Published in final edited form as:

J Surg Res. 2018 March ; 223: 155–164. doi:10.1016/j.jss.2017.11.015.

Critical Intestinal Cells Originate from the Host in Enteroid-Derived Tissue Engineered Intestine

Barrett P. Cromeens^a, Yijie Wang^a, Yanchun Lui^a, Jed Johnson^b, and Gail E. Besner^a

^aDepartment of General Pediatric Surgery, Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH, 43205, USA

^bNanofiber Solutions, Inc., 4389 Weaver Court North, Hilliard, OH 43026

Abstract

Background—Enteroid-derived tissue engineered intestine (TEI) contains intestinal subepithelial myofibroblasts (ISEMF) and smooth muscle cells (SMC). However, these cell types are not present in the donor enteroids. We sought to determine the origin of these cell types and to quantify their importance in TEI development.

Materials and Methods—Crypts from pan-EGFP or LGR5-EGFP mice were used for enteroid culture and subsequent implantation for the production of TEI. TEI from pan-EGFP enteroids was labeled for smooth muscle α -actin (SMA) to identify ISEMFs and SMCs and green fluorescent protein (GFP) to identify cells from pan-EGFP enteroids. FISH for the Y-chromosome was applied to TEI from male LGR5-EGFP enteroids implanted into female NOD/SCID mice. To identify chemotactic effects of intestinal epithelium on ISEMFs, a Boyden chamber assay was performed.

Results—Immunofluorescence of TEI from pan-EGFP enteroids revealed GFP-positive epithelium with surrounding SMA positivity and no co-localization of the two. FISH of TEI from male LGR5-EGFP enteroids implanted in female NOD/SCID mice revealed that only the epithelium was Y-chromosome positive. Chemotaxis assays demonstrated increased ISEMF migration in the presence of enteroids (983 ± 133) compared to either Matrigel alone (357 ± 36) or media alone (339 ± 24) ($p = 0.05$).

Corresponding author: Gail E. Besner, MD, Department of Pediatric Surgery, FOB6135, Nationwide Children's Hospital, 700 Children's Drive, Columbus, Ohio 43205, USA, Gail.Besner@nationwidechildrens.org, Phone: (614) 722-3930, FAX: (614) 722-3903.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Contributions: Barrett Cromeens was involved with all aspects of design, experimentation, data analysis, and writing of the manuscript. Yijie Wang and Yanchun Liu were involved with experimentation, data analysis, and manuscript writing. Jed Johnson was involved with the experimental design, data analysis, and manuscript writing. Gail Besner was involved with all the previously mentioned domains.

Disclosure Statement: Jed Johnson is a founder of Nanofiber Solutions, Inc., a biotechnology company that manufactures bioabsorbable scaffolds for tissue engineering applications. While we have utilized Nanofiber Solutions scaffolds in other research, no Nanofiber Solutions products were utilized in this work. All other authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article. Neither funding source held any role in the study design, data collection, analysis, or interpretation, writing of the report, or the decision to submit for publication.

Conclusion—Lack of GFP/SMA co-localization suggests that ISEMFs and SMCs are derived from host animals. This was confirmed by FISH which identified only epithelial cells as being male. All other cell types originated from host animals. The mechanism(s) by which these cells are recruited is unknown, however, Boyden chamber assays indicate a direct chemotactic effect of intestinal epithelium on ISEMFs.

Keywords

Tissue engineering; short bowel syndrome; intestine; enteroids; epithelial culture; intestinal subepithelial myofibroblast

1. INTRODUCTION

Stem cells are the progenitors of healthy tissue and reside in specific anatomic and functional locations known as the stem cell niche. While stem cells differentiate into the healthy cells that comprise complex tissues, it is not the stem cells themselves but the complex interplay of these cells within their niche that determine restitution and maintenance. With increased interest in stem cells as therapeutic targets, understanding the determinant relationships within the niche is of paramount importance for therapeutic success.¹

The intestine is lined by epithelium comprised of differentiated cells, including enterocytes, enteroendocrine cells, goblet cells, and Paneth cells, all derived from intestinal stem cells which reside in the crypt domain. Like many other stem cell compartments, the intestinal stem cell has been the subject of increasing interest in clinical applications for intestinal pathologies.^{2–4} This increased interest has resulted in focused research in understanding the intestinal stem cell niche composed of Paneth cells, extracellular matrix, and varying pericryptal cell types.^{5–8} Of particular importance within this niche are the intestinal subepithelial myofibroblasts (ISEMF) which are aligned below the basement membrane and concentrated around the crypts.⁹ The ISEMFs express numerous non-canonical Wnt signals which are implicated in epithelial proliferation and differentiation.^{10, 11} The importance of ISEMFs is underscored in studies performed using three-dimensional epithelial culture systems known as enteroid cultures. Enteroid cultures are purely epithelial cultures that are maintained without the mesenchymal niche through exogenous replacement of critical growth factors such as R-spondin which contribute to Wnt/ β -catenin signaling. Co-culturing of enteroids with a feeder layer of ISEMFs results in larger, more robust enteroids that proliferate at a faster rate. Additionally, in the presence of ISEMFs, enteroids continue to grow and proliferate in the absence of some of these exogenous factors.¹²

In the production of tissue engineered intestine (TEI), the importance of each component of healthy functioning intestine is critical. The main goal of producing TEI is to correct short bowel syndrome, where patients have lost the majority of their bowel resulting in loss of absorption of vital nutrients.^{13–17} Ideally, this would require the development of an entirely functional length of intestine from the patient's native tissue in order to replace that which has been lost. In order to do so, all components critical to normal intestinal function must be present. One method of TEI production utilizes cultured enteroids to seed onto scaffolds and

implant into host animals. Using these general methods, the TEI produced has been shown to contain all the major epithelial lineages.^{12, 18–20} Additional components have also been identified such as ISEMFs and smooth muscle cells (SMC).²⁰ The presence of ISEMFs and SMCs within the TEI was unexpected, since the enteroids from which the TEI was grown are a purely epithelial culture and these supportive cells are not present at the time of implantation. This suggests that these critical cell types are either transdifferentiated from donor epithelial lineages through an epithelial-mesenchymal transition or are recruited from the host animal.

In this study, we investigated the origins of ISEMFs and SMCs in enteroid-derived TEI. We set out to determine if these cell types were being recruited from the host animal or transdifferentiated from donor epithelium. Additionally, we investigated whether the supplementation of ISEMFs to TEI produces more robust epithelial growth, analogous to that seen with *in vitro* studies. We hypothesized that supplementing TEI with additional ISEMFs will result in a more robust product.

2. MATERIALS AND METHODS

2.1 Animal Use

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals, with the approval of our Institutional Animal Care and Use Committee (IACUC) under protocol # AR12-00001. These experiments were performed with LGR5-EGFP transgenic mice (n=20; male = 11/female = 9), pan-EGFP transgenic mice (n=3; all male), or Non-Obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice (n=43; male = 16; female = 27) depending on the experimental design. All mice were bred in house. The LGR5-EGFP and pan-EGFP colonies were obtained from The Jackson Laboratory (Bar Harbor, ME). The NOD/SCID colony was obtained from Charles River Laboratories (Wilmington, MA). All enteroid cultures were obtained from either LGR5-EGFP mice or pan-EGFP mice depending on experimental design. All seeded scaffolds were implanted into NOD/SCID mice.

2.2 Isolation of Crypts and Culture of Enteroids

The methods used for the isolation of crypts and subsequent enteroid culture have been described previously.²⁰ In brief, small intestine was obtained from donor mice, opened longitudinally, minced, and washed with phosphate buffered saline (PBS) (Corning, Manassas, VA) until clean. The minced tissue was then incubated in 2 mM ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Waltham, MA) in PBS at 4°C for 30 min. After the incubation, the tissue underwent cycles of agitation by trituration to release mucosal fragments and settling to capture the mucosal fragment-containing supernatant until no further fragments were identified in the supernatant. The supernatant was then filtered through a 70 µm sieve (BioDesign Inc. of New York, Carmel, NY) to separate the crypts from the larger fragments. The crypt fraction underwent a series of washes and were resuspended in growth factor reduced Matrigel (Corning, Manassas, VA) at 100–200 crypts/50 µL of Matrigel. They were then plated into 24-well culture plates at 50 µL/well. Each well was incubated with 500 µL of complete crypt culture medium at 37°C

and changed every 3–4 days. The medium contained Advanced DMEM/F12 (Thermo Fisher Scientific, Waltham, MA), 2mM GlutaMAX, 10 mM HEPES, 100 U/mL penicillin/100 µg/mL streptomycin, 1× N2 supplement, 1× B27 supplement (all from Thermo Fisher Scientific, Waltham, MA), 1mM N-Acetylcysteine (Sigma, St. Louis, MO), 50 ng/mL HB-EGF (R&D Systems, Minneapolis, MN), 100 ng/mL Noggin (R&D Systems, Minneapolis, MN), and 500 ng/mL R-spondin (Sino Biological Inc., Beijing, China).

2.3 ISEMF Isolation and Culture

Donor mice underwent laparotomy and small intestine from the ligament of Treitz to the terminal ileum was excised. The intestine was opened longitudinally and the villi were scraped from the surface using a glass cover slip. The intestine was then minced and washed sequentially with PBS until no gross debris could be identified. The tissue was then digested in a 30 mL sterile solution of serum-free DMEM (Thermo Fisher Scientific, Waltham, MA) with 150 U/mL of collagenase type I (Worthington Biochemical Corp., Lakewood, NJ), and 1 U/mL of dispase (Worthington Biochemical Corp., Lakewood, NJ) for 30 min at 37°C. Once complete, the digestion was stopped by the addition of 10% fetal bovine serum (FBS) (Atlanta Biologics, Flowery Branch, GA). The solution was then triturated by pipetting up and down through a 10 mL pipette for 30 sec. The mixed cell and tissue fragment suspension was then filtered through a 15 µm sieve (BioDesign Inc. of New York, Carmel, NY) to capture single cells and small cell clusters. The solution of cells and cell clusters was pelleted at 300 *g* for 7 min and the supernatant discarded. The pelleted cells were washed in DMEM and centrifuged in this fashion three times. The washed cells were seeded into T-75 flasks and cultured in DMEM supplemented with 10% FBS, 10 µg/mL insulin (Sigma, St. Louis, MO), 10 µg/mL transferrin, 100 U/mL penicillin/100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA), and 2 ng/mL epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN), and cultured at 37°C in a humidified 5% CO₂ incubator. Growth media was changed every 2–3 days. Once the flasks reached 80% confluence, the flasks were passaged in a 1:3 ratio using 0.05% trypsin/EDTA (Thermo Fisher Scientific, Waltham, MA) until the majority of cells were released and then neutralized using growth media containing FBS. All cells used for these studies were from passages 2–5.

2.4 Scaffold Fabrication, Seeding, and Implantation

The details of scaffold fabrication, seeding, and implantation have been previously described.²⁰ In brief, scaffolds were fabricated from polyglycolic acid (PGA) biofelt (Biomedical Structures, Warwick, RI) by wrapping the felt around a 3 mm diameter mandrel and coating with 5% polylactic acid (PLLA) (Sigma, St. Louis, MO) to produce 5 mm × 3 mm internal diameter tubular scaffolds. Enteroids at 10–14 days in culture were mechanically released from matrigel, washed, and pelleted via centrifugation three times. Phase contrast microscopy was utilized to quantify the enteroids in solution, and the enteroids were pelleted a final time. They were then resuspended to seed 50 µL of enteroid/Matrigel solution onto each scaffold at a concentration of 20 enteroids/mm³ of scaffold material. Seeded scaffolds were implanted into the peritoneal cavity of immunocompromised NOD/SCID mice via midline laparotomy for a total of 4 weeks of *in vivo* incubation.

2.5 ISEMF Characterization

ISEMFs were characterized using a combination of IF and flow cytometry. For IF, ISEMFs were plated in 8-well culture slides (Thermo Fisher Scientific, Waltham, MA) with 10,000 cells/well and allowed to adhere overnight at 37°C. The cells were then fixed using 4% paraformaldehyde (PFA) for one hour at room temperature. They were then incubated at 4°C overnight with primary antibodies against either smooth muscle alpha actin (SMA) (1:500; Thermo Fisher Scientific, Waltham, MA) and vimentin (1:500; Millipore, Billerica, MA) or SMA and desmin (1:500; Abcam, Cambridge, MA). The slides were then incubated with Alexa Fluor 568 and Alexa Fluor 488 secondary antibodies (Life Technologies, Carlsbad, CA), mounted using Vectashield with DAPI, and imaged using a Zeiss 710 confocal microscope (Peabody, MA). For flow cytometry, ISEMFs were first removed from tissue culture dishes and washed in flow wash buffer (1% BSA and 0.1% sodium azide in PBS). An equal number of cells ($0.5\text{--}1 \times 10^6$) were subjected to flow cytometry analysis. Briefly, cells were fixed and permeabilized using an intracellular staining/permeabilization kit (Biolegend, San Diego, CA) according to the manufacturer's recommendation. The cells were then blocked with ChromPure mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 15 min followed by primary antibody labeling at room temperature for 30 min using SMA (Thermo Fisher Scientific, Waltham, MA), desmin (Abcam, Cambridge, MA), and vimentin (Millipore, Billerica, MA). The cells were washed and then labeled with the fluorescent conjugated secondary antibodies phycoerythrin (PE) (Cell Signaling Technology, Danvers, MA), allophycocyanin conjugate (APC) (Santa Cruz Biotechnology, Santa Cruz, CA) or fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, West Grove, PA) for 30 min. Cells were washed and measured with a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (version 7.6.5 Ashland, OR).

2.6 Histology, Immunofluorescence (IF), and Fluorescence *in situ* Hybridization (FISH)

All TEI was explanted and fixed with 10% neutral buffered formalin overnight. After fixation, scaffolds were transected across the axis of the lumen and paraffin embedded with the cut surface down. In experiments where neomucosa was quantified, 5 µm sections were taken at 300 µm intervals through the length of the implant. Each section underwent H&E staining to confirm the presence of TEI defined by the presence of any simple columnar epithelium. A representative section from each specimen was further analyzed via light microscopy with ImageJ software for the quantity of neomucosa present. For each section, the basal membrane of all the simple columnar epithelium was traced and quantified in µm. For origin experiments utilizing IF and FISH, three 5 µm sections were taken at 150 µm intervals. The first section at each level was stained with H&E to confirm the presence or absence of neomucosa, and subsequent sections at each level were subjected to IF or FISH.

To identify the origins of ISEMFs and SMCs, a combination of IF and FISH was used. IF was used to detect co-localization of SMA positive cells (ISEMFs/SMCs) and cells of donor origin indicated by green fluorescent protein (GFP). Slides underwent antigen retrieval by boiling in a pressure cooker at 120°C at 15 psi for 30 min while submerged in a 10 mM Na-Citrate buffer (pH 6.0). They were then incubated overnight at 4°C overnight with primary antibodies for GFP (1:100; Abcam, Cambridge, MA) and SMA (1:500; Thermo Fischer

Scientific, Waltham, MA). The slides were then incubated with Alexa Fluor 568 and Alexa Fluor 488 secondary antibodies (Life Technologies, Carlsbad, CA), mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and imaged using confocal microscopy. FISH was used to detect the distribution of male cells that had been implanted to female recipients. Explanted TEI was sectioned in 5 μ m sections and baked at 65°C overnight. After deparaffinization, slides were treated with 30% sodium bisulfide in 2 $\times$ saline-sodium citrate (SSC) buffer at 45°C for 10 min and the proteins were digested with 2.5 μ g/ml proteinase K at 45°C for 20 min. Slides were then dehydrated in 70%, 85% and 100% ice-cold ethanol for 2 min each. The slides were then dried at 37°C on a hot plate and 10 μ l of mouse Y-chromosome paint probe (Creative Bioarray Inc, Shirley, NY) were applied to the slides. Chromosomal DNA and the Y-Chromosome probe were denatured on a hot plate at 90°C for 8 min followed by hybridization at 37°C humidified chamber overnight. The next day, the slides were washed in 2 $\times$ SSC/0.3% NP-40 at 73°C solution for 2 min followed by 2 $\times$ SSC/0.1% NP-40 wash at room temperature for 30 sec. Finally, slides were counterstained and mounted with DAPI containing Vectashield (Vector Laboratories, Burlingame, CA) and fluorescent images were obtained using confocal microscopy.

2.7 Experimental Design

2.7.1 ISEMF and SMC Origins—To determine the origin of ISEMFs and SMCs in TEI, two separate experiments were performed. This was first investigated by utilizing enteroids obtained from pan-EGFP mice where all cells express GFP. The protocol for establishing enteroid culture (section 2.2) was performed three separate times using pan-EGFP mice to obtain enough enteroids for experimentation. Enteroids were seeded onto 10 scaffolds and implanted into the peritoneal cavities of ten NOD/SCID mice as described in section 2.4. All scaffolds were explanted after 4 weeks and evaluated for the presence of TEI. They were further analyzed with IF for SMA and GFP. All cells derived from donor enteroids should express GFP while all cells derived from NOD/SCID hosts should not.

To confirm the findings from the first study, enteroids obtained from only male LGR5-EGFP mice were utilized. The protocol for establishing enteroid culture (section 2.2) was performed three separate times using male LGR5-EGFP mice to obtain enough enteroids for experimentation. Enteroids were seeded onto 12 scaffolds and implanted into the peritoneal cavities of female NOD/SCID mice as described in section 2.4. All scaffolds were then explanted after 4 weeks and evaluated for the presence of TEI, and were further analyzed with FISH for the Y-chromosome. All cells derived from donor enteroids should be positive for the Y-chromosome while all cells derived from the NOD/SCID female hosts should not.

2.7.2 ISEMF Chemotaxis Assay—Since the exact mechanism(s) by which ISEMFs established themselves within enteroid-derived TEI were not known, we next sought to determine if there was a direct chemotactic effect of the seeded enteroids on ISEMFs. To investigate this, we performed a Boyden chamber chemotaxis assay using 24-well plates with permeable polyester (PET) 8.0 μ m pore BioCoat Control transwell inserts (Corning Life Sciences, Tewksbury, MA). Enteroid cultures were established per the protocol described in section 2.2. Cultures were established four separate times from LGR5-EGFP mice, and utilized at 10–14 days of culture for the assay. ISEMF cultures were established

per the protocol described in section 2.3 four separate times from LGR5-EGFP mice. The chemotaxis assay was performed with three experimental groups: 1) Enteroid growth media; 2) Enteroid growth media + Matrigel; and 3) Enteroid growth media + Matrigel + Enteroids. The groups defined above were placed into the bottom wells of the Boyden chamber. For wells containing media + Matrigel, a fresh bead of Matrigel was plated. For wells containing media + Matrigel + enteroids, the enteroids at 10–14 days of culture were mechanically released from their Matrigel, washed, and re-plated with 500 enteroids/well in fresh Matrigel in the Boyden chamber. Each bottom chamber received 0.75 mL of enteroid growth media without the growth factors noggin, R-spondin, and HB-EGF. All transmembrane wells were seeded with 100,000 ISEMFs/well in a total of 0.5 mL of enteroid growth media in which the same growth factors had been withheld. The chambers were cultured at 37°C in a 5% CO₂ humidified incubator for 24 h. After incubation, the top of each transmembrane well was cleaned with a cotton tipped applicator to remove cells that did not cross the transmembranes. The transmembranes were then fixed and stained using a Diff Quick staining kit (Thermo Fisher Scientific, Waltham, MA). The cells that remained on the bottom of the transmembrane were then quantified using light microscopy by summing the number of cells in 5 randomly selected fields at 10× magnification. Each well counted as n=1. This experiment was performed with three or four wells per experimental group on four separate occasions, with n=13 for each group, using fresh enteroid and ISEMF cultures each time.

2.7.3 ISEMF Co-seeding for Production of TEI—To determine the potential benefits of ISEMF *in vivo*, ISEMFs were co-seeded with enteroids and implanted to produce TEI. The protocol for establishing enteroid culture (section 2.2) was performed four separate times using LGR5-EGFP mice to obtain adequate enteroids for experimentation. The protocol for establishing ISEMF culture (section 2.3) was performed two separate times using LGR5-EGFP mice to obtain enough ISEMFs for co-seeding. All enteroids were seeded at 20 enteroids/mm³ of scaffold material. There were three experimental groups: 1) Enteroids alone (n=11); 2) Enteroids + 2,500 ISEMFs/mm³ of scaffold material (n=11); and 3) Enteroids + 50,000 ISEMFs/mm³ of scaffold material (n=9). All implants were evaluated with H&E staining for the presence and the quantity of TEI produced after 4 weeks of *in vivo* incubation.

2.8 Statistical Analyses

To compare the number of ISEMFs that migrated across the chemotaxis assay membranes between the three treatment groups, and to compare the length of neomucosa produced from the three treatment groups in the ISEMF co-seeding experiments, a Kruskal-Wallis analysis was performed. *P* values of less than 0.05 were considered statistically significant. To perform pairwise comparisons between every two of the treatment groups, the Mann-Whitney test was performed. Gender of the animal subjects was not considered in the analyses. GraphPad Prism 6 (La Jolla, CA) was utilized in all statistical analyses.

3. RESULTS

3.1 ISEMF and SMC Origins

Seeding pan-EGFP enteroids onto scaffolds produced TEI in 6/13 (46%) implants. Each implant that was positive for TEI underwent IF for SMA and GFP. All scaffolds showed the same pattern of labeling (Figure 1A). The simple columnar epithelium was ubiquitously positive for GFP. The surrounding stroma showed positivity for SMA. However, there was never any co-localization of the two signals.

Seeding exclusively male LGR5-EGFP enteroids onto scaffolds with implantation into female NOD/SCID recipients only, produced TEI in 11/12 (92%) implants. Each implant that was positive for TEI underwent FISH for the Y-chromosome. All scaffolds showed the same pattern of chromosome labeling (Figure 1B). The simple columnar epithelium was ubiquitously positive for the Y-chromosome. There was never any signal identified in the surrounding stroma where ISEMFs and SMCs would be located.

3.2 ISEMF Chemotaxis Assay

Prior to performing chemotaxis assays, we confirmed the identity of the primary cultures established as ISEMFs. Immunofluorescence was performed on three separate ISEMF cultures co-labeled for SMA and vimentin (Figure 2A), and for SMA and desmin (Figure 2B). The putative ISEMF cells were consistently SMA positive, vimentin positive, and desmin negative, as expected for ISEMFs. These findings were confirmed by flow cytometry using the same three markers, confirming that the cells were SMA positive, vimentin positive, and desmin negative compared to isotype controls (Figure 3).

In the chemotaxis assay, when only media was present, an average of 339 ± 24 cells crossed the semi-permeable transmembrane, representing the baseline movement of the ISEMFs across the membrane. In wells with media + Matrigel, an average of 357 ± 36 ISEMFs crossed the transmembrane, which was not significantly different from the media alone group ($p > 0.05$). This indicated that there were no chemotactic effects from the growth factor reduced Matrigel. However, when enteroids were present in the Matrigel, there were an average of 983 ± 133 cells crossing the transmembrane, which was significantly higher than the media alone or media + Matrigel groups ($p < 0.0001$) (Figure 4). This indicates that enteroids exert a chemotactic effect on ISEMFs.

3.3 ISEMF Co-Seeding for Production of TEI

Seeding with enteroids alone produced TEI in 8/10 (80%) scaffolds. Co-seeding of enteroids + 2,500 ISEMFs/mm³ of scaffold material was similarly successful at producing TEI in 8/11 (73%) scaffolds. However, co-seeding of enteroids + 50,000 ISEMFs/mm³ of scaffold material produced TEI in only 2/9 (22%) scaffolds. A similar trend was seen when quantifying the amount of neomucosa produced in each group. Seeding with enteroids only produced an average of $8,618 \pm 3,056$ μ m of neomucosa. Seeding with enteroids + 2,500 ISEMFs/mm³ of scaffold material produced an average of $4,192 \pm 2,809$ μ m of neomucosa, which was not significantly different from seeding with enteroids only ($p = 0.21$). However, co-seeding with enteroids + 50,000 ISEMFs/mm³ of scaffold material produced an average

of $443 \pm 350 \mu\text{m}$ of neomucosa, which was significantly less than seeding with either enteroids only, or co-seeding with enteroids + 2,500 ISEMFs/ mm^3 of scaffold material ($p=0.03$) (Figure 5).

4. DISCUSSION

There are numerous methods of TEI production, each with their own advantages and disadvantages. One of the most well established and extensively investigated methods utilizes the seeding of bio-absorbable scaffolds with organoid units from the digestion of whole intestine.^{13, 21–28} Organoid units are composed of crypt domains surrounded by the mesenchymal niche. Because these are derived from the digestion of whole tissue, included in the seeded material are other derivatives such as smooth muscle, endothelial cells, and neural elements. Using this amalgam of digested tissue results in a robust product that contains all of the major components of intestine including an organized and robust epithelium, sheets of smooth muscle, mesenchyme, lymphatics, capillary networks, and neural elements.^{23, 27–31} While this TEI is robust in structure, it requires the digestion of substantial quantities of healthy intestine which patients with SBS cannot provide. This has led others to look at expandable sources of seeding material.

Enteroids are intestinal epithelial cultures that can be obtained from intestinal crypts or derived from pluripotent stem cells.^{32, 33} These enteroids can be derived from a minimal starting material such as a simple biopsy, passaged, and indefinitely expanded logarithmically *in vitro*, making them an attractive candidate as a tissue source for TEI development.^{12, 20, 33–35} Using enteroids, TEI has been produced that contains a healthy and complete epithelium, smooth muscle, connective tissue, mesenchymal niche components, and capillary networks.^{12, 20, 36, 37} Paramount to the ability to produce a robust TEI from enteroids is the presence of ISEMFs.

It has been shown *in vitro* that enteroids are better established and grow larger when grown in the presence of ISEMFs. Moreover, critical growth factors can be replaced with the presence of a feeder layer of ISEMFs.^{12, 18, 37} Further *in vivo* experimentation where enteroids were implanted into the subcutaneous pocket of mice demonstrated that without ISEMF supplementation, there was no appreciable growth of TEI. However, when the enteroids were co-seeded with ISEMFs, there was continued epithelial growth and the presence of ISEMFs within the TEI.^{12, 18} We have previously shown that TEI can be produced without the addition of ISEMFs in the seeded material if the enteroids are implanted in the peritoneal cavity, suggesting a positive effect of proximity to peritoneal contents.²⁰ In addition to producing TEI without the addition of ISEMFs, we noted that ISEMFs were present along the basilar surface of the healthy epithelium. Since the seeded enteroids were purely epithelial, the ISEMFs had to either be recruited into the TEI from the host or transdifferentiated from donor epithelial elements.

In the current study we used pan-EGFP donor mice to establish enteroids in which all cells express GFP, and host mice in which none of their cells express GFP. Using IF we were able to show that only the epithelium of the TEI expressed GFP while the ISEMFs did not. The lack of expression of GFP in the ISEMFs indicated that these cells were recruited from the

host animals. To rule out the possibility that the expression of GFP may have been lost through the process of epithelial-mesenchymal transition, we sought to confirm these results by implanting male enteroids into female recipients with labeling of the TEI produced for the Y-chromosome. Y-chromosomes were only identified in the epithelium, with no labeling in the surrounding stroma where ISEMFs are located. This confirmed that these critical cells were being recruited from the host and not transdifferentiated from the epithelium of donor cells.

The mechanism(s) by which the ISEMFs are recruited from the host are not known at present. Previous TEI research suggests that enteroids physically isolated from the peritoneal contents do not survive while enteroids implanted in the peritoneal cavity allow for ISEMF recruitment, indicating possible paracrine effects.^{12, 18} For these reasons, we investigated whether enteroids themselves had a chemotactic effect on ISEMFs. The *in vitro* Boyden chamber assay performed in this study demonstrated a definitive chemotactic effect between enteroids and ISEMFs. How this relationship expresses itself *in vivo* is not yet understood. Whether through paracrine or humoral effects and whether these cells are directly migrating from surrounding tissues is not clear and requires further study. What is clear is that the intestinal epithelium and ISEMFs maintain an important relationship that results in critical cell recruitment and appropriate anatomic apposition in order to maintain healthy epithelial tissue in the TEI produced.

In vitro experimentation has shown that the growth of enteroids is enhanced by co-culturing with ISEMFs, and that ISEMFs enhance the engraftment and growth of enteroids on bioabsorbable scaffolds.¹⁹ With mounting evidence that ISEMFs are critical to the successful development of TEI, we next sought to determine whether co-seeding of enteroids and ISEMFs would positively impact the growth of enteroid-derived TEI in our model. We reasoned that providing these critical cell types immediately at the time of implantation would bypass the time needed to recruit and engraft these cells on TEI implants, thus having potentially positive benefits to the epithelium. However, in the current study, scaffolds seeded with enteroids + ISEMFs showed both decreased numbers of scaffolds successfully growing healthy epithelium, as well as a significantly decreased amount of epithelium when there was positive TEI growth. This finding was unexpected given the previous data showing that TEI implanted into a subcutaneous pocket only grew with the supplementation of ISEMFs to the implants.^{12, 18} While ISEMF supplementation appears to be critical in an environment devoid of normal mesenchymal influence, it does not appear to offer additional benefit in the peritoneal cavity where the implants are influenced by the surrounding healthy viscera and peritoneal fluid. This highlights the complexity of the relationship between the epithelium and the ISEMFs. While ISEMFs are critical for epithelial survival, simply adding ISEMFs in an environment where they are already present does not necessarily offer additional benefit. What is not clear is why there seems to be a detrimental effect from the addition of ISEMFs. Presumably, when these cells are added to the scaffold and implanted in the peritoneal cavity, only a certain percentage would survive. An appreciable portion theoretically undergo apoptosis due to the trauma of passaging, seeding, and implanting into the peritoneal cavity, an environment devoid of the controlled supplementation of necessary growth factors. We speculate that the death of these supplemented ISEMFs may alter the otherwise healthy signaling cascade that results in the

recruitment of ISEMFs and healthy maintenance of the epithelium. However, further studies are needed to determine the mechanisms resulting in decreased ISEMF production.

There are limitations to the current study. There was appreciable variability between mouse strains regarding successful production of TEI. Most of our experiments utilized the LGR5-EGFP mouse strain. When implanting enteroids alone from LGR5-EGFP donor mice we obtain TEI in >80% of attempts. To identify the origins of ISEMFs however, we utilized Pan-EGFP donor mice. Using these donor mice, we were only able to obtain TEI in 46% of attempts. While the reason for this substantial decrease in success is not known, unique biological characteristics of the enteroids from Pan-EGFP mice appear to be present. However, we do not believe that this variability affects the main point of our results, since we confirmed recruitment of ISEMFs using Y-chromosome labeling on gender specific implantation in the LGR5-EGFP mouse strain. Although there is variability in these mouse strains regarding success of TEI growth, evidence of ISEMF recruitment is shown across both strains. Regarding the potential mechanisms behind ISEMF recruitment, the Boyden chamber studies indicate a chemotactic effect between enteroids and ISEMFs. However, the specificity of this relationship is uncertain. Although there was increased migration of ISEMFs in the presence of enteroids, it is unknown if this effect is unique to the interaction of ISEMFs with enteroids or if enteroids show this effect with other cell types. Further testing using other fibroblast cell lines may shed light on the specificity of this relationship. Moreover, while this effect is seen *in vitro*, it may not reflect what is occurring in the recruitment of ISEMFs into TEI *in vivo*. Leveraging the power of transgenic mice in future studies will hopefully shed light on the mechanisms by which critical cell types are recruited to TEI.

5. CONCLUSIONS

Through the use of IF and FISH, we have been able to demonstrate that the epithelium in enteroid-derived TEI is derived from donor enteroids. Although the donor enteroids are purely epithelial, the TEI derived from these enteroids contains ISEMFs and SMCs. We have demonstrated that these critical cell types are recruited from the host animal and not transdifferentiated from the donor enteroids. Although the mechanism(s) by which this recruitment occurs *in vivo* is unknown, we demonstrated a significant chemotactic effect of the enteroids on ISEMFs. While the presence of ISEMFs in the health of TEI is critical, their beneficial interaction with the epithelium is more complex than simply being present, as evidenced by co-seeding experiments. Supplementation of additional ISEMFs to this enteroid-derived TEI model in which ISEMFs are recruited from the host does not appear to offer additional benefit. This underscores the complexity of TEI development, in which simply adding the necessary components together to create complex tissues is not sufficient. Success is dependent upon dynamic cellular interactions that require further understanding as TEI models are optimized for translational research.

Acknowledgments

Funding for this study was obtained through The Research Institute at Nationwide Children's Hospital (GEB) and from NIH R43DK107168 (JJ, GEB).

References

1. Scadden DT. The stem-cell niche as an entity of action. *Nature*. 2006; 441:1075–1079. [PubMed: 16810242]
2. Sato T, Clevers H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science*. 2013; 340:1190–1194. [PubMed: 23744940]
3. Holmberg FE, Seidelin JB, Yin X, Mead BE, Tong Z, et al. Culturing human intestinal stem cells for regenerative applications in the treatment of inflammatory bowel disease. *EMBO Mol Med*. 2017; 9:558–570. [PubMed: 28283650]
4. Gehart H, Clevers H. Repairing organs: lessons from intestine and liver. *Trends Genet*. 2015; 31:344–351. [PubMed: 25989898]
5. Murrow LM, Weber RJ, Gartner ZJ. Dissecting the stem cell niche with organoid models: an engineering-based approach. *Development*. 2017; 144:998–1007. [PubMed: 28292846]
6. Tan DW, Barker N. Intestinal stem cells and their defining niche. *Curr Top Dev Biol*. 2014; 107:77–107. [PubMed: 24439803]
7. Yen TH, Wright NA. The gastrointestinal tract stem cell niche. *Stem Cell Rev*. 2006; 2:203–212. [PubMed: 17625256]
8. Tesori V, Puglisi MA, Lattanzi W, Gasbarrini GB, Gasbarrini A. Update on small intestinal stem cells. *World J Gastroenterol*. 2013; 19:4671–4678. [PubMed: 23922464]
9. Roulis M, Flavell RA. Fibroblasts and myofibroblasts of the intestinal lamina propria in physiology and disease. *Differentiation*. 2016; 92:116–131. [PubMed: 27165847]
10. Gregorieff A, Pinto D, Begthel H, Destree O, Kielman M, et al. Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology*. 2005; 129:626–638. [PubMed: 16083717]
11. Powell DW, Pinchuk IV, Saada JI, Chen X, Mifflin RC. Mesenchymal cells of the intestinal lamina propria. *Annu Rev Physiol*. 2011; 73:213–237. [PubMed: 21054163]
12. Lahar N, Lei NY, Wang J, Jabaji Z, Tung SC, et al. Intestinal subepithelial myofibroblasts support in vitro and in vivo growth of human small intestinal epithelium. *PLoS One*. 2011; 6:e26898. [PubMed: 22125602]
13. Grant CN, Grikscheit TC. Tissue engineering: a promising therapeutic approach to necrotizing enterocolitis. *Semin Pediatr Surg*. 2013; 22:112–116. [PubMed: 23611615]
14. Guner YS, Chokshi N, Petrosyan M, Upperman JS, Ford HR, et al. Necrotizing enterocolitis--bench to bedside: novel and emerging strategies. *Semin Pediatr Surg*. 2008; 17:255–265. [PubMed: 19019294]
15. Spurrier RG, Grikscheit TC. Tissue engineering the small intestine. *Clin Gastroenterol Hepatol*. 2013; 11:354–358. [PubMed: 23380001]
16. Ladd MR, Nino DF, March JC, Sodhi CP, Hackam DJ. Generation of an artificial intestine for the management of short bowel syndrome. *Curr Opin Organ Transplant*. 2016; 21:178–185. [PubMed: 26867049]
17. Hong SN, Dunn JC, Stelzner M, Martin MG. Concise Review: The Potential Use of Intestinal Stem Cells to Treat Patients With Intestinal Failure. *Stem Cells Transl Med*. 2016
18. Lei NY, Jabaji Z, Wang J, Joshi VS, Brinkley GJ, et al. Intestinal subepithelial myofibroblasts support the growth of intestinal epithelial stem cells. *PLoS One*. 2014; 9:e84651. [PubMed: 24400106]
19. Shaffiey SA, Jia H, Keane T, Costello C, Wasserman D, et al. Intestinal stem cell growth and differentiation on a tubular scaffold with evaluation in small and large animals. *Regen Med*. 2015 In Press.
20. Cromeens BP, Liu Y, Stathopoulos J, Wang Y, Johnson J, et al. Production of tissue-engineered intestine from expanded enteroids. *J Surg Res*. 2016; 204:164–175. [PubMed: 27451883]
21. Levin DE, Barthel ER, Speer AL, Sala FG, Hou X, et al. Human tissue-engineered small intestine forms from postnatal progenitor cells. *J Pediatr Surg*. 2013; 48:129–137. [PubMed: 23331805]

22. Sala FG, Matthews JA, Speer AL, Torashima Y, Barthel ER, et al. A multicellular approach forms a significant amount of tissue-engineered small intestine in the mouse. *Tissue Eng Part A*. 2011; 17:1841–1850. [PubMed: 21395443]
23. Duxbury MS, Grikscheit TC, Gardner-Thorpe J, Rocha FG, Ito H, et al. Lymphangiogenesis in tissue-engineered small intestine. *Transplantation*. 2004; 77:1162–1166. [PubMed: 15114078]
24. Grant CN, Mojica SG, Sala FG, Hill JR, Levin DE, et al. Human and mouse tissue-engineered small intestine both demonstrate digestive and absorptive function. *Am J Physiol Gastrointest Liver Physiol*. 2015; 308:G664–677. [PubMed: 25573173]
25. Kaihara S, Kim S, Benvenuto M, Kim BS, Mooney DJ, et al. End-to-end anastomosis between tissue-engineered intestine and native small bowel. *Tissue Eng*. 1999; 5:339–346. [PubMed: 10477856]
26. Kim SS, Kaihara S, Benvenuto MS, Choi RS, Kim BS, et al. Effects of anastomosis of tissue-engineered neointestine to native small bowel. *J Surg Res*. 1999; 87:6–13. [PubMed: 10527698]
27. Liu Y, Rager T, Johnson J, Enmark J, Besner GE. Enriched Intestinal Stem Cell Seeding Improves the Architecture of Tissue-Engineered Intestine. *Tissue Eng Part C Methods*. 2015; 21:816–824. [PubMed: 25603285]
28. Liu Y, Nelson T, Cromeens B, Rager T, Lannutti J, et al. HB-EGF embedded in PGA/PLLA scaffolds via subcritical CO₂ augments the production of tissue engineered intestine. *Biomaterials*. 2016; 103:150–159. [PubMed: 27380441]
29. Tavakkolizadeh A, Berger UV, Stephen AE, Kim BS, Mooney D, et al. Tissue-engineered neomucosa: morphology, enterocyte dynamics, and SGLT1 expression topography. *Transplantation*. 2003; 75:181–185. [PubMed: 12548119]
30. Perez A, Grikscheit TC, Blumberg RS, Ashley SW, Vacanti JP, et al. Tissue-engineered small intestine: ontogeny of the immune system. *Transplantation*. 2002; 74:619–623. [PubMed: 12352876]
31. Choi RS, Riegler M, Pothoulakis C, Kim BS, Mooney D, et al. Studies of brush border enzymes, basement membrane components, and electrophysiology of tissue-engineered neointestine. *J Pediatr Surg*. 1998; 33:991–996. discussion 996–997. [PubMed: 9694083]
32. Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature*. 2011; 470:105–109. [PubMed: 21151107]
33. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. 2009; 459:262–265. [PubMed: 19329995]
34. Fuller MK, Faulk DM, Sundaram N, Shroyer NF, Henning SJ, et al. Intestinal crypts reproducibly expand in culture. *J Surg Res*. 2012; 178:48–54. [PubMed: 22564827]
35. Mahe MM, Sundaram N, Watson CL, Shroyer NF, Helmrath MA. Establishment of human epithelial enteroids and colonoids from whole tissue and biopsy. *J Vis Exp*. 2015
36. Watson CL, Mahe MM, Munera J, Howell JC, Sundaram N, et al. An in vivo model of human small intestine using pluripotent stem cells. *Nat Med*. 2014; 20:1310–1314. [PubMed: 25326803]
37. Jabaji Z, Sears CM, Brinkley GJ, Lei NY, Joshi VS, et al. Use of collagen gel as an alternative extracellular matrix for the in vitro and in vivo growth of murine small intestinal epithelium. *Tissue Eng Part C Methods*. 2013; 19:961–969. [PubMed: 23566043]

Figure 1. ISEMF and SMC origin studies

A) Immunofluorescence for SMA and GFP on TEI grown from pan-EGFP enteroids. The simple columnar epithelium is positive for GFP (green arrows), indicating that the epithelial cells are derived from the pan-EGFP-positive enteroids that were seeded onto the TEI implants. SMA (red arrows) indicates the presence of ISEMFs and SMCs surrounding the epithelium as expected. There is no co-localization of signals, indicating that the ISEMFs and SMCs are derived from the host animal. B) FISH for the Y-chromosome on TEI grown from male enteroids implanted into female hosts. The simple columnar epithelium is positive for the Y-chromosome (red arrows) indicating that the epithelial cells are derived from the male donor enteroids. There is no Y-chromosome signal in the surrounding stroma, indicating that all other cells are derived from the host. Scale bars = 100 μ m.

Figure 2. Characterization of primary ISEMF cultures using immunofluorescence

Cultured ISEMFs were labeled for SMA (A) and for vimentin (B). The cells were ubiquitously positive for both SMA and vimentin with diffuse co-localization of both signals (C), as would be expected for ISEMFs. ISEMFs were also labeled for SMA (D) and for desmin (E). While all of the cells were again positive for SMA, there were no cells positive for desmin, and thus no co-localization of these signals (F), as expected for ISEMFs.

Figure 3. Characterization of primary ISEMF cultures using flow cytometry

A) Single channel readout for cells labeled with SMA, vimentin, and desmin. There is clear positive signal for both SMA and vimentin, and negative signal for desmin, as expected for ISEMFs. B) Dot plot for SMA and vimentin co-labeling of isotype controls (left) and ISEMFs (right). Isotype controls are negative for both antibodies, whereas over 75% of the cultured cells are SMA/vimentin positive. C) Dot plot for desmin and vimentin co-labeling of isotype controls (left) and ISEMFs (right). Isotype controls are negative for both antibodies, whereas nearly 95% of the cultured cells are vimentin positive and desmin negative.

Figure 4. ISEMF Chemotaxis assay

Representative images of the transmembrane inserts from each treatment group: A) Media + Matrigel + enteroids; B) Media + Matrigel; C) Media alone. Grossly, more cells have migrated across the transmembrane in the presence of enteroids compared to the other two groups. D) Quantification of the number of migrated cells in each group. Significantly more cells migrate across the membrane when enteroids are present. n = 13 for each experimental group. * $p < 0.0001$.

Figure 5. Effect of ISEMF co-seeding on the production of TEI

Neomucosal length of TEI was quantified for each treatment group. All implants were seeded with 20 enteroids/mm³ of scaffold and implanted into the peritoneal cavity of NOD/SCID mice for four weeks. Scaffolds were seeded with either: enteroids only (control); enteroids + 2,500 ISEMFs/mm³ of scaffold; or enteroids + 50,000 ISEMFs/mm³ of scaffold. TEI was explanted and analyzed using H&E staining. Positivity was defined as the presence of simple columnar epithelium, consistent with the presence of intestinal mucosa. Neomucosal length was quantified on a representative section using ImageJ software by tracing the basal surface of the epithelium. Scaffolds seeded with enteroids only demonstrated more neomucosa than scaffolds co-seeded with either 2,500 or 50,000 ISEMFs/mm³ of scaffold. * $p < 0.05$.