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Tissue engineering for the treatment of short bowel syndrome in children

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Abstract

Short bowel syndrome is a major cause of morbidity and mortality in children. Despite decades of experience in the management of short bowel syndrome, current therapy is primarily supportive. Definitive treatment often requires intestinal transplantation, which is associated with significant morbidity and mortality. In order to develop novel approaches to the treatment of short bowel syndrome, we and others have focused on the development of an artificial intestine, by placing intestinal stem cells on a bioscaffold that has an absorptive surface resembling native intestine, and taking advantage of neovascularization to develop a blood supply. This review will explore recent advances in biomaterials, vascularization, and progress towards development of a functional epithelium and mesenchymal niche, highlighting both success and ongoing challenges in the field.

Introduction

The clinical problem of short bowel syndrome

Short bowel syndrome (SBS) refers to the condition in which limited intestinal mucosa cannot meet the nutritional needs of the patient via enteral absorption (1,2). The diagnosis of SBS relies on an assessment of the loss of intestinal length (1–3), in combination with poor enteral absorption (4), dependence on total parenteral nutrition (TPN) (5–7), or some combination of these factors. (1,2,8). The epidemiology of SBS remains difficult to define due to variation in diagnostic criteria, study population (pediatric versus adult), and the length of the follow-up period among studies. In one of the largest population-based studies of SBS (3), the overall incidence was found to be 0.02% of all live births, and 2.2% of

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neonatal intensive care unit (NICU) admissions. Notably, the incidence among premature infants (<37 weeks gestational age) was 100× higher than among infants >37 weeks. Further, there is an increased incidence associated with low birth weight (0.7 in very low birth weight, and 1.1% in extremely low birth weight) babies (1).

Causes of short bowel syndrome and the need for innovative therapies

Among the pediatric population, leading causes of SBS include necrotizing enterocolitis (NEC), intestinal atresia, gastroschisis, and malrotation with volvulus (2,7,9,10), with NEC representing about 96% of cases in the low birth weight NICU population (1).

Complications of SBS are many, and include cholestasis resulting in liver failure, bowel dilation resulting in bacterial overgrowth, and sepsis with its related complications, which may arise both from central-line associated bacterial infection [CLABSI] and bacterial overgrowth. (2,5,7,10–12). The overall mortality of SBS is estimated at 27.5% to 37.5% over a follow up period of 2–5 years (1,2,7,8,13), with leading causes of death being hepatic failure and sepsis (2,7,9,11,13). Given that the inability to achieve enteral autonomy is one of the leading predictors of mortality (2,7,9), current therapy for SBS aims to restore enteral autonomy. Present treatment options focus on either increasing absorption (via adaptation and intestinal reversal procedures) or restoring intestinal length (via intestinal lengthening procedures and transplant.). Despite decades of experience with TPN supplementation and optimization of surgical techniques and transplantation, half of all patients with SBS will never attain enteral autonomy. The annual mortality in SBS is 15–30% (3,8,12.) Given the limited success and high morbidity of current therapy, novel treatment approaches are clearly needed. As will be reviewed in the following sections, the development of an artificial intestine derived from the patient's own intestinal stem cells and incorporated into a novel bioscaffold that recruits an endogenous blood supply may represent an attractive option for the treatment of children with SBS.

Strategies for the development of an artificial intestine

The goals of developing an artificial intestine include the provision of appropriate absorptive epithelium, barrier and immune functions, and motility. In addition, the artificial intestine would be autologous in order to achieve enteral independence without the need for immunosuppressive drugs. In support of the success of this approach, the modern era of tissue engineering began in 1988 with Joseph Vacanti and Robert Langer demonstrating growth of pancreatic and intestinal tissue on a bioabsorbable scaffold implanted into the omentum of rats (14). Since this early finding, there has been a large body of research focused on the development of a tissue engineered intestine. The basic requirements for an engineered intestine include a source of stem cells with the capacity to grow and differentiate into a mature and absorptive mucosal surface (Figure 1), a bioscaffold capable of supporting cellular growth (Figure 2–4), a niche for engraftment and growth of the tissue, and vascularization of the new tissue. Additional challenges include meeting the large surface area needed for enteral autonomy and optimization of gastrointestinal motility. Each of these challenges will be discussed with a focus on successes and opportunities which lie ahead.

Biomaterials for the development of an artificial intestine

The production of an effective and biocompatible scaffold on which cells can grow and be transplanted as a graft is a critical step in the development of artificial intestine. A successful bioscaffold will require the following features: a material capable of supporting stem cell attachment, proliferation, and differentiation; support of angiogenesis and vascular ingrowth; approximation of mechanical and electrical properties of the intestine so as to facilitate operative insertion, and incorporation without an excessive acute inflammatory or chronic immune response. Available scaffolds fall into one of three categories: decellularized organs, biological materials, and synthetic polymers.

i. decellularized tissue—Decellularized scaffolds have been used for a variety of tissue engineering applications (15–20), and decellularized intestine has garnered attention as a potential scaffold for engineered intestine(21–23). Totonelli *et al.* demonstrated the adherence of intestinal epithelial cells, preservation of extracellular components, and angiogenic properties of a seeded decellularized intestinal scaffold. Nowocin *et al.* demonstrated the ability to implant seeded decellularized pig intestine scaffolds into rats without evidence of an inflammatory response. Other decellularized tissues such as acellular dermal matrix (ADM) and small intestinal submucosa have also been demonstrated to support intestinal mucosal ingrowth when anastomosed with native intestine (24–26). With all decellularized scaffolds, tissue source is a consideration. Additionally, some recent evidence (27) suggests that human embryonic stem cells (hESCs) do not grow on decellularized porcine tissue, and human intestinal organoids (HIOs) lose their intestinal phenotype on decellularized porcine tissue *in vivo*.

ii. biologically based scaffolds—Biologically-based scaffolds are typically created from components of tissue, such as collagen, or other naturally occurring materials like silk or chitosan hydrogel (28–33). Small intestinal submucosal components and collagen carry the advantage of maintaining some components of the naturally occurring extracellular matrix, but allow more customization of the design of the final construct. Chitosan has been used with favorable biocompatibility, degradation, and abundant reactive groups useful for pharmaceuticals. Recent publications have reported successful creation of polarized epithelium and supportive mesenchyme in an *in vitro* model with silk scaffolds (29). Potential advantages of silk include low immunogenicity and the ability to tune its degradation and mechanical properties.

iii. synthetic scaffolds—The most widely-reported class of scaffold in the current literature is synthetic scaffolds. Specifically, polyglycolic acid (PGA), polylactic acid (PLA), poly-ε-caprolactone (PCL), polylactic-co-glycolic acid (PLGA), and other combinations are frequently used, often coated with a second polymer or matrix protein such as collagen, fibronectin, or matrigel (34–43) (Figure 2–4). The primary advantage of synthetic polymers is the ability to synthesize nearly unlimited quantities and tune mechanical, structural, and degradation properties based upon rational polymer design. Disadvantages include the potential for chronic inflammation and complement activation, which is seen particularly in the polyesters. Recently, in collaboration with the March lab, we have created novel intestinal scaffolds using synthetic PLGA that mimics the microarchitecture of the intestinal

villus, and demonstrated effective growth of enteroids and macrophages, as well as recreation of the crypt-villus architecture (43). We further demonstrated that these scaffolds were capable of regenerating the anorectal mucosa following mucosectomy in dogs. Additionally, PLGA has been shown to support growth of human enteroids, with successful transplantation into immunocompromised murine hosts (43–45). Recently, we have begun investigating the use of poly(glycerol sebacate) (PGS) synthetic scaffolds, which have been shown to support the growth of murine and porcine intestinal epithelium. (Figure 1)

Vascularization of the implanted scaffold

Vascularization is a critical step in the production of an artificial intestine. Implantation of artificial intestine into a recipient relies on cellular nourishment via diffusion until a vascular supply is achieved. In a series of experiments with autotransplantation of intestinal organoid units, (34,38,40,46), Vacanti's group reported successful vascularization of artificial intestine with production of neomucosa on *PLGA* scaffolds in P7 rat omentum after 3 weeks. We have corroborated these findings in our studies in dogs (43). Sites of engraftment in which vascularization can be successfully achieved include the omentum (34–37,43) or renal capsule (27,29,47). However, vascularization sufficient for surgical anastomosis of an isolated segment of artificial intestine in either the omentum or renal capsule remains a challenge. Gardner-Thorpe (34) *et al.* investigated angiogenesis in a rat model of omental vascularization and determined that while the mucosa and submucosa of artificial intestine increased in size, vascularity was limited to capillary growth without evidence of formation of the larger vessels present in native intestine. Further, they found that various growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were higher in native compared to artificial intestine. In a subsequent study (48), Rocha *et al.* found that PGA scaffolds seeded with VEGF microspheres and implanted into the omentum of rats resulted in higher VEGF levels, increased proliferation, and increased capillary density compared to empty constructs. Similarly, Minardi *et al.* (49) found improved vascularization and epithelial growth of tissue when scaffolds incorporated with slow-release platelet-derived growth factor were used. Another approach to vascularizing tissue engineered constructs is pre-vascularization. Pre-vascularization has demonstrated some success in cardiac and other muscular tissue engineering, but in spite of more robust capillary network formation, such tissues still lack adequate vascular architecture for surgical anastomoses with the native circulatory system. Thus, these systems rely instead on vascular budding and ingrowth to survive, which is explained in part from our finding that endothelial stem cells are recruited to the site of graft implantation (43). Ju, *et al.*, recently published a method of prevascularization using scaffolds pre-seeded with autologous endothelial cells and smooth muscle. Using this technique, they demonstrated fully cellularized vascular constructs capable of contractility. Their grafts demonstrated high degree of patency and structural integrity and minimal immune reaction in host tissue (50). Future directions may thus employ artificial intestinal constructs in which autologous endothelial stem cells are pre-woven into the graft (50,51).

Achieving an intact epithelial lining within the artificial intestine

The most important goal in the development of an artificial intestine is restoration of enteral autonomy. In order to achieve this, a functional mucosa capable of absorption must be

developed. A large body of work has been performed showing that intestinal crypt stem cells can differentiate into the four types of differentiated intestinal mucosa: goblet cells, enteroendocrine cells, paneth cells, and columnar mucosal cells (52,53). Perhaps the largest advance occurred through work of Hans Clever *et al.*, who first identified a reliable intestinal epithelial stem cell marker, LGR5, and demonstrated that crypt-villus units can be generated from a single LGR5+ stem cell (54,55). We now understand that LGR5 is a co-receptor for the canonical Wnt pathway, which is activated through binding of R-spondin proteins (56–60). Many investigators, including our group (35,36,43,45,61–63), have reported successful generation of intestinal tissue using an intestinal source of stem cells (Figure 2). While Clever *et al.* demonstrated that crypt-villus units can be generated from a single LGR5+ stem cell (enteroids), many methods of intestinal generation involve the isolation of multicellular units derived from the intestinal crypt, termed organoids, using the method of Evans *et al.* (64), rather than individual stem cells (Figure 1). The primary distinction between enteroids and organoids is the inclusion of mesenchymal cellular components in organoids, whereas enteroids consist of primarily intestinal epithelial cells.

Mesenchymal components required for generation of an artificial crypt-villus axis

The mesenchymal elements of the intestine have been found to play a critical role in creation and maintenance of the epithelial niche. This niche is maintained by intestinal subepithelial mesenchymal cells (ISEMCs), whose functions include the maintenance of the basement membrane and regulation of epithelial cell function via signaling pathways involving VEGF, Wnt, R-spondin, and stem cell factor. Multiple studies have suggested that the presence of myofibroblasts, macrophages, and smooth muscle cells enhance the growth and differentiation of artificial intestinal epithelium (56,57,67–69). Various authors have addressed the issue of mesenchymal support by utilizing native mesenchyme. In a series of papers from 1999–2000, Vacanti's group reported production of neomucosa with autotransplantation of intestinal organoid units on *PLGA* scaffolds in P7 rat omentum after 3 weeks (38,40,46). In additional studies, Grikscheit *et al.* have demonstrated neomucosa in rat pups with good crypt-villus architecture, collagen-rich submucosa and basement membrane, actin-positive muscle fibers in the muscularis propria, and microarchitecture including microvilli (34,39). Multiple experiments by Vacanti's group suggested that, following a period of growth in the omentum, anastomosis with native intestine enhanced growth of artificial intestine. When compared to artificial intestine that remained in the omentum without anastomosis, the anastomosed artificial intestine demonstrated superior villus height, crypt depth, and proliferation (38,46,70). It is unclear the degree to which this improved growth is attributable to lateral ingrowth of native intestinal mucosal and mesenchymal components versus restoration of the mesenchymal niche resulting in improved growth of transplanted cells. Other groups have recreated the mesenchymal niche by adding back specific growth factors, such as Wnt, Noggin, R-spondin, and epidermal growth factor (EGF) and demonstrated the ability to grow and expand enteroids *in vitro* for extended periods of time (55,71–73). Our group has focused on a combinatorial approach to establish the intestinal niche in epithelial cell culture utilizing a combination of Wnt, Noggin, R-spondin, EGF, macrophages, myofibroblasts, and commensal bacterial cultures resulting in a more robust epithelialization and vascularization of artificial intestine grafts (43).

Enteric Nervous System and Motility

In order for an artificial intestine to function as an autonomous, nutrient absorbing apparatus, it must attain some degree of innate peristaltic activity. To achieve this, various labs have focused on the ability to develop a functional enteric nervous system (ENS) (69,74–76). Pachnis' group identified (RET+) cells serving as multipotent progenitors capable of inducing colonization of aganglionic bowel with neurons and glia (77), and Schafer *et al.* published a technique for isolation of neurospheres from the ENS (78). When isolated and transplanted into aganglionic mice, these neurospheres differentiated into neurons and glia with evidence of synapse formation which were capable of contraction (79,80). In artificial intestine, multiple groups have demonstrated evidence of neuronal components after patch graft with collagen-based (33,67) or PLGA-based constructs (37).

More recently, neuronal components have been successfully harvested from humans (74, 76, 81.) Wieck et al demonstrated successful engraftment of human and murine neurospheres into tissue engineered colonic tissue with successful differentiation of neuronal and glial components (74). These findings suggest that co-culture of organoids with neurospheres may re-create functional neuronal networks and achieve intestinal motility as recently described by Workman *et al.* (82).

Putting it all together: manufacturing an artificial intestine

Several laboratories have integrated many of the above findings in order to manufacture an artificial intestine which appears to histologically resemble the native intestine. Grickscheit's group has shown that intestinal-derived organoid units can produce a histologically normal appearing epithelium, mesenchymal components, and neuronal components in models using omental implantation (44,45). Sala *et al.* reported one of the first large animal models with successful production of artificial intestine from autologous intestinal cells (37). In this model, autologous jejunal cells from 6 week old Yorkshire swine were seeded onto PLGA scaffolds and vascularized in the omentum. At 7 weeks, they were found to have crypt-villus architecture similar to native intestine. They also identified enterocytes, goblet cells, and enteroendocrine cells. They found ganglion cells between longitudinal and circular muscle layers and in the submucosa. One potential limitation to the organoid unit is its requirement for derivation from full-thickness intestine, raising the question as to whether a partial thickness biopsy could re-create all layers of the intestine or whether mesenchymal and neuronal components would need to be added back individually. Additionally, it is unclear whether the neuronal component is preserved after multiple expansions (72) and cryopreservation (44). Given these challenges, several groups have turned to a second potential source for intestinal tissue, namely HIOs, which may be generated from hESCs or induced pluripotent stem cells (iPSCs). Multiple groups have described a system in which hESCs and iPSCs can be differentiated into three-dimensional intestinal tissue with epithelium and mesenchyme (27,83–86).

Recent publications (27,84) have implanted HIOs into the mouse kidney capsule and demonstrated evidence of epithelial and mesenchymal tissue formation *in vivo*. After implantation for 6 weeks, these HIOs produced crypt-villus architecture with central capillary networks, demonstrated the presence of all epithelial cell types in addition to

multiple mesenchymal cell types, specifically ISEMFs, fibroblasts, and smooth muscle cells (84). Notably, when using HIOs alone, Finkbeiner *et al.* found an absence of S100b+ and NeuN+ cells. They were able to reproduce these cells by adding organoid units (27). This suggests that current methods of HIO differentiation may not be able to reproduce functional neuronal networks and may require the addition of organoid units or neurospheres. Workman *et al.* (2016) recently published methods to combine HIOs and iPSC-derived neural crest cells (NCCs) with the achievement of a functional enteric nervous system in mice (82).

Determining the functional capacity of artificial intestine

While multiple studies have demonstrated the ability to form artificial intestine that histologically resembles native intestine, the question remains, does it work? Several studies have evaluated the functionality of artificial intestine constructs. Specifically, in one study, rats underwent a colectomy with end ileostomy with the addition of an implantation of artificial intestine via an anastomosis in a side-to-side fashion proximal to the end ileostomy. The rats that received artificial constructs showed attenuated weight loss, slowed stool transit times, and increase in serum bile acids compared to controls (39). Another study reported that engineered intestine implantation resulted in improved recovery after massive small bowel resection with implanted rats regaining more of their preoperative weight (98%) than controls (76%). In addition, rats receiving engineered constructs had increased stool transit times (35). Avansino *et al.* also demonstrated improvement of bile acid malabsorption using ileal stem cell transplantation onto jejunal scaffolds after mucosectomy (87). Follow-up studies by Dr. Grikscheit's lab showed engineered intestine could be developed using human tissue as an organoid source when seeded onto PLGA scaffolds and implanted into the omenta of irradiated NOD/SCID mice. At four weeks, tissue evaluation revealed the presence of all four small intestinal epithelial cell types. Additionally, they noted presence of fibroblasts and neuromuscular elements. Immunostaining for the human isotype of β_2 microglobulin confirmed that these identified components were from the human cell source (45). In a subsequent study, using human organoid units, Grant *et al.* demonstrated the presence of sodium chloride secretory transporters in the basolateral membrane of the intestinal epithelium, as well as sodium glucose transport proteins (SGLT-1), suggesting that absorption theoretically could occur (62). Watson *et al.* (84) recently demonstrated successful engraftment of collagen-embedded HIOs into the kidney capsule of SCID mice. At six weeks, they demonstrated all intestinal cell lineages with normal architecture, proliferation, and differentiation along the crypt-villus axis. They further noted the presence of lamina propria, muscularis mucosa, submucosa, and smooth muscle layers after engraftment. They observed the presence of brush border enzymes such as alkaline phosphatase, lactase, glucose transporter 2 (GLUT2), and sucrose-isomaltase. Recent work by Workman *et al.* (82) presented novel methods of differentiating NCCs from human iPSCs. They presented an experimental model combining HIOs and neural crest cells derived from iPSC-neural crest cells (iPSC-NCCs) into spheroid aggregates, which were cultured under three-dimensional growth conditions for 28 days and then engrafted into mice for 6–10 weeks. Using this method, they produced not only mature vascularized intestinal tissue with submucosal and myenteric layers of smooth muscle fibers, but also found

evidence of organized neuronal ganglia located in close proximity to the submucosal and myenteric layers of smooth muscle. Functional tests of this tissue *in vitro* revealed coordinated calcium efflux in the ENS cells in response to potassium chloride and organized, ENS-dependent contractile waves in response to electrical stimulus. While investigation is ongoing into the ability to create a fully mature and functional enteric neuromuscular system, this study shows promise for the future development of a fully-functional artificial intestine.

Strategies towards clinical usage of the artificial intestine

With multiple successes in functional animal models, the field has begun to turn an eye towards clinical applicability. The ultimate goal in human treatment would be to obtain a human source of tissue, either full thickness intestine at the time of initial NEC resection or in a second surgery, biopsied intestinal tissue at a later date, or from iPSCs. To limit the amount of starting material required, several labs have described reliable methods for expansion of intestinal epithelial cells in culture (88). This would allow repopulation of an entire graft with a small amount of starting tissue. Besner and colleagues recently reported successful artificial intestine production using expanded enteroids from murine intestinal biopsies, confirming that small amounts of starting material can be used to generate enough cells for an entire graft (72). In a further bridge to clinical applicability, Spurrier *et al.* (44) have reported methods of successfully cryopreserving organoid units, suggesting that intestinal organoid units could be harvested and preserved until needed. It is unclear whether the neuronal component was preserved in these experiments. Recent work by Dr. Helmrath's group (82) holds promise for the potential to add in the neural component prior to implantation.

As noted above, an absorptive epithelium requires the development of an intact blood supply in order to transport nutrients from the gut lumen to the blood stream. This may be achieved in part through either recruitment of endogenous endothelial stem cells (48,89,90) or the use of a combination of pre-vascularization and prefabricated constructs with the artery/arteriole/capillary vascular hierarchical structure already present (50,51,91). For development of motility, barriers to overcome include the development of an independently functioning neuromuscular plexus, for which iPSC-NCCs hold promise (82). An alternative approach might be the use of battery powered implantable pumps to re-create peristaltic movement. Finally, while recent successes with human tissue in small animal models have been promising, large animal models are still needed. While various investigators, including our own group, have utilized swine and dogs, such studies are expensive, time intensive, and more prone to the development of significant inflammation after transplantation compared to rodent studies. Ongoing large animal trials are necessary to confirm the applicability of previous work in a model more similar to the human child and to ultimately gain Food and Drug Administration (FDA) approval for use.

Design of clinical trials using the artificial intestine

Once successful products are proven in animals, human trials will still face significant challenges. The FDA regulatory process will require that all fabrication and manufacturing

processes are safe. It will also be important to define which population may be expected to benefit most from implantation of an artificial intestine. Initial studies should focus on those in whom medical management has failed and in whom intestinal transplantation is not an option, for instance due to an anatomic or genetic barrier. In the ideal workflow setting, autologous stem cells would be obtained as described above, expanded *in vitro* on an appropriate scaffold, re-implanted into the host, and then connected to the native gut to allow for enteric absorption. Following the exciting work in human usage of vascular (92) and bladder (93) grafts, it is reasonable to have optimism that such success may also one day be seen for the artificial intestine.

Conclusions

Based upon studies performed over the past several decades, that which was once thought to be science fiction i.e. the generation of a functional artificial intestine – may finally be inching closer to reality. With recent advances in our ability to isolate, grow, and differentiate intestinal stem cells from the host, an increased understanding of the intestinal niche required to maintain the artificial intestinal mucosa, and novel techniques for the generation of an intact blood supply, it appears that the major elements for a functional absorptive intestine are achievable. With ongoing investigation into machine driven motility and endogenous neural elements, there is additional optimism that peristalsis may also be achieved. Taken together, through the work of many labs around the world, it is reasonable to believe that an artificial intestine may one day be developed for children with SBS, thus alleviating suffering and providing hope to these fragile patients.

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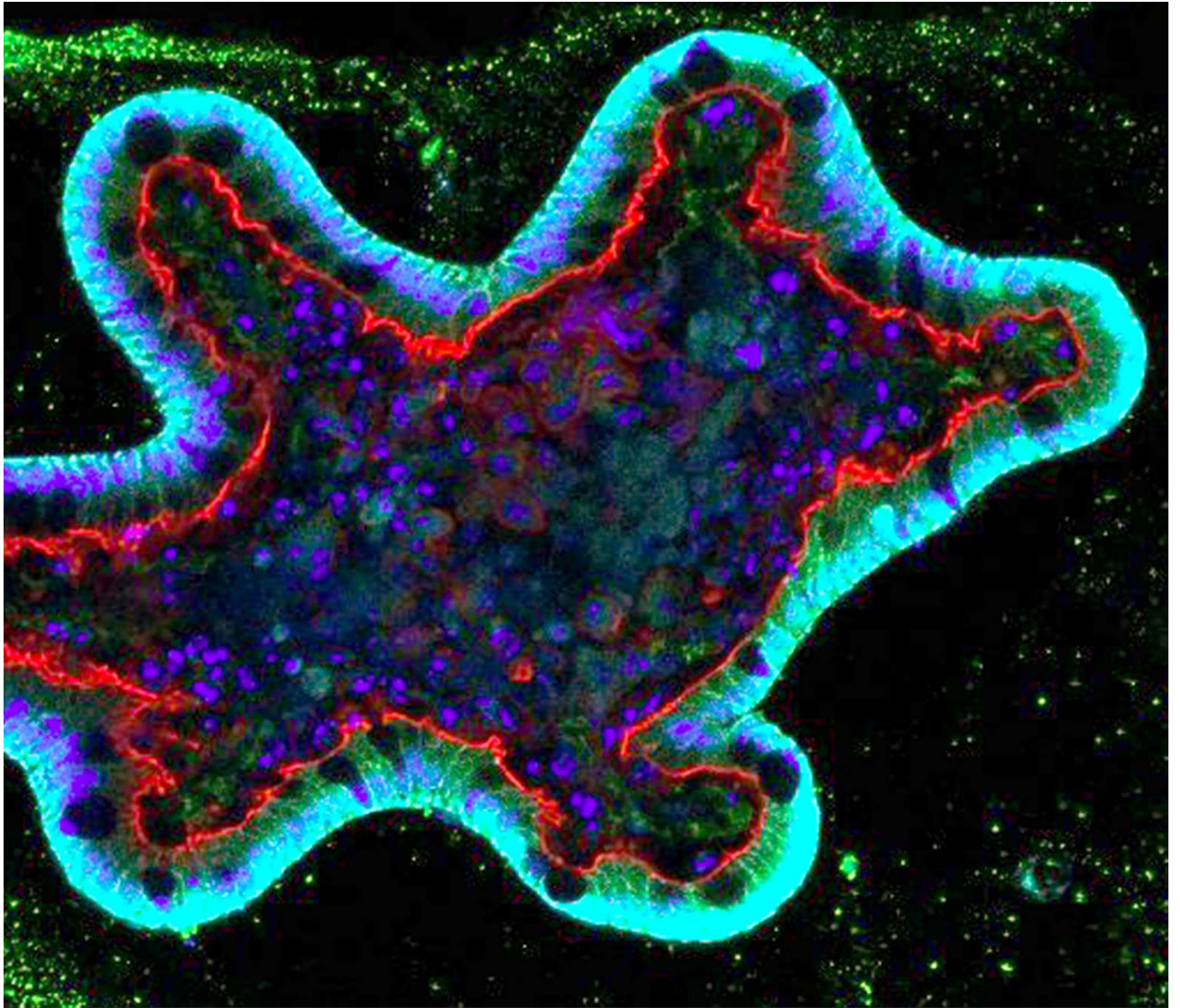


Figure 1. Confocal micrograph revealing the growth of a mini-gut (enteroid) in culture as a precursor to the development of an artificial intestine. Green - ki67, cyan – e-cadherin, red – phalloidin, blue – DAPI.

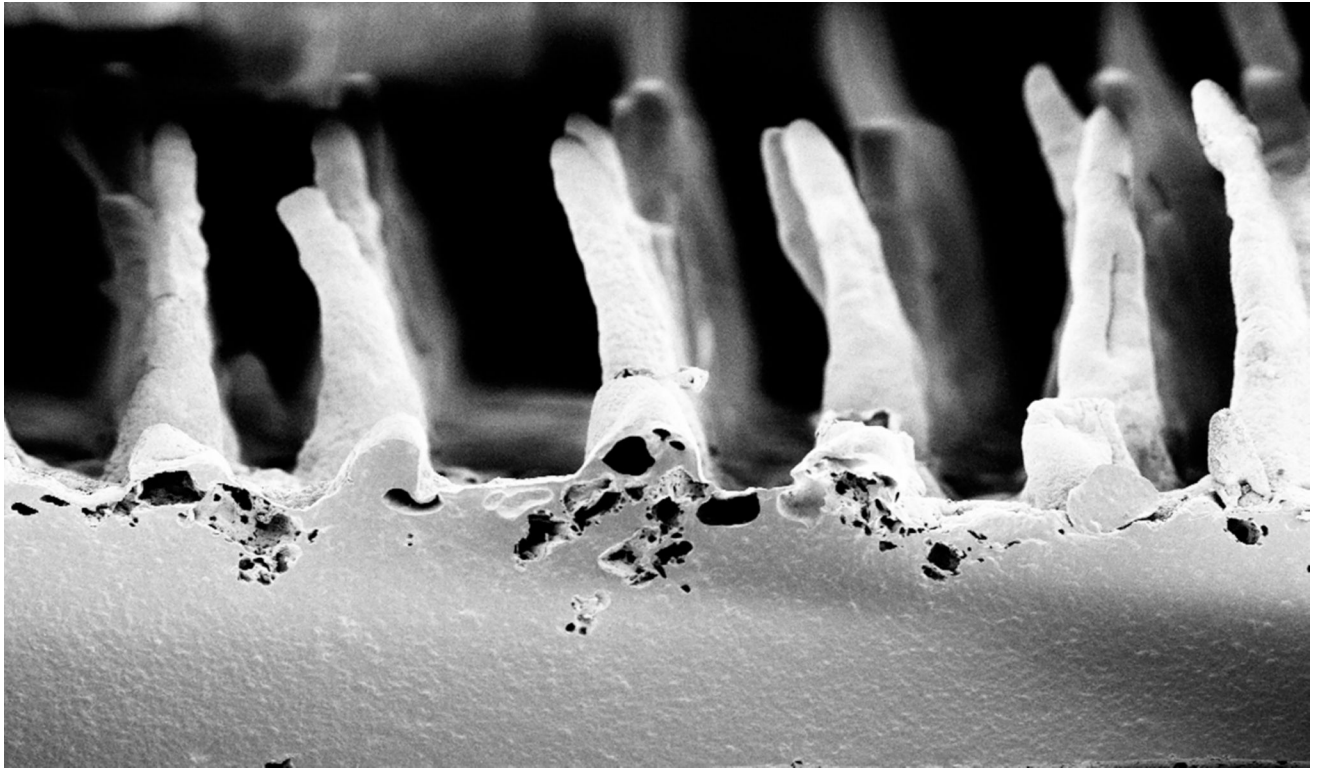


Figure 2. Scanning electron micrograph showing a synthetic (Poly(glycerol) sebacate (PGS) scaffold with an architecture that mimics the native intestinal crypt-villus architecture in cross section.

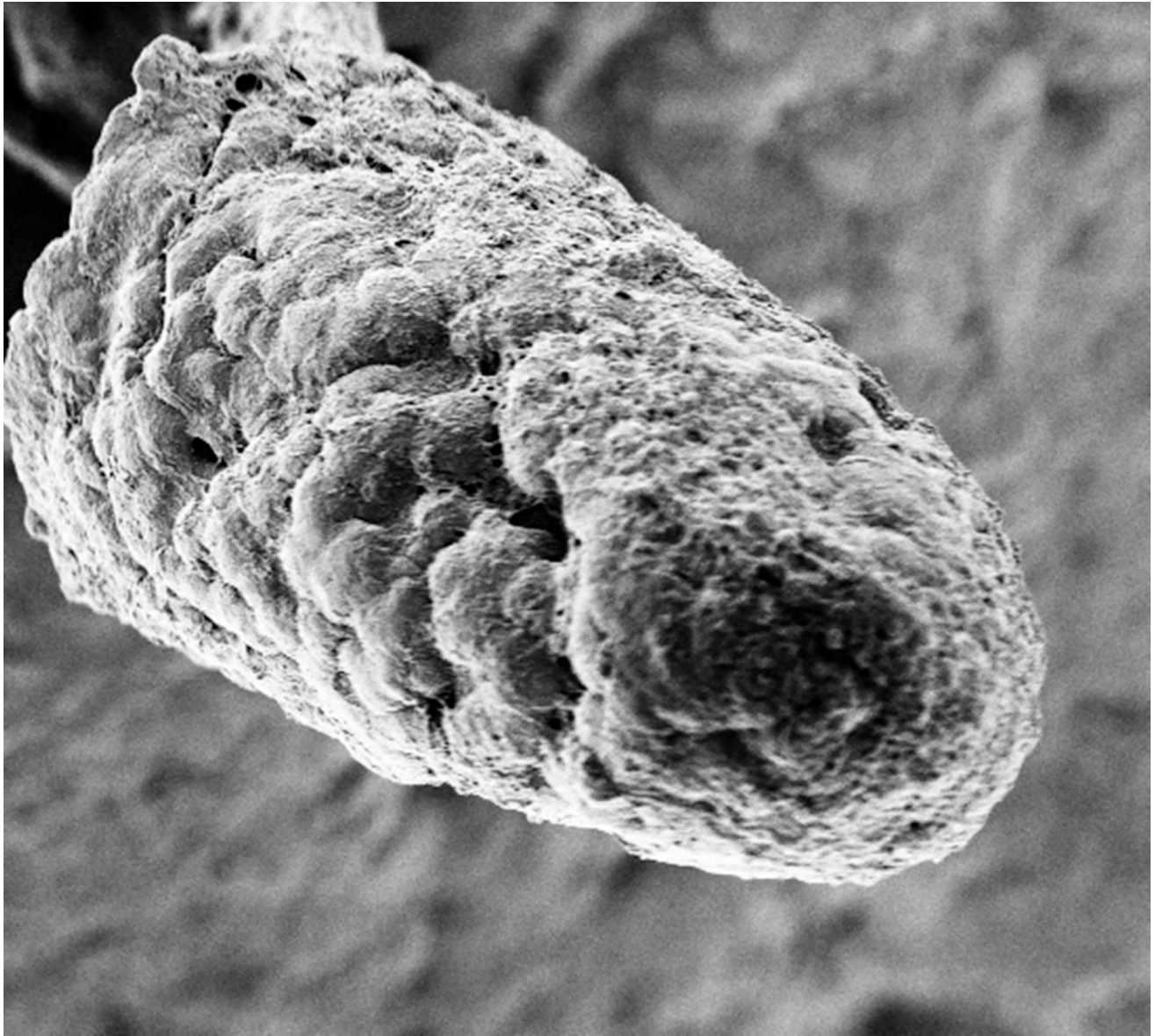


Figure 3. Scanning electron micrograph showing attached cells on a synthetic villus prior to implantation into the host omentum.

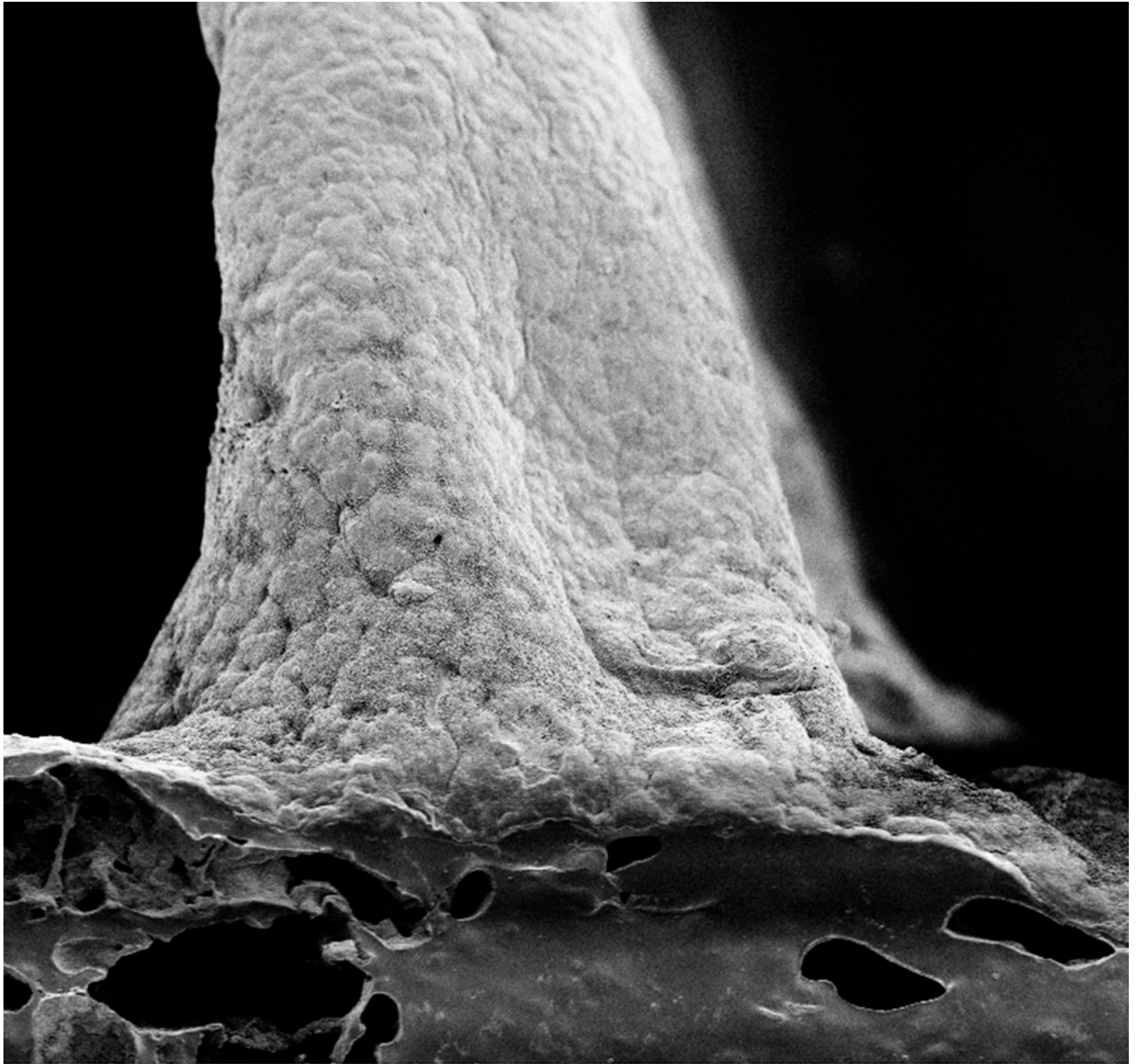


Figure 4.
Scanning electron micrograph showing the intestinal stem cells covering a synthetic villous
at the villus base.
a. PGS scaffold supports growth of intestinal epithelium