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Production of Tissue Engineered Intestine from Expanded Enteroids

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Abstract

Background—Short bowel syndrome (SBS) is a life-threatening condition with few solutions. Tissue engineered intestine (TEI) is a potential treatment, but donor intestine is a limiting factor. Expanded epithelial surrogates termed enteroids may serve as a potential donor source.

Materials and Methods—To produce TEI from enteroids, crypts were harvested from mice and enteroid cultures established. Enteroids were seeded onto polymer scaffolds using Matrigel or culture medium, and implanted in immunosuppressed mice for 4 weeks. Histology was analyzed using Periodic Acid Schiff (PAS) staining and immunofluorescence (IF). Neomucosa was quantified using ImageJ software. To determine if TEI could be produced from enteroids established from small intestinal biopsies, 2×2mm pieces of jejunum were processed for enteroid culture, enteroids were expanded and seeded onto scaffolds, and scaffolds implanted for 4 weeks.

Results—Enteroids in Matrigel produced TEI in 15/15 scaffolds, whereas enteroids in medium produced TEI in 9/15 scaffolds. Use of Matrigel led to more neomucosal surface area compared to media (10,520±2,905 μm *vs.* 450±127 μm, $p<0.05$). Histologic examination confirmed the presence of crypts and blunted villi, normal intestinal epithelial lineages, intestinal subepithelial myofibroblasts, and smooth muscle cells. Crypts obtained from biopsies produced an average of 192±71 enteroids. A single passage produced 685±58 enteroids, which was adequate for scaffold seeding. TEI was produced in 8/9 scaffolds seeded with expanded enteroids.

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Conclusions—Enteroids can be obtained from minimal starting material, expanded *ex vivo*, and implanted to produce TEI. This method shows promise as a solution to the limited donor intestine available for TEI production in patients with SBS.

Keywords

Tissue engineering; Short bowel syndrome; Intestine; Enteroids; Epithelial culture; intestinal stem cells

1. Introduction

Short bowel syndrome (SBS) is a vexing clinical condition with no ideal solution. Patients with SBS have typically lost over 50-75% of their normal small bowel length. This vastly decreases the functional absorptive area of the intestine, often resulting in weight loss, dehydration, and vitamin deficiencies. The management of patients with SBS is primarily designed to mitigate these complications. Repletion of nutritional and fluid deficiencies is achieved with parenteral nutrition (PN). Although lifesaving, PN is associated with substantial risks including venous thrombosis, central line associated blood stream infections, cholestasis, and liver failure.[1, 2] Surgical therapies such as the Bianchi procedure and serial transverse enteroplasty have been developed to lengthen the remaining small bowel. Although successful in select cases, not all patients are candidates and the procedures often fail.[3, 4] The only current clinical approach that replaces lost intestine is heterotopic small bowel transplantation. This modality can be successful in select cases; however, the 5-year survival remains low at 55% and is associated with the difficulties and complications of long term immunosuppression.[5] A proposed solution to the shortcomings of current SBS treatment is the development of tissue engineered intestine (TEI).[6-8]

Ideally, TEI would utilize the patient's native tissue to grow a completely functional and immunologically compatible length of intestine to replace the intestine that has been lost. Currently, the most commonly used animal model of TEI utilizes organoid units containing intestinal crypts and their mesenchymal niche that are seeded onto bioabsorbable scaffolds and implanted into the peritoneal cavity of a host animal. Utilizing these general methods, TEI has successfully been produced in rats, mice, swine, and dogs.[9-12] Additionally, human TEI has been produced by implanting human organoid units into immunosuppressed mice.[13] The TEI created utilizing these models has been shown to contain the epithelial, mesenchymal, neural, and smooth muscle components critical to functioning intestine.[10, 12-22]

Despite the quality of the TEI currently produced, limitations regarding a healthy tissue source for TEI production hinder translational application. In the described methods, a substantial quantity of healthy intestine must be digested to obtain an adequate number of organoid units to produce TEI. Moreover, the TEI produced is relatively small and unpredictable in size, ranging from a few millimeters to 2 cm in diameter. In order to replace the long lengths of intestine needed for SBS patients, even greater quantities of starting material would likely be needed. However, patients with SBS have little healthy intestine to spare for the production of TEI.

Sato et al.,[23, 24] have previously demonstrated that intestinal stem cell (ISC) containing crypts can be harvested and established in a 3-D culture system to produce intestinal epithelial surrogates known as enteroids. Enteroids contain crypt and villus domains with all of the terminally differentiated epithelial lineages. They can be passaged for greater than 18 months while maintaining their phenotype. They have also been shown to expand *in vitro* in a logarithmic fashion and maintain their expansion capacity even after cryopreservation.[25] These unique characteristics make enteroid culture a potential clinically applicable tissue reservoir for the production of TEI in patients with SBS.

Therefore, we hypothesized that TEI can be grown from minimal starting material comparable to that which a SBS patient could provide, when utilizing *ex vivo* enteroid culture and expansion. To test this hypothesis, we first established a reproducible seeding and implantation protocol to reliably produce TEI from enteroids. We then investigated the possibility that crypts could be obtained from minimal starting material to produce TEI.

2. Materials and Methods

2.1 Animal Use

All animal procedures were performed with the approval of our Institutional Animal Care and Use Committee (Protocol #AR12-0001). All enteroid cultures were established from male (n=13) and female (n=10) LGR5-EGFP transgenic mice bred in house. The colony was established from breeders obtained from The Jackson Laboratory (Bar Harbor, ME). All seeded scaffolds were implanted into male (n=38) or female (n=44) Non-Obese Diabetic/ Severe Combined Immunodeficiency (NOD/SCID) mice bred in house. This colony was established from breeders obtained from Charles River Laboratories (Wilmington, MA).

2.2 Crypt Isolation and Enteroid Culture

Donor intestine for enteroid culture was obtained from LGR5-EGFP transgenic mice in which LGR5+ ISC located in the crypts express green fluorescent protein, allowing them to be tracked throughout all steps of experimentation. After euthanasia, a laparotomy was performed and the proximal half of the small intestine starting two centimeters distal to the pylorus was removed. We utilized the proximal intestine due to previous *in vitro* studies showing the crypts from the proximal intestine were more efficient at producing enteroids.[25] The lumen was opened longitudinally and enteric contents rinsed away with phosphate buffered saline (PBS) (GE Life Sciences, Marlborough, MA). Villi were scraped away using a glass coverslip and the remaining tissue was minced and washed to remove debris. Once clean, the tissue was incubated in 2 mM ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Waltham, MA) in PBS for 30 min at 4°C. After incubation, tis sue fragments were allowed to settle and the EDTA was discarded. The tissue was resuspended in ice-cold PBS and agitated by trituration for 30 sec to release fragments of mucosa including crypts. The remaining tissue was allowed to settle and the supernatant containing the mucosal fragments was filtered through a 70 μm sieve (BioDesign Inc. of New York, Carmel, NY). The sieve removed the larger mucosal fragments including remaining villi and allowed the crypts to flow through. The process of resuspension, trituration, and filtration was repeated until mucosal fragments were no longer identified in the supernatant. The

flow-through from each fractionation and filtration were combined and pelleted at 300 *g* for five min. at 4°C. Pelleted crypts were then re suspended in basic crypt medium consisting of Advanced DMEM/F12 supplemented with 2 mM GlutaMAX, 10 mM HEPES, and 100 U/mL penicillin/100 μg/mL streptomycin (all from Thermo Fisher Scientific, Waltham, MA), and pelleted at 100-150 *g* for 2 min. to remove single cells in the supernatant. This process was repeated three times. Prior to the final centrifugation, crypt density was determined.

After crypt isolation was completed, pelleted crypts were resuspended in growth factor reduced Matrigel (Sigma, St. Louis, MO) at a density of 100-200 crypts/50 μL of Matrigel. The cold Matrigel-crypt suspension was then plated onto pre-warmed 24-well culture plates with 50 μL of Matrigel per well. Plates were placed in an incubator at 37°C for 15 min to allow the Matrigel to completely solidify, and then each well received 500 μl of complete enteroid culture medium consisting of basic crypt medium supplemented with 1 mM N-Acetylcysteine (Sigma, St. Louis, MO), 1× N2 supplement (Thermo Fisher Scientific, Waltham, MA), 1× B27 supplement (Thermo Fisher Scientific, Waltham, MA), 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). Media was changed every 3-4 days during culture.

2.3 Scaffold Fabrication, Seeding, and Implantation

Polyglycolic acid (PGA) Biofelt (2 mm thick; 60 mg/cm^3 density) (Biomedical Structures, Warwick, RI) was utilized for scaffold production. An 8 mm × 30 mm section of PGA Biofelt was wrapped around a 3 mm diameter mandrel to produce 3 cm long × 3 mm internal diameter (ID) scaffolds. Once on the mandrel, a 5% polylactic acid (PLLA) (Sigma, St. Louis, MO) in chloroform solution was sprayed onto the exterior using an atomizer. Once the PLLA coating was dry, scaffolds were soaked in 100% ethanol for 30 min and then washed three times with PBS. Scaffolds were then collagen coated by soaking them in a 0.4 mg/mL type I bovine collagen solution (Advanced BioMatrix, Carlsbad, CA). Scaffolds were then removed from the mandrels, cut to the desired implantation length of 5 mm, and sterilized with ethylene oxide gas.

All enteroids were seeded onto 5 mm long × 3 mm ID PGA tubular scaffolds. Enteroids at day 10-14 of culture were released from the Matrigel by aspirating through a p1000 pipette tip and resuspended in basic crypt media. Enteroids were centrifuged at 200 *g* for 2 min. and supernatants discarded. This process of resuspension and centrifugation was repeated twice to wash away any dead cells. Before the final centrifugation, the number of enteroids in solution was quantified using phase contrast microscopy. Pelleted enteroids were resuspended in either growth factor reduced Matrigel or complete enteroid culture media. Sterile PGA scaffolds were pre-warmed to 37°C and the enteroids were seeded on to the scaffolds using 50 μL of seeding medium to a seeding density corresponding to the desired treatment group. Seeded scaffolds were maintained at 37°C until implantation into imm unocompromised NOD/SCID mice. Mice were anesthetized using isoflurane anesthesia and a midline laparotomy performed. Seeded scaffolds were placed into the peritoneal cavity, the

midline laparotomy was closed in layers, and scaffolds were allowed to incubate for 4 weeks.

2.4 Histology and Immunofluorescence (IF)

For enteroids, beads of Matrigel were fixed for two hours in 10% formalin and paraffin embedded. Progressive 5 μm sections were taken and stained with hematoxylin and eosin (H&E) to determine their morphology. For TEI, explanted scaffolds were fixed overnight in 10% formalin. Each specimen was bisected perpendicular to the axis of the lumen, and paraffin embedded with the cut surface facing down. Progressive 5 μm sections were taken at 300 μm intervals through the length of the implant. Sections were stained with H&E to determine the presence of TEI, and to assess the architecture and quantify of neomucosa present. An implant was considered positive for TEI if any section was identified that contained simple columnar epithelium consistent with intestinal epithelium. To determine the amount of neomucosa produced, a representative slide from each implant was selected for further analysis. Using ImageJ software, the basilar surface of all identifiable simple columnar epithelium was outlined and the length quantified.

To identify the components present in TEI, a combination of Periodic Acid-Schiff (PAS) staining and IF was used. PAS staining was used to identify goblet cells as noted by the characteristic bright pink staining of mucin filled vacuoles. IF was used to identify LGR5+ ISC, Paneth cells, enterochromaffin cells, enterocytes, intestinal subepithelial myofibroblasts (ISEMFs), and smooth muscle cells. IF was also used to identify ISEMFs and smooth muscle cells in cultured enteroids. We did not study the epithelial components of enteroids as these have been previously identified.[24] Antigen retrieval was performed by boiling the slides in 10 mM Na-Citrate buffer (pH 6.0) in a pressure cooker (120°C; 15 psi) for 30 min. Slides were incubated at 4°C overnight with the following prima ry antibodies: green fluorescent protein (1:100; Abcam, Cambridge, MA), lysozyme (1:500; Abcam), chromogranin A (1:200; Abcam), villin (1:500; Thermo Fisher Scientific, Waltham, MA), smooth muscle alpha actin (1:500; Thermo Fisher Scientific), vimentin (1:500; Millipore, Billerica, MA), and desmin (1:500; Abcam, Cambridge, MA). After incubation in primary antibodies, Alexa Fluor 568 and Alexa Fluor 488 secondary antibodies (Life Technologies, Carlsbad, CA) were used for labeling, and the slides mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Images of enteroids were acquired using differential interference contrast (DIC) microscopy (Leica DMI 4000B, Buffalo Grove, IL). Light microscopy images of tissue were obtained using an Olympus SZX7 microscope or an Olympus BX51 microscope (Center Valley, PA). Fluorescent images were obtained using a Zeiss 710 confocal microscope (Peabody, MA).

2.5 Experimental Design

To investigate the potential use of enteroids as a tissue source for TEI production we needed to: 1) identify a reliable enteroid seeding medium for TEI growth, 2) determine the optimal enteroid seeding density for TEI growth, and 3) establish that TEI could be produced from minimal starting material.

2.5.1 Enteroid Seeding Medium—To determine a reliable enteroid seeding medium for TEI growth, enteroids were seeded onto PGA scaffolds using either growth factor reduced Matrigel or complete enteroid culture media. The protocol for establishing enteroid culture (section 2.2) was performed ten separate times using ten different mice to obtain enough enteroids for completion of the experiments. Fifteen scaffolds were seeded and implanted in both the Matrigel and complete enteroid culture media arms as described in section 2.3. All implants were evaluated histologically for the presence of TEI and the quantity of neomucosa produced four weeks after implantation.

2.5.2 Enteroid Seeding Density—To determine the optimal seeding density for TEI growth, enteroids were seeded onto PGA scaffolds at 10, 20, and 30 enteroids/mm^3 of scaffold material. The protocol for establishing enteroid culture (section 2.2) was performed ten separate times using ten different mice to obtain enough enteroids for completion of the experiments. Enteroids were seeded at the desired seeding density with Matrigel, and implanted in NOD/SCID mice using the methods described in section 2.3. Fifteen scaffolds were seeded at 10 enteroids/mm^3, fifteen scaffolds at 20 enteroids/mm^3, and thirteen scaffolds at 30 enteroids/mm^3. All implants were evaluated for the presence of TEI and the quantity of neomucosa produced four weeks after implantation. These specimens were additionally analyzed using PAS staining and IF to identify TEI components.

2.5.3 Enteroid Expansion—To determine if enteroids represent a clinically viable tissue reservoir for TEI production, enteroid culture was established from minimal starting material, comparable in size to an endoscopic biopsy, expanded *ex vivo* for scaffold seeding, and implanted to grow TEI. Nine 2 × 2 mm pieces of proximal mouse jejunum were obtained from three different LGR5-EGFP mice. Each piece of tissue was processed individually to establish enteroid culture using the methods described in section 2.2. Enteroids were passaged for expansion to obtain enough material to seed scaffolds for implantation. Passaging was performed every 7-14 days by aspirating the enteroids through a 30 gauge needle to break them into small enteroid fragments. The fragments were then resuspended in fresh Matrigel and re-plated in 24-well culture plates. Enteroids were quantified before and after passage using DIC microscopy (Leica DMI 4000B) to establish passage efficiency. Once enough enteroids were obtained for seeding, scaffolds were seeded and implanted for four weeks using the methods described in section 2.3. All implants were evaluated for the presence of TEI.

2.6 Statistical Analyses

To compare the length of neomucosa produced from the two different seeding mediums, the Mann-Whitney test was used for analysis. P values less than 0.05 were considered significant. To compare the length of neomucosa produced from the three different seeding mediums, the Kruskal-Wallis test was used for analysis. P values less than 0.05 were considered significant. Pairwise comparisons between every two seeding densities were performed using the Mann-Whitney test. Bonferroni correction was used for multiple comparisons and p values less than 0.017 were considered significant. Gender of the donor or host animals was not considered in analysis. All analyses were performed using GraphPad Prism 6 (La Jolla, CA).

3. Results

3.1 Enteroid Seeding Medium

Seeding of crypts in Matrigel produced TEI in 15/15 (100%) attempts, whereas seeding with complete enteroid culture medium produced TEI in only 9/15 (60%) attempts (Figure 1A). Regarding the quantity of neomucosa produced, when seeded with Matrigel, implants grew an average of 10,520 ± 2,905 μm of neomucosa which was significantly greater than the average of 450 ± 127 μm when seeded with complete enteroid culture media ($p \leq 0.05$) (Figure 1B). The architecture of scaffolds seeded with Matrigel ranged from a simple columnar epithelium to crypt domains with overlying blunted villi (Figure 2). Figure 2 also demonstrates how neomucosal length was outlined for the quantification shown in Figure 1B.

3.2 Enteroid Seeding Density

Seeding enteroids at 20 enteroids/mm^3 of scaffold material produced TEI in 15/15 (100%) attempts compared to 14/15 (93.3%) attempts at 10 enteroids/mm^3 and 8/13 (61.5%) attempts at 30 enteroids/mm^3 (Figure 3A). Regarding the quantity of neomucosa produced, when seeded at 20 enteroids/mm^3 implants grew an average of 14,896 ± 2,974 μm of neomucosa which was significantly greater than the average of 5,970 ± 1,753 μm or the average of 2,461 ± 943 μm grown in the 10 and 30 enteroids/mm^3 groups, respectively ($p \leq 0.01$) (Figure 3B). There were no significant differences between the amount of neomucosa produced in the 10 or 30 enteroids/mm^3 seeding groups. The histologic architecture in this experiment is shown in Figure 2. Seeding at 20 enteroids/mm^3 of scaffold material offered the highest likelihood of producing TEI and produced the greatest quantity of neomucosa.

3.3 Enteroid Expansion from Minimal Starting Material

Crypts were successfully obtained from each of the nine fragments of LGR5-EGFP intestine and grown into enteroids (Figure 4). Each fragment initially produced an average of 192 ± 71 enteroids. The nine colonies of established enteroids were passaged after 14 days of culture, yielding an average of 685 ± 58 enteroids. This provided an approximate passage ratio of 1 : 3.5. Only a single passage was necessary to obtain enough crypts to seed a single PGA scaffold in each of the nine attempts. TEI was produced from expanded enteroids in 8/9 (88.9%) attempts. The architecture observed in these nine specimens was consistent with that demonstrated in Figure 2.

3.4 Components of Enteroids and TEI

For TEI, a combination of PAS staining and IF identified LGR5+ ISC as well as all of the terminally differentiated epithelial cell lineages including goblet cells, Paneth cells, enterocytes, and enterochromaffin cells (Figure 5). LGR5+ ISC were demonstrated by staining for GFP, which identifies the LGR5+ ISC and confirms that the TEI produced is derived from the LGR5-EGFP donor enteroids that were seeded onto the scaffolds. IF also identified the presence of ISEMFs and smooth muscle cells in TEI but not in cultured enteroids (Figure 6).

4. Discussion

The ability to produce TEI has significantly advanced in recent years. Intestinal organoid units obtained from healthy intestine have been shown to produce a complex intestinal tissue containing the major components of normal intestine. Organoids obtained from rodents, dogs, swine, and humans have been used to produce TEI.[9-12] Moreover, short segments of TEI produced by these methods have been anastomosed as interposition intestinal grafts without detrimental effects, and with some evidence that the TEI produced may help to reverse the effects of SBS when anastomosed in line in an animal model of SBS.[26] However, there remain multiple challenges to translational application, including an adequate source of intestinal tissue for TEI production. Organoid units for TEI production are obtained by the digestion of healthy intestinal tissue. To avoid the need for immunosuppression, autologous organoid units need to be obtained. However, SBS patients have limited expendable healthy intestine. A reliable and expandable donor tissue reservoir needs to be identified. Given the ability for long term maintenance and logarithmic expansion *in vitro*, we investigated the use of enteroid culture as a potential tissue reservoir for the production of TEI.

On examination of the seeding substrate, TEI from enteroids seeded in complete enteroid culture medium only was inconsistently produced, and when it was present the quantity of neomucosa was limited. However, when seeded using Matrigel, TEI was always present with substantially increased neomucosa. Others have noted similar findings when seeding organoid units in Matrigel for the production of TEI.[27] The advantages provided by Matrigel are unclear, but there are several potential benefits. Matrigel is a sterile extract of the Englebreth-Holm-Swarm mouse sarcoma that contains numerous proteins and basement membrane components including laminins, type IV collagen, and growth factors.[28] The growth factors present are numerous and include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF). Matrigel has been shown to enhance differentiation in numerous cell lines *in vitro* and to promote angiogenesis and adipogenesis.[28, 29] Although we utilized growth factor reduced Matrigel, which has been processed to remove the bulk of these proteins, they are not depleted.[30, 31] Potential benefits of Matrigel in the production of TEI include: 1) enhanced cell attachment through the viscous nature of the seeding substrate as well as basement membrane proteins including type IV collagen and laminins; 2) the differentiation and proliferative effects on the epithelium from the contained growth factors; and 3) the angiogenic properties of Matrigel potentially providing enhanced blood supply. Further investigation is necessary to determine Matrigel's beneficial effects.

We have demonstrated significant effects on TEI production with variable enteroid seeding densities. Seeding with 20 enteroids/mm^3 of scaffold was the optimal seeding density found. In standard cell culture, it is well known that low seeding density significantly decreases cell survival in many cell lines, especially in primary cell cultures. This may be due to decreased cell-derived signaling at low cell densities.[32] It is possible that this same effect occurs when enteroids are seeded at low densities *in vivo*. The general effect of inadequate seeding density has been observed in other areas of tissue engineering. In models of bone, cartilage,

and adipose tissue engineering, seeding densities that are too low produce less tissue, and increasing the concentration of starting material leads to an increase in the amount of engineered tissue produced.[33-35] On the other hand, seeding densities that are too high have also been shown to hinder proliferation, decrease differentiation, and alter protein expression, resulting in less desirable tissue properties.[36, 37] These studies underscore the importance of identifying optimal seeding densities. To our knowledge, we are the first to demonstrate the importance of optimizing seeding density in any model of TEI. Further studies are needed to better understand how variable seeding densities alter TEI production and phenotype.

Others have previously shown that enteroids cultured *in vitro* can be utilized to grow neomucosa *in vivo* [38]. However, the architecture was limited to relatively small, isolated cysts of simple columnar epithelium. Additionally, they were unable to grow any neomucosa unless they were seeded with enteroids co-cultured with ISEMFs. In the data presented here, TEI could be produced in every attempt when paired with an ideal seeding medium and optimal seeding density. Moreover, the organization of the neomucosa more closely recapitulated crypt-villus architecture, and contained all of the expected intestinal epithelial cell lineages. This was achieved in the absence of ISEMF co-culture. The TEI produced also contained ISEMFs and smooth muscle cells, even though these cell types were not identified in the enteroids seeded onto the scaffolds. We speculate that this is may be due to the location of scaffold implantation. In the models that required the use of co-cultured ISEMFs, scaffolds were implanted in subcutaneous pockets.[38-41] We utilized the peritoneal cavity as an incubator for the production of TEI. It is possible that an intraperitoneal location provides local paracrine signaling effects to the implants by way of proximity to the surrounding viscera, which contain a robust mesenchymal niche in the form of peri-crypt ISEMFs. The presence of ISEMFs and smooth muscle cells in the TEI, despite their absence in the seeded enteroids, suggests that either: 1) the intraperitoneal location allows their recruitment into the TEI, or 2) trans-differentiation occurs from an epithelial lineage into the other cell types. The mechanism by which this occurs is not known and further investigation into the source of the identified ISEMFs and smooth muscle cells through lineage tracing will be performed in the future.

With a reliable method for TEI production from enteroids established, we then investigated their potential as a clinically applicable tissue reservoir. We demonstrated that a single small piece of intestine comparable in size to an endoscopic biopsy had the ability to establish enteroid cultures. The number of enteroids obtained from a single biopsy was only enough to grow in a single 50 μL drop of Matrigel. Despite having very few enteroids to work with initially, passaging and expansion were successful. Only one passage was necessary to reach the number of enteroids needed for scaffold seeding in mice. Coupling the findings we have demonstrated here with the fact that enteroids can be maintained for many months and expanded logarithmically, enteroid expansion can be easily scaled up to the size and length of scaffold required for human applications. Figure 7 illustrates how the methods established here may ultimately apply to patients with SBS.

Our study demonstrates that enteroids are a potentially viable tissue source for TEI production. Although this method shows great potential, it is not without limitation. The

growth of TEI from enteroids was not adequate when seeded in simple enteroid growth medium and required the use of Matrigel as a seeding substrate. Due to its origin from a mouse tumor, it is unlikely that Matrigel will be approved for clinical use. Studies investigating type I collagen as an alternative for intestinal epithelial growth have been met with some success.[39, 40] However, optimal alternatives to Matrigel are yet to be determined. There are also limitations with the complexity of the TEI we have produced. Although all of the necessary epithelial components as well as ISEMFs and smooth muscle cells are present, the architecture and organization of the TEI is not optimal. There is crypt-villus architecture, however, the villi are blunted and do not reach the lengths that are typical of native intestine. Although it appears that ISEMFs and smooth muscle cells are being recruited into the TEI, they are disorganized and do not recapitulate the laminated nature of native intestine. There is already some evidence showing that co-seeding with other cell lineages improves the quantity of the epithelium and the overall quality of TEI in other models.[38, 41, 42] Further efforts are needed to establish methods for organized co-seeding of enteroids with other cell types such as ISEMFs, smooth muscle cells, and neural stem cells in order to augment the architecture and organization of the TEI produced. Although we have demonstrated the presence of cell lineages consistent with native intestine, we still need to investigate the function of the TEI produced. Further investigation of functional components including the presence of disaccharidases and ion and nutrient transporters is necessary to determine whether this TEI has absorptive function. Lastly, we have begun evaluating different polymer scaffolds and architectures to further help direct differentiation and neomucosa formation.[22, 43]

5. Conclusions

In conclusion, we have established a reliable method of TEI production utilizing *ex vivo* enteroid cultures. We have demonstrated the importance of an optimal seeding density to maximize tissue production. With these methods established, we were able to show proof of concept that TEI can be produced from the limited starting material that a patient with SBS can provide. We conclude that *ex vivo* enteroid cultures represent a potential tissue source for the production of TEI. Further studies of enteroid cultures are underway to maximize the translational capabilities of this technique in the production of TEI for clinical use.

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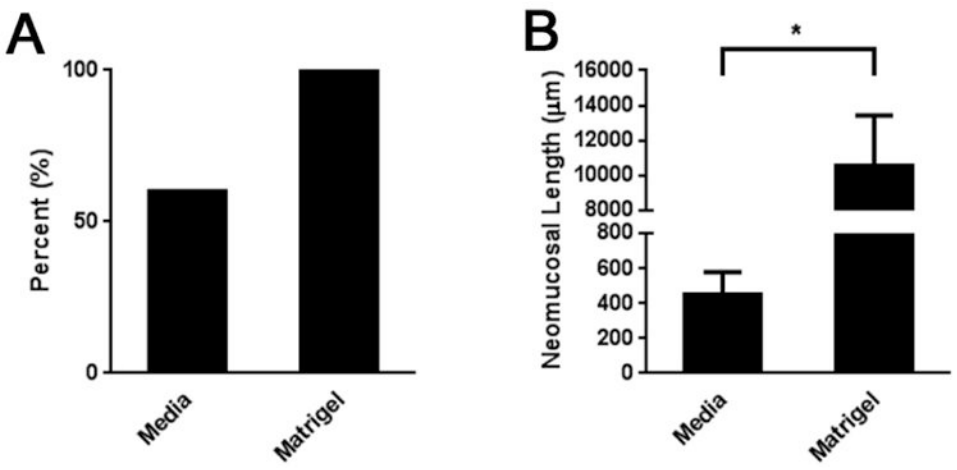

Figure 1. Effects of enteroid seeding substrate on the production of TEI

A) Percent positivity of TEI. B) Neomucosal length of TEI. Enteroids were seeded onto PGA scaffolds after 11-14 days in culture using either complete enteroid culture medium or Matrigel. Scaffolds were then implanted into the peritoneal cavity of NOD/SCID mice four weeks after which they were explanted and analyzed using H&E staining. Positivity was defined as the presence of simple columnar epithelium consistent with intestinal mucosa. Neomucosal length was quantified on a representative section using ImageJ software by tracing the basal surface of all simple columnar epithelium. n = 15 for both media and Matrigel groups. *$p \leq 0.05$.

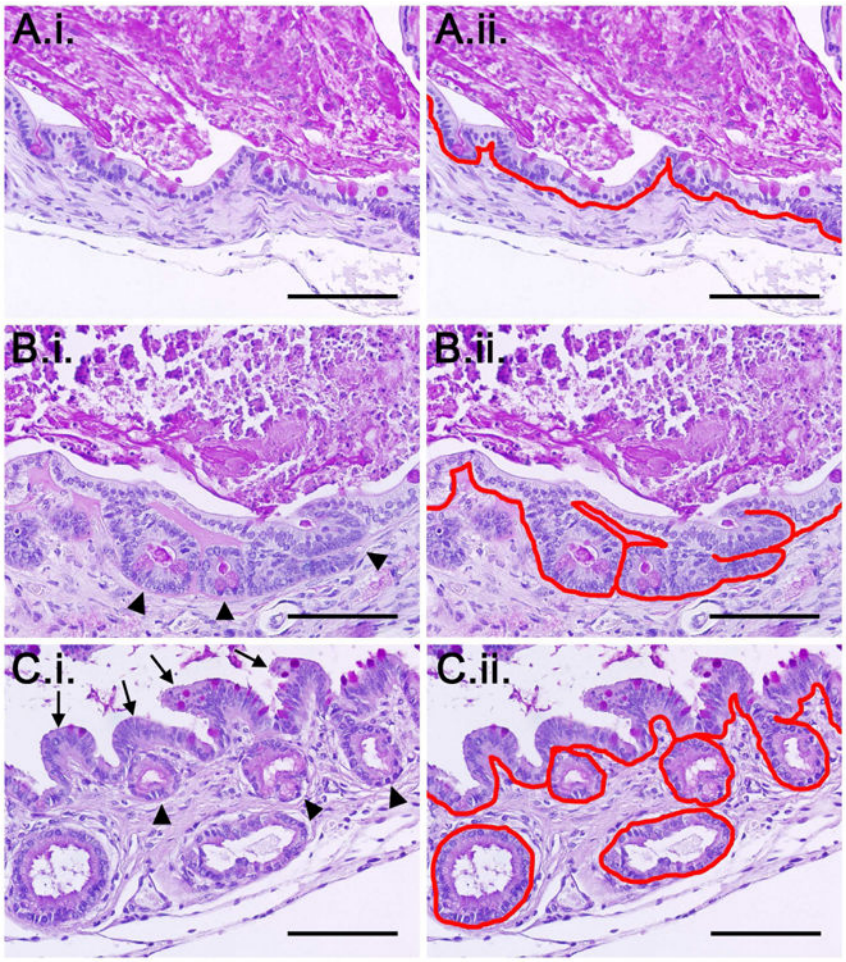

Figure 2. Architecture of TEI produced from enteroid seeding

Shown are representative images demonstrating: A.i) simple columnar epithelium, B.i) flat simple columnar epithelium with underlying crypt domains (arrowheads), and C.i) crypt domains (arrowheads) with overlying blunted villi (arrows). A.ii-C.ii) Respective images of the varying types of neomucosa with the basement membrane traced in red to demonstrate how the neomucosa was quantified. All images are stained PAS. The bright pink staining in the lumen represents retained cellular debris and mucin. Scale bars = 100 μm.

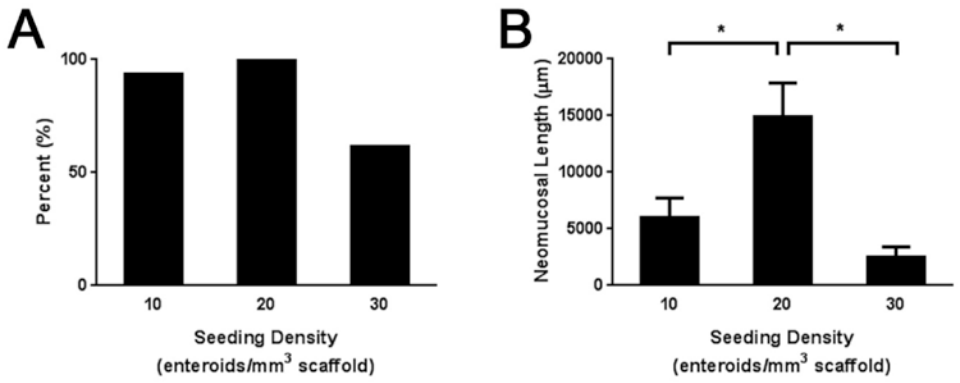

Figure 3. Effect of enteroid seeding density on the production of TEI

A) Percent positivity of TEI. B) Neomucosal length of TEI. Enteroids were seeded to PGA scaffolds after 11-14 days in culture at 10, 20, or 30 enteroids/mm^3 of scaffold. The scaffolds were then implanted into the peritoneal cavity of NOD/SCID mice four weeks after which they were explanted and analyzed using H&E staining. Positivity was defined as the presence of simple columnar epithelium consistent with intestinal mucosa. Neomucosal length was quantified on a representative section using ImageJ software by tracing the basal surface of all simple columnar epithelium. n = 15 for 10 and 20 enteroids/mm^3; n = 13 for 30 enteroids/mm^3. *$p \leq 0.01$.

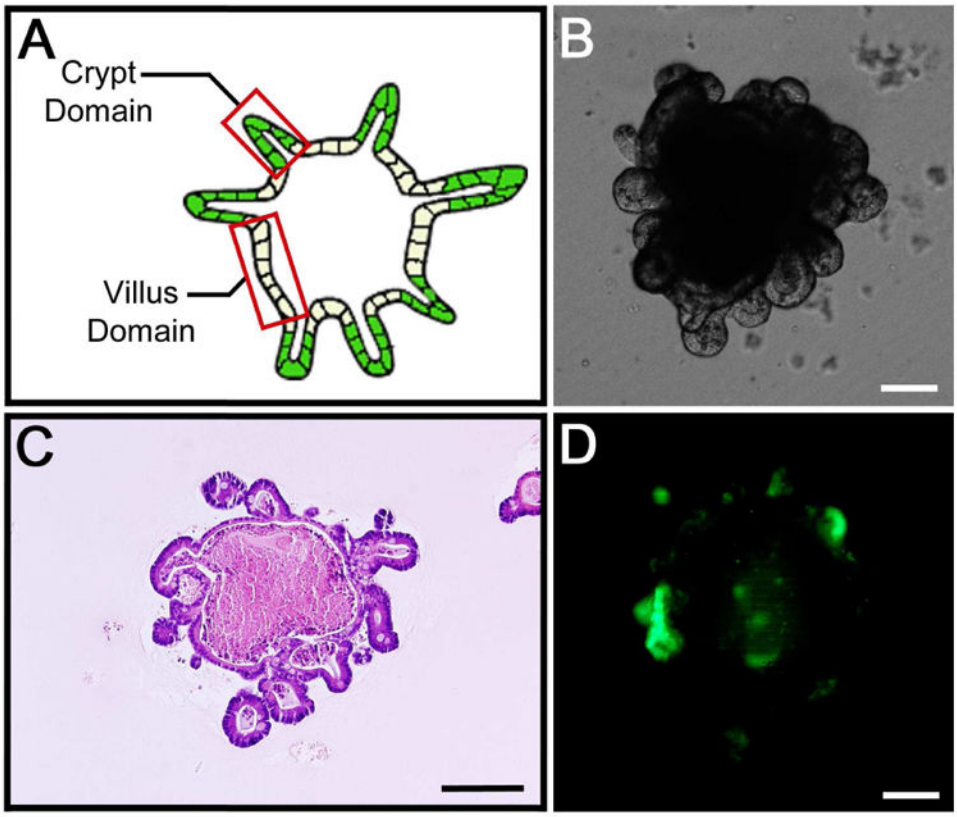

Figure 4. LGR5+ enteroids

A) Illustration of a typical enteroid demonstrating the crypt domains as represented by the peripherally oriented buds and the villus domains as represented by the epithelium between the crypt domains. B) DIC microscopy showing a typical enteroid after 11 days in culture. Note the peripherally oriented buds corresponding to the crypt domains. C) H&E staining of a typical enteroid after 14 days in culture with peripherally oriented buds consistent with crypt domains. Note luminal contents consistent with cellular debris and mucin. D) Fluorescence microscopy confirming that the peripheral buds of the enteroids represent crypt domains that contain LGR+EGFP ISC (green). Scale bars = 100 μm.

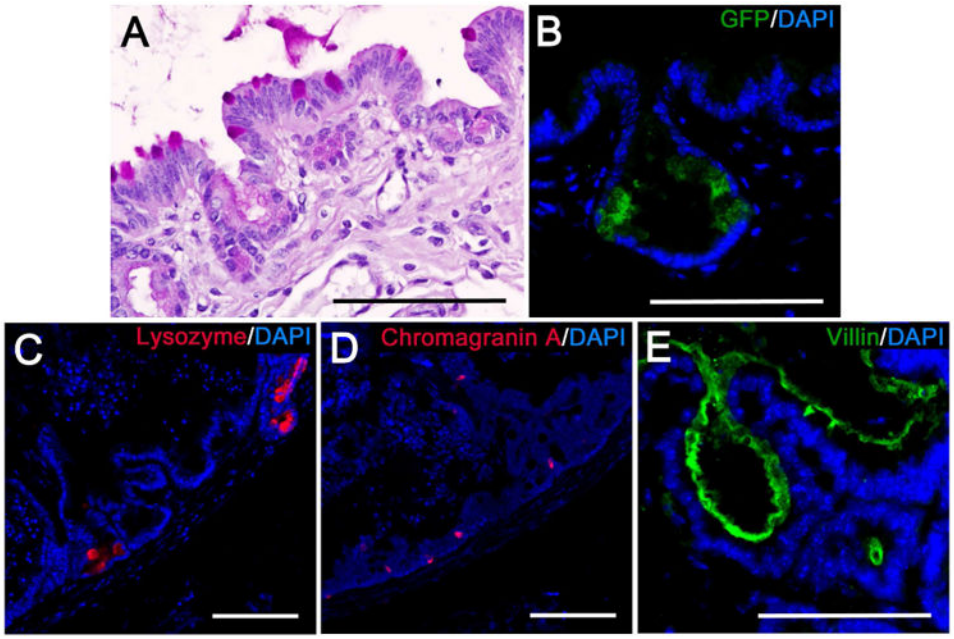

Figure 5. TEI contains ISCs and differentiated intestinal epithelial cells

Shown are representative images demonstrating: A) PAS staining to identify goblet cells as noted by mucin filled vacuoles that stain bright pink, B) IF labeling for green fluorescent protein (GFP) (green) to identify LGR5+ ISC in crypt domains. C) IF labeling of lysozyme (red) to identify Paneth cells, D) IF labeling of chromagranin A to identify enterochromaffin cells (red), E) IF labeling of villin in the brush border to identify enterocytes (green). For all IF images, nuclei labeled with DAPI are represented in blue. Scale bars = 100 μm.

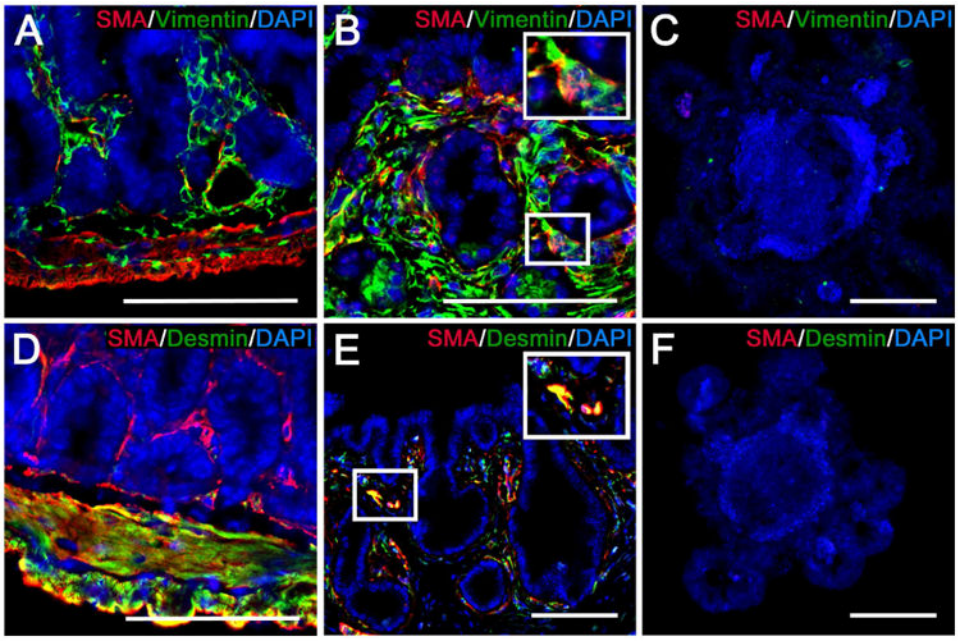

Figure 6. TEI contains ISEMFs and smooth muscle cells

Shown are representative images from native intestine (A, D), TEI (B, E), and enteroids (C, F) that have been subjected to IF labeling for the identification of ISEMFs and smooth muscle cells. A-C) ISEMFs are identified by co-localization of labeling for smooth muscle alpha actin (SMA) (red) and vimentin (green). D-F) smooth muscle cells are identified by co-localization of labeling for SMA (red) and desmin (green). A) Native intestine shows strong SMA and vimentin co-localization in the submucosal layers only, indicative of ISEMFs. There is some vimentin staining in the muscularis that occurs in the mesenchymal stroma between the longitudinal and circular layers. B) TEI demonstrates the same SMA and vimentin co-localization surrounding the crypt domains. C) SMA and vimentin are not evident in enteroids. D) Native intestine shows strong SMA and desmin co-localization in the muscularis, indicative of smooth muscle cells. There is SMA labeling in the submucosa without desmin co-localization, consistent with ISEMFs. E) TEI demonstrates similar SMA and desmin co-localization surrounding all areas of the epithelium, however, it appears scattered and less organized than that observed in native intestine. F) SMA and desmin are not evident in enteroids. Scale bars = 100 μm.

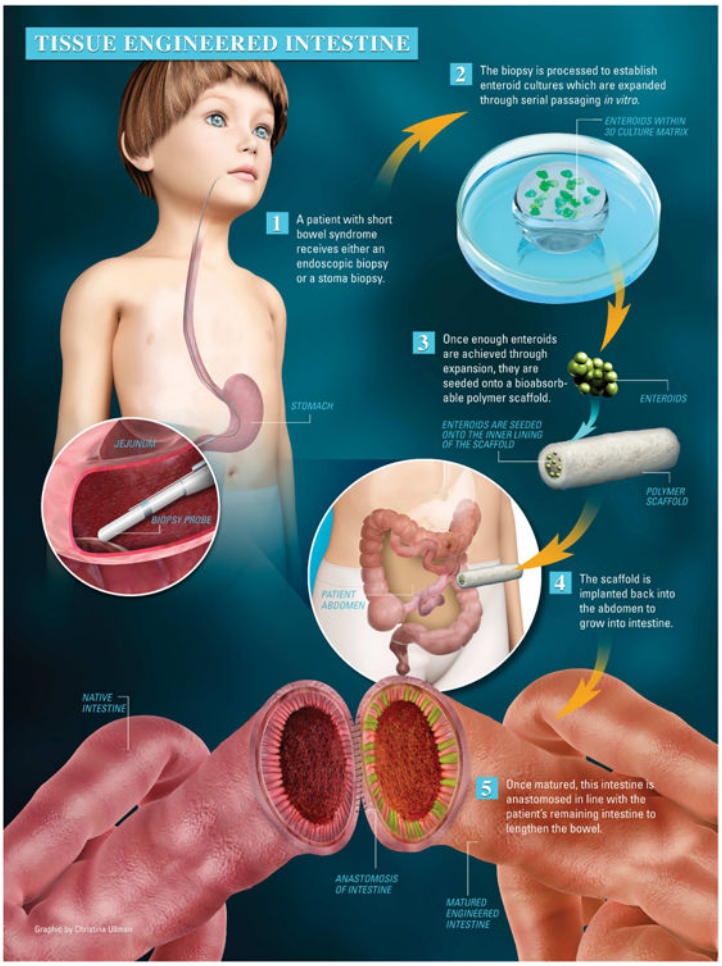

Figure 7. The potential use of enteroid culture in human tissue engineered intestine

1) A patient with SBS undergoes either an endoscopic or stoma biopsy. 2) The biopsy is processed to establish enteroid cultures which can be expanded as needed for the length of intestine required. 3) The appropriate number of enteroids are seeded onto a bioabsorbable scaffold of appropriate length and, 4) implanted back into the peritoneal cavity of the same SBS patient. 5) Once matured, the TEI is anastomosed with the patient's remaining native bowel to lengthen and reverse the effects of SBS.