by selecting colon distal to the visible transition point of obstruction. Immunofluorescence staining of the proximal end of this resected colon confirmed aganglionosis (Fig. 5B, C). One group of murine aganglionic OU was seeded onto scaffolds and implanted alone into β-actin/eGFP hosts. The other group was supplemented with β-actin/eGFP neurospheres on the scaffold before implantation into either immunocompetent CAG::mRFP1 mice for lineage tracing or NOD/SCID mice to determine the outcome of *in vivo* neurosphere culture. Both the β-actin/eGFP and CAG::mRFP1 strains of mice are maintained in a C57BL/6 background.

FIG. 3. Neurons in murine and human TEC include key motor and sensory neuronal subtypes. In native murine and human colon, ChAT and nNOS mark a large proportion of enteric neurons, particularly in the myenteric ganglia (**A, B, I, J**). Murine and human TEC also demonstrate ChAT⁺ and nNOS⁺ neurons both subjacent to the epithelium and in areas deeper in the mesenchyme (**E, F, M, N**). VIP⁺ neurons, likely vasosecretomotor neurons, were observed in the submucosal ganglia in the relatively small submucosal space of the native murine colon (**C, arrow**) as well as in the submucosal ganglia of native human colon (**K, arrow**). In murine and human TEC, VIP⁺ neurons were also observed (**G, O, arrows**). Calbindin staining identified intrinsic primary afferent sensory neurons, and was observed in the myenteric (**D**) and submucosal (**L**) ganglia in native murine and human colon. Although less numerous, calbindin⁺ neurons were also found in murine and human TEC, typically in close association with the epithelium (**H, P**). All scale bars 100 μ m. ChAT, choline acetyltransferase; VIP, vasoactive intestinal polypeptide. Color images available online at www.liebertpub.com/tea

Four weeks after implantation, murine TEC with and without neurosphere supplementation contained intestinal epithelium with all differentiated epithelial cell types and surrounding smooth muscle (Supplementary Fig. S1A–J; Supplementary Data are available online at www.liebertpub.com/tea). TEC derived from Ednrb^{-/-} aganglionic OU alone demonstrated relatively poor epithelial growth (Fig. 5D), a partial contribution of non-ENS mesenchymal cells from the host, and scant to absent neuronal and glial staining (Fig. 5E, F). In contrast, the TEC supplemented with GFP neurospheres demonstrated improved epithelial structures such as crypt organization (Fig. 5G). Enterocytes, goblet cells, and enteroendocrine cells were all GFP (Supplemen-

tary Fig. S1F–H). A large amount of GFP⁺/α-SMA⁻ staining surrounding the epithelium (Supplementary Fig. S1I) was positive for neuronal and glial markers, indicating that neurosphere-derived ENS progenitor cells had survived and differentiated within this construct (Fig. 5H, I). Of all Tuj-1⁺ elements, 49% ± 25% were also GFP⁺, comparable to the percentage of GFP⁺ neurons seen in TEC derived from β-actin/eGFP OU. Similarly, 44% ± 15% of all s100b⁺ elements were also GFP⁺. Additionally, various neuronal subtypes were present, as identified by GFP⁺/Tuj-1⁺/ChAT⁺ and GFP⁺/Tuj-1⁺/nNOS⁺ subepithelial cells (Supplementary Fig. S1K, L). The neurosphere-derived elements were found mostly within the smooth muscle and

FIG. 4. Neurospheres derived from β -actin/eGFP murine colon differentiate into neurons and glia. Early neurospheres before second purification exhibit typical spherical morphology, are free floating, and retain GFP signal (**A**, **B**). Neurospheres consisted of many cells expressing the enteric nervous system progenitor cell marker p75 alone (45–60%) as well as glial cells coexpressing p75 and GFAP (17–32%) (**C**), before mixing with aganglionic OU. Many cells also express the neuronal marker Tuj-1 (25–45%) (**D**), but few express the smooth muscle marker α -SMA (1–5%) (**E**). Neurospheres in prodifferentiation culture conditions gave rise to neurons (**F**) and glia (**G**), many of which were positive for Ki67 (arrows). Scale bars (**A**–**E**) 100 μ m; (**F**, **G**) 25 μ m. α -SMA, alpha smooth muscle actin. Color images available online at www.liebertpub.com/tea

beneath the epithelium, where neural projections appeared to encircle the epithelium.

To generate human TEC, full-thickness aganglionic rectosigmoid colon (Fig. 6A) was obtained from a 7-week-old male patient with HD undergoing initial resection. The

clinical pathologist's diagnosis was verified by immunofluorescence staining showing aganglionosis (Fig. 6B, C). Again, the resulting aganglionic human OU were separated into two groups, those with and without supplemental β -actin/eGFP neurospheres. Both groups were seeded onto

FIG. 5. Neurospheres populate murine TEC derived from aganglionic colon with neurons and glia. The distal segment of resected colons from *Ednrb*^{-/-} mice (**A**) demonstrated no ganglia when evaluated for neurons (**B**) and glia (**C**). TEC created from this aganglionic colon, but not supplemented with neurospheres was implanted in β -actin/eGFP hosts. The epithelium within this TEC was flat and simple (**D**). Mesenchyme demonstrated host contribution, but no neurons (**E**) or glia (**F**) from either the donor or the host. Aganglionic *Ednrb*^{-/-} OU supplemented with GFP⁺ neurospheres and implanted within either CAG::mRFP1 or NOD/SCID gamma host resulted in TEC with more complex architecture (**G**) surrounded by large clusters of neurons and glia that retained GFP (**H, I**), as confirmed by costaining of GFP with Tuj-1 and S100b, respectively (high magnification *insets* in **H, I**). Arrows in (**H, I**) point to areas magnified in *insets*. *Inset* scale bars 25 μ m, all other scale bars 100 μ m. Color images available online at www.liebertpub.com/tea

scaffolds and implanted into the omentum of NOD/SCID mice. Regardless of whether neurospheres were supplemented, aganglionic OU generated human TEC that contained all differentiated epithelial cell types and surrounding smooth muscle with proliferation present in both the epithelium and mesenchyme (Supplementary Fig. S2A–J). In both groups, staining for β -2 microglobulin, which is specific for human cells, demonstrated that the epithelium was of human origin, but the mesenchyme was derived from both host and donor (Fig. 6E, H). This is consistent with our previous work.^{30,32}

Human TEC derived from aganglionic HD OU, but not supplemented with neurospheres demonstrated a paucity of ENS elements (Fig. 6E, F). In contrast, aganglionic HD OU supplemented with neurospheres contained a more complex epithelium (Fig. 6G) with GFP enterocytes, goblet cells, and enteroendocrine cells (Supplementary Fig. S2F–H). The epithelium was surrounded by GFP⁺ neuronal and glial staining (Fig. 6H, I). Further staining for ChAT and nNOS revealed that the neuronal progenitors had differentiated into various subtypes of neurons (Supplementary Fig. S2K, L). Of the GFP⁺ cells with a strand-like appearance, some were S100b⁺ whereas some were α -SMA⁺ (Fig. 6I), although the

α -SMA staining was weaker than GFP⁺/SMA⁻ cells (Supplementary Fig. S2I).

Discussion

Populating aganglionic TEC with neurosphere-derived ENS progenitor cells is a promising new strategy to correct the absence of the ENS, which occurs in multiple clinical conditions. The survival and differentiation of these ENS progenitor cells into neurons and glia is essential to correct intestinal aganglionosis. This study identified neurons and glia in TEC that was formed from donor cells obtained from murine and human aganglionic large intestine when supplemented with neurospheres derived from normally innervated colon. In our *in vivo* model, the postnatal-derived ENS progenitor cells remained viable at 4 weeks, consistent with other published results,²⁸ providing further support for this approach to cellular replacement as a durable therapy.

Both TEC derived from ganglionated colon and aganglionic TEC with added neurospheres contain glia, neurons, and mesenchyme rich in smooth muscle. Meaningful regeneration of the ENS within TEC is supported by the presence of cellular markers that indicate further differentiation toward

FIG. 6. Neurospheres populate human TEC derived from aganglionic colon with neurons and glia. The aganglionic margin of resected Hirschsprung disease colon (**A**) demonstrated no ganglia when evaluated for neurons (**B**) and glia (**C**). TEC created from this aganglionic colon formed multiple lumens (**D**) lined by β 2-microglobulin staining epithelium of human origin, mesenchyme of mixed host/donor origin, absent neuronal staining (**E**), and scant glial staining (**F**). OU supplemented with GFP⁺ neurospheres resulted in TEC with more complex epithelium (**G**) enveloped by neuronal structures of neurosphere origin (**H**) as confirmed by costaining of Tuj-1 and GFP on higher magnification *insets*. Neurosphere-derived glial cells and projections also enveloped the epithelium (**I**). An enlarged view of these glial cells confirming costaining of S100b and GFP is featured in the *inset*. Arrows in (**H**, **I**) point to areas magnified in *insets*. *Inset* scale bars 25 μ m, all other scale bars 100 μ m. Color images available online at www.liebertpub.com/tea

distinctive neuronal subtypes. Although ChAT may be found in other neuronal subtypes (including subpopulations of secretomotor neurons), the vast majority of ChAT myenteric neurons are excitatory motor neurons, and calbindin is uniquely expressed in intrinsic primary afferent (sensory) neurons. The nNOS expression is primarily seen in inhibitory motor neurons. Essentially all VIP⁺ submucosal neurons are secretomotor/vasodilator neurons, although VIP may also be expressed in myenteric inhibitory motor neurons.^{12,36} Interneurons and intestinofugal neurons represent a small proportion of ENS neurons, but they play important roles in intrinsic reflex circuitry and feedback signaling to the CNS, respectively.

Given the limitations of employing several antibodies simultaneously, we interpret ChAT⁺ neurons to indicate likely excitatory motor neurons, nNOS⁺ neurons to indicate likely inhibitory motor neurons, calbindin⁺ neurons to indicate intrinsic primary afferent neurons, and VIP⁺ neurons to indicate potential secretomotor/vasomotor neurons. Within both TEC derived from normal colon and TEC derived from aganglionic colon supplemented with neurospheres, ChAT⁺ and nNOS⁺ neurons were identified (Fig. 3A, B, E, F, I, J, M,

N, and Supplementary Figs. S1K, L and S2K, L) predominantly within the smooth muscle. VIP⁺ neurons (Fig. 3C, G, K, O) and calbindin⁺ neurons were also observed (Fig. 3D, H, L, P). Thus, for the first time, evidence of key ENS neuronal subtypes is demonstrated in both human and murine TEC, including TEC generated from aganglionic colon supplemented with neurospheres.

Interestingly, the TEC derived from our human aganglionic OU supplemented with β -actin/eGFP mouse-derived neurospheres contained some GFP⁺SMA⁺ cells. Neurosphere-derived SMA⁺ cells have been described by other groups using similar culture methods^{20,37} and may represent either greater differentiation potential of the neurosphere progenitor cells or incomplete purification of the culture. Although the organization of these mesenchymal components is comparatively disorganized, this study has taken the initial steps to demonstrate that OU contain essential elements for mesenchymal regeneration, including neural elements. Our laboratory has previously shown that in a rat model, after TEC was put in continuity with luminal contents, significant remodeling of the mesenchyme resulted in normalization of the muscle layer.³¹ Consequently, this initial step

of validating murine TEC enables further investigations with transgenic animals.

HD is attributed to defects in genes in the *RET*, endothelin, and other pathways involved in neural crest cell migration, survival, and differentiation.³⁸ Previous studies have shown that when transplanted into normal gut, the ENS stem cells in neurospheres can migrate, proliferate, and differentiate.^{28,39} However, there are concerns that the mesenchymal milieu of aganglionic colon deficient in these factors may not support interventions for neo-innervation. Aganglionic TEC with added neurospheres demonstrated ENS progenitor cell survival and differentiation. Clusters of neuronal and glial cells were present in previously aganglionic colon, and the GFP colocalization confirmed their neurosphere origin (Figs. 5H, I and 6H, I). In the *Ednrb*^{-/-}-derived murine TEC supplemented with GFP neurospheres, the presence of ENS elements is less surprising since *Ednrb* is expressed on ENS precursors and not in the surrounding mesenchyme. With regard to the human TEC, *Ednrb* mutations are responsible for only a small percentage of HD and the genotype of the HD patients in this study is unknown. Yet, our data demonstrate that the HD mesenchymal milieu is not always prohibitive to neurosphere-derived ENS progenitor cells. This is consistent with the published results showing that ENS progenitor cells can migrate, differentiate, and function when transplanted as neurospheres into aganglionic mouse colon in vitro.²² In tissue-engineered intestine, the process of tissue engineering itself may further encourage ENS migration, proliferation, and differentiation through close proximity of cell types that may be disordered in diseased development.

Notably, the ENS is known to have important modulatory functions on intestinal epithelium and in our data, neurosphere-derived Tuj-1⁺ and S100b⁺ cells were found largely in close association with the epithelium, often enveloping the epithelium in their projections. We observed that the epithelium of aganglionic TEC supplemented with neurospheres exhibited more complex crypt architecture than observed in aganglionic TEC alone, although at baseline, even TEC derived from normal colon can develop epithelium of varying histological complexity. However, even in the absence of neurospheres, aganglionic TEC contained all differentiated epithelial cell types and smooth muscle (Supplementary Figs. S1A–D, F–I and S2A–D, F–I), and proliferation was seen throughout the epithelium and mesenchyme (Figs. 1E, J and 2E, J). These findings suggest that the neurosphere-derived neurons and glia may promote more robust epithelial regeneration, but are not required for epithelial cell differentiation, smooth muscle formation, or epithelial and mesenchymal proliferation. This is consistent with a study demonstrating that parasympathetic innervation in the salivary gland increases branching morphogenesis and regulates the balance between stem cell proliferation and differentiation.⁴⁰ Similarly, another study has shown that adding neurospheres to the intestine of rats recovering from necrotizing enterocolitis increased the villus height.⁴¹ Glucagon-like peptide 2 has been found to activate intestinal epithelium through receptors on enteric neurons, thereby modulating epithelial cell activity and promoting growth.⁴² Further regulation of mucosal proliferation and apoptosis in response to injury also occurs through enteric neuronal serotonin signaling,⁴³ calcitonin gene-related peptide,⁴⁴

enteric glial-derived *s*-nitrosoglutathione,⁴⁵ enteric glial-derived neurotrophic factor,⁴⁶ and enteric glial-derived pro-EGF.⁴⁷

Several other genetic defects are implicated in HD,³⁸ and there is evidence that nongenetic causes, such as *in utero* exposures or vitamin A deficiency,⁴⁸ can result in HD-like phenotypes.⁴⁹ To assess this study's generalizability, future experiments will explore the potential of neurosphere supplementation in TEC derived from aganglionic colon of various etiologies. Moreover, tissue-engineered esophagus,^{50,51} stomach,^{52,53} and small intestine^{52,53–55} have been formed in highly similar protocols to the process employed to form TEC, and neurosphere supplementation for enteric neuropathies involving each of these tissue-engineered structures remains to be explored. Because these neurons participate in complex physiological processes, further work is required to optimize ENS organization in TEC, assess motor function, identify the reproduction of neuronal reflex pathways, and evaluate proper interfacing of secretomotor and vasomotor neurons with the epithelium and vasculature.

Enteric neuropathies represent a formidable clinical challenge with suboptimal treatment options. As demonstrated in our study with HD, combining tissue engineering and ENS cell replacement therapy offers a fundamental shift in the therapeutic philosophy of enteric neuropathies: correction of the underlying pathology. Advantages of this approach include the autologous sourcing of OU from necessarily resected colon coupled with the potential autologous endoscopic sourcing of neurospheres from normally innervated segments of the gastrointestinal tract. Autologously acquired donor tissue would avoid the morbidity of cellular allografts and potential need for lifelong immunosuppression.⁵⁶ Furthermore, this strategy does not require lentiviral transfection, as seen in induced pluripotent stem cell protocols, or embryonic stem cells with its attendant ethical limitations.⁵⁷

Human and murine TEC exhibit a differentiated epithelium, smooth muscle, glia, and key enteric neuronal subtypes. TEC created from aganglionic colon can be populated with ENS progenitor cells that differentiate into neurons and glia. This approach may represent a new solution for enteric neuropathies, particularly HD.

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Disclosure Statement

No competing financial interests exist.

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