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Tissue Engineering in the Gut: Developments in Neuromusculature

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Abstract

The complexity of the gastrointestinal (GI) tract lies in its anatomy as well as in its physiology. Several different cell types populate the GI tract adding to the complexity of cell sourcing for regenerative medicine. Each cell layer has a specialized function in mediating digestion, absorption, secretion, motility and excretion. Tissue engineering and regenerative medicine aim to regenerate the specific layers mimicking architecture and recapitulating function. Gastrointestinal motility is the underlying program that mediates the diverse functions of the intestines, as an organ. Hence, the first logical step in GI regenerative medicine is the reconstruction of the tubular smooth musculature along with the drivers of their input, the enteric nervous system. Recent advances in the field of GI tissue engineering have focused on the use of scaffolding biomaterials in combination with cells and bioactive factors. The ability to innervate the bioengineered muscle is a critical step to ensure proper functionality. Finally, in vivo studies are essential to evaluate implant integration with host tissue, survival and functionality. In this review, we will focus on the tubular structure of the GI tract, tools for innervation and finally evaluation of in vivo strategies for GI replacements.

Keywords

Intestinal tissue engineering; Neo-innervation; Enteric nervous system; Smooth muscle; GI motility; Neurogastroenterology

1. Introduction: Defining the biological problem

The gastrointestinal (GI) tract is a continuous tubular organ responsible for transport and digestion of food, absorption of nutrients and excretion of waste. The activity of the GI tract

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is a summation of several complex cell types that include smooth muscle cells, neurons, glia, interstitial cells and different kinds of intestinal epithelial cells. The outer layer of the GI tract is composed of 2 types of smooth muscle tissues; circular and longitudinal smooth muscle. The sphincters of the GI tract allow unidirectional and directed flow of luminal contents. Apart from the smooth musculature, the GI tract contains several kinds of intestinal epithelial cells that mediate absorption and secretion within the gut. Smooth muscle tissues are the primary effectors of motility in the gut, mediating the movement of luminal content. The function of the muscle tissue is dictated by the enteric nervous system (ENS), which is the intrinsic innervation of the gut. Several classes of functional neurons (sensory, motor, secretory, etc.) and glia are present in the ENS, with a diversity paralleled only by the central nervous system (1). The ENS is responsible for the variety of gastrointestinal motor patterns produced in different parts of the gut, as well as the coordination of function between various segments of the gut. The interstitial cells of Cajal are additionally also implicated in pacemaking function within the gut (2), rounding out the primary players responsible for gastrointestinal motility.

Gastrointestinal motility can be altered post-natally due to disease, damage, surgical or obstetric trauma, and age. Congenital defects of GI motility include but are not limited to Hirschsprung's disease, intestinal pseudoobstruction and achalasia (3). While the therapeutic mainstay for motility disorders has remained pharmacological, surgical correction also does not provide a long-lasting solution. Regenerative medicine seeks to replace GI segments preferably using the patient's own cells while employing the optimal route of delivery. Advances in biomaterials and tissue engineering have catapulted regenerative medicine strategies forward, bringing them closer to the bedside.

This review will focus on regenerative medicine strategies aimed at the restoration of the neuromuscular anatomy and/or function of the neuromusculature of the GI tract. The review highlights both biomaterial-based and cell transplantation-based methods. Finally, a future perspective is provided indicating the complexities of sourcing and maintaining phenotypes of many constituent cells, neo-innervation and neo-vascularization.

2. Tissue engineering of GI tubular organs: Where do we start?

2.1. Anatomy and function

Tissue engineering the GI tract has fundamental challenges that one would encounter when faced with most biological systems – anatomic and physiological complexity. The complexity of the GI tract lies in the different cell layers that exist within the tract. These cells work in coordination in order to respond appropriately to different stimuli. In GI tissue engineering, each of the different cell types must be considered. The first question that arises in any tissue engineering application is the appropriate source of cells. Can the several cell types required to duplicate physiological complexity be sourced? If yes, can they be sourced in adequate numbers from a biopsy, which is preferably minimally invasive?

2.1.1. Musculature—The GI tract is a complex, highly regulated multilayered system. Although the muscularis propria is divided into several different layers, its complexity is determined by its anatomy. For example, different muscle types exist along the length of the

esophagus. The first one third is composed of skeletal muscle and the lower one third is composed of both circular and longitudinal smooth muscle. The middle part of the esophagus is a mixture of both skeletal and smooth muscle. The variety of muscle types is essential to ensure swallowing and propulsion of food into the stomach. In addition to the circular and longitudinal smooth muscle layers that make up the gastric musculature, another oblique smooth muscle layer exists (4). Taking into account the different muscle types and their orientation is critical when engineering GI segments. In the small and large intestine, the muscular layer is divided again into longitudinal and circular muscle layers. The basic unit of intestinal musculature is the smooth muscle. It receives regulatory inputs from different levels in order to perform its contractile functions. Smooth muscle contraction is initiated by membrane depolarization, which activates voltage-gated calcium channels and leads to calcium (Ca^{2+}) influx into the cell. Entry of Ca^{2+} stimulates complex signaling cascades within the smooth muscle cell, leading to contraction. Different intracellular proteins are involved in mediating the contractile response via a series of phosphorylation and dephosphorylation (G proteins, phospholipases, calmodulin, myosin light chain kinase, Rho kinases, and phosphatases). Although similar pathways exist for both circular and longitudinal smooth muscle layers, mechanisms for Ca^{2+} mobilization differ between the two. Depolarization of smooth muscle is controlled at different cellular levels in the GI tract. Contractile response is divided into 2 phases; initial and sustained contractions. A balance between phosphorylation and dephosphorylation events is responsible for the occurrence of the 2 phases. A complete regeneration of the musculature of the GI tract requires the maintenance of all the intracellular pathways. Smooth muscle cells can also be classified as phasic or tonic smooth muscle based on the contents of contractile protein isoforms and their levels of expression. A higher level of contractile protein expression is observed in smooth muscle cells present in the high-pressure zones of the GI tract, namely the sphincters of the GI tract. For example, circular smooth muscle of the lower esophageal sphincter (LES) or the Internal Anal Sphincter (IAS) differ significantly from the smooth muscle of the esophageal body or the colo-rectum respectively (5, 6).

2.1.2. Epithelium and defense functions—The epithelium of the gut performs various critical functions such as enzyme secretion, nutrient absorption, and acts as a physical barrier to perform a highly sophisticated defense function. Secretion and absorption functions require a large surface area, which is provided by finger-like villi structures that face the luminal side of the gut. The epithelial monolayer is characterized by apico-basolateral polarity and is divided into several specialized cell types; enteroabsorptive cells, goblet cells, Paneth cells and neuroendocrine cells (7).

Polarity of the epithelium is essential for function, it allows cells to sense and respond to stimuli (8). The epithelium differs in structure and function along different parts of the GI tract (9). Apart from nutrient absorption, the epithelium also provides a defense function. The gut has to have an appropriate defense system capable of protecting itself from commensal bacteria as well as foreign antigens in its luminal content. A single layer of epithelial cells makes up the epithelial barrier as a primary defense wall. A coordinated interaction between the different epithelial cells contributes to the defense function of the epithelium. The integrity of the epithelial barrier is maintained by tight junctions. In addition to tight

junctions, adherens junctions and gap junctions are also involved in cell-cell interaction. What makes the epithelial barrier more complex is the fact that it is a dynamic structure that is continuously renewed in the context of epithelial cell shedding and proliferation (10, 11). Thus, the gut is vulnerable and is a potential site for infection, inflammatory diseases and loss of the epithelial barrier integrity. Part of the regeneration process must involve generating a polarized epithelium with tight junction integrity, to reinstate mucosal function.

2.1.3. Regulatory apparatus—The regulatory apparatus of the GI tract is multilayered – with input arising from the intramural innervation, integrated inputs from the central and autonomic nervous system as well as input from ICCs. Ultimately, GI physiology is a complex addition and interpretation of several signals that lead to smooth muscle motor activity, mucosal secretion/transport, local blood flow/vasodilation, and intestinal immune and endocrine function. The enteric nervous system (ENS), is entirely contained within the musculature of the GI tract and is arranged within ganglionated plexi. The myenteric plexus is anatomically located between the circular and longitudinal muscle layers, and extends the full length of the digestive tract from the esophagus to the rectum. The submucosal plexus lies between the mucosa and the inner circular muscle layer. Together, the neurons of the ENS regulate diverse functions like control muscle activity, secretory activity of intestinal glands, motility of the blood vessels, sensory functions that include reflex pathways. Depending on the region of the gut, the size and compositional diversity of the ganglia within the plexi vary. For example, the submucosal plexus is far more obvious in the small and large intestine than in the stomach and the number and neuron density of myenteric ganglia are higher in the colon near the mesenteric attachment (12–14). Different classes of enteric neurons exist with diverse neurochemical coding. Neurotransmitters in the intestine are similar to those of the CNS, and include acetylcholine, tachykinins, serotonin, nitric oxide, purines, and several neuropeptides (15). Motor neurons are responsible for excitatory and inhibitory transmission to smooth muscle of both the muscularis externa and muscularis mucosa. Neuro-neuronal transmission is mediated by 2 types of interneurons; ascending and descending interneurons. While ascending interneurons are thought to mediate propulsive reflexes, descending interneurons have been implicated in local motility reflexes, secretomotor reflexes as well as conducting migrating myoelectric complexes. The basis for these functions arises in complex neurochemical coding for these neurons. Intrinsic sensory neurons (intrinsic primary afferent neurons IPANs) are responsible for several reflexes that mediate mucosal chemo-sensing, mechano-sensing and stretch responsiveness. Intrinsic secretomotor neurons are controlled both by local reflex circuits and from sympathetic input outside of the ENS. Extrinsic vasodilation is known to occur through sensory neurons that originate in the dorsal root ganglia with processes directed towards the GI microvessels (16, 17). Submucosal secretomotor neurons have also been implicated in neurogenic vasodilation and control of mucosal blood flow (16, 18). Although a majority of the neuronal input arises from nerves of local origin, afferent sensory inputs from the intestine are transmitted to the CNS via the nodose ganglia of the vagus or the dorsal root ganglia. For example, sensations of fullness from the stomach are transmitted through this pathway (19). Efferent impulses from the CNS are primarily parasympathetic in nature from the vagus and pelvic nerves, and sympathetic from prevertebral ganglia. An example of integrated neurogenic response is the vago-vagal reflex in the esophagus that mediates swallowing-induced peristalsis. In addition

to the neural input, smooth muscle intrinsic electrical activity is mediated by the interstitial cells of Cajal (ICC). Three populations of ICCs have been identified in the human colon using Kit immunohistochemistry. These include the ICCs along the submucosal surface of the circular muscle, ICCs within the deep muscular layers and ICCs in the myenteric region (20). The role of ICCs in neurotransmission, slow wave electrical activity and functions in the gut have been reviewed extensively (21, 22). ICCs have a syncytial pacemaker activity through which they regulate smooth muscle slow wave electrical activity. Significant improvement has been made in the past decade for the isolation of ICC populations (23–25). Incorporation of these cells during regeneration of the intestine might allow more efficient neurotransmission and timely peristaltic activity. Small steps are being made towards re-capturing the diversity of the intrinsic innervation to the gut as well, which will be discussed in detail in Section 4.

3. Tissue engineering the GI tract: how can the physical structure be achieved?

Once the biological challenges have been defined, a materials and structural question arises. Despite the fact that tubular organs have simple geometry, their structural and mechanical properties are complex. Esophagus is a long muscular tube that mediates the transport of food to the stomach via peristalsis. Stomach functions as a reservoir that grinds and mixes food. The small and large intestines are tubular structures that function to enhance absorption of nutrients, transit of food and finally excretion of waste via coordinated rhythmic peristaltic waves. The GI segments are interspersed with sphincters that ensure unidirectional flow and prevent any backflow. If anatomic, structural and physiology complexity were to be achieved, even one-step at a time, or focused on single cell layers, the next rate-limiting step in tissue engineering is neo-vascularization. Successful implantation necessitates cell survival and maintenance of biological functions post implantation. Vascularization guarantees transport of oxygen and nutrients and excretion of waste products. Several tissue engineering approaches can be considered to facilitate vascularization including delivery of angiogenic factors or pre-vascularization. Vascularization strategies have been discussed extensively in several reviews (26, 27), and will not be a subject of the current review.

3.1 Biomaterials

A wide spectrum of biomaterials can be used to fabricate tubular scaffolds that mimic the GI tubular organs. Natural as well as synthetic biomaterials have been investigated in GI regenerative medicine for their ability to support cell attachment, proliferation and differentiation. In any tissue engineering application, scaffolds are characterized by the extent and degrees of biocompatibility, porosity and pore sizes, and mechanical properties, among several other specific material properties (28). Those characteristics dictate the kind of interaction cells will have with the material in terms of cell behavior, alignment, phenotype maintenance and function in a three-dimensional setting. The key point for successful tissue regeneration and function is to mimic the native alignment of the cells. Early studies failed to restore the proper alignment of different layers of the GI tract using cell-seeded scaffolds (29). Described within this section are instances where specific

biomaterial interactions were used for achieving cellular alignment during the tissue engineering process.

3.1.1 Natural materials—Collagen, a major component of the extracellular matrix, is one of the most common natural biomaterials used in the field of GI tissue engineering. Collagen scaffolds were fabricated and evaluated for smooth muscle attachment and growth in gastric and intestinal regeneration (30–33). Additionally, collagen was used as a coating material in gastric reconstruction (34). Collagen supported cell attachment and differentiation; however, it failed to support the recapitulation of the native architecture. Chitosan is another natural polymer commonly used in tissue engineering applications (35, 36). We have demonstrated the biocompatibility of chitosan in GI applications (37). Intestinal smooth muscle cells attached, proliferated and maintained their contractile phenotype when cultured on chitosan-collagen membranes.

Our group has bioengineered circular smooth muscle tissue constructs using fibrin gel (38). The smooth muscle cells were aligned circumferentially in a pattern that mimics the orientation of circular smooth muscle cells in native GI tract. We have also bioengineered composite tubular scaffolds made of chitosan and collagen. We fabricated highly porous scaffolds with different lengths and diameters. A lumen was also created to obtain a hollow tube that could be used to replace tubular organs of the GI tract. The advantage of using chitosan is its ability to be immobilized to glycosaminoglycans (GAG) (35) such as heparan sulfate, which is an abundant GAG in intestinal extracellular matrix (ECM) (39). We placed several bioengineered smooth muscle tissue constructs around the chitosan scaffolds and demonstrated their physiological functionality in response to potassium chloride (KCl), acetylcholine (Ach) and vasoactive intestinal peptide (VIP). When designing these experiments towards clinical translation, a normalized comparison to native tissue for kinetics and magnitude of physiological responses will be a requirement. Physiological analyses must include mandates to resolve phasic or tonic activity of the smooth muscle type under investigation. Additionally, the underlying mechanical properties of the biomaterial scaffold need to be evaluated, in order to elucidate the evolution of the structure-function relationship of physiologically functional smooth muscle. Moving forward, a critical review of the biomaterial itself also needs to be under taken – for instance, how much remodeling does the material allow, how much infiltration does the scaffold support, and what kind of immunogenic responses would be acceptable for the material to be considered safe for transplantation.

Investigations have then shifted towards modifying the structure and chemistry of the scaffolds. OptiMaix collagen scaffolds were fabricated by unidirectional porous structures which allowed directional smooth muscle growth (40). Totonelli et. al. have successfully optimized a decellularization protocol using rat small intestine to completely remove cellular component and preserve the native architecture of the intestine (41). The maintenance of architecture helps guiding the cells to orient themselves on the matrix. Our group has also bioengineered longitudinal smooth muscle tissues (42). Uniaxial smooth muscle alignment was facilitated by substrate microtopographies. The bioengineered tissues mimicked the alignment of the longitudinal layer of the small and large intestine. The tissues also demonstrated physiological functionality (contraction and relaxation).

3.1.2 Synthetic materials—Synthetic materials also represent strong candidates due to their biocompatibility, biodegradability, mechanical properties and ease of fabrication. However, due to the lack of binding domains in synthetic polymers, addition of natural polymers has been investigated to enhance cell attachment and survival (43). In a recent study, poly(L-lactide-co-caprolactone) (PLLC) scaffolds were electrospun and immobilized with fibronectin to enhance cell attachment and growth for esophageal reconstruction (44). Synthetic polymers were also investigated in their ability to regenerate different layers in the stomach. Polyglycolic acid (PGA) scaffolds were coated with collagen and seeded with organoid units (34). Epithelial and muscularis layers were regenerated; however the native architecture was not recapitulated. Recently, a tissue engineered small intestine was formed using polyglycolic acid scaffolds coated with collagen and seeded with post-natally derived progenitor cells (45). The scaffolds were implanted in the omentum of mice for a period of 28 days. Immunostaining studies demonstrated the presence of differentiated epithelial cells, muscularis layer and nerve tissue. The use of organoid units to regenerate segments of the GI tract requires further optimization in regards to the source of the units, the age of the donor and the initial level of differentiation of the units.

3.1.3 Mechanical characterization of tissues—Studying the mechanical properties of GI tissues is valuable in providing insight in the developmental stages of regenerative medicine based therapies; it allows us to (i) understand the biophysical mechanisms behind the transport of luminal contents; (ii) understand the pathophysiology of diseases associated with an underlying change in the mechanical properties of the tissue; and (iii) to develop scaffolds suitable for replacements. Extensive mechanical studies have been conducted on tissue engineered tubular organs like blood vessels (46–48), but little emphasis is directed to the study of the mechanics of GI tissues. Previous tensile strength studies on rats showed a trend of increased tensile strength from proximal to distal colon (49). This change is correlated to the exposure of the colon to higher stress as the fecal pellets become more solid. Egorov *et al.* reported tensile properties of human esophagus, stomach, small and large intestine (50). In this report, the small and large intestines were studied as multi-layered structures. It was concluded from this study that the mechanical strength of the intestinal wall is predominantly determined by the submucosa and muscularis layers with little contribution from the serosa and the mucosa. Another study compared the mechanical behavior of the whole esophagus to the mucosa alone, implicating a mucosal contribution to the strength of the esophageal wall (51). Another study has shown a decrease in tensile strength of the colon as a function of age (52). This is correlated to a change in the connective tissue composition change with age. Recently, a new technique has been employed to measure mechanical properties of the esophageal wall as a way to provide therapy for patients (53). The study demonstrated a decrease in esophageal distensibility and esophageal compliance in patients with eosinophilic esophagitis. The outcome of this study helps correlate the mechanical properties of the esophageal wall with tissue remodeling and fibrosis in pathological conditions. Distinguishing the mechanics of the gut wall between the different layers helps in the design and synthesis of the scaffolds to match the native tissue.

4 Regenerative medicine and neo-innervation

Gastrointestinal function is predominantly controlled by the ENS, which is considered as the intrinsic innervation of the GI tract. ENS consists of an enormous diversity of enteric neurons as well as enteric glia, both of which derive from the neural crest.

Neurodegenerative conditions are characterized by the loss of the neuronal circuitry which results in dysmotility in the GI tract.

4.1 Stem cell transplantation

Neural stem cell transplantation provides a cell-based therapy to reinstate innervation in segments of the GI tract. The main goal of cell transplantation is to restore neuronal function in cases of neurogenic disorders. However, the long-term fate of the transplanted cells requires further investigations. It is ideal to obtain sufficient quantities of stem cells from an easily accessible source, using minimally invasive procedures (54). Different sources of neural stem cells are being actively investigated, including the central nervous system and the enteric nervous system. Delivery of stem cells is another factor that needs to be taken into account. Progress in tissue engineering and regenerative medicine provides different materials as delivery vehicles for the cells. Delivery of the cells must ensure target-specificity and complete repopulation with the specific neuronal subtypes appropriate for the location along the GI tract. Availability of cell source, viability and differentiation of the stem cells post-transplantation are critical factors that must be accounted for when considering clinical use.

4.1.1 Enteric nervous system as a source for progenitor cells—Techniques for isolation of enteric neural progenitor cells from embryonic, fetal, post-natal and adult rodent as well as from human GI tract have already been established (55–61). They can be isolated from full thickness, muscularis and mucosal biopsies with the ability to differentiate into mature neurons. Recently, neural crest progenitor cells were isolated from neonatal rats and were delivered to a denervated colon. The cells differentiated into neurons and glia and were able to restore motility (62). In another study, fetal and post-natal neural progenitor cells derived from mice intestines were transplanted into the external muscle layer of mice distal colon (63). The transplanted cells migrated, proliferated and differentiated into excitatory and inhibitory motor neurons. Although motility studies were not performed in these animals, there was no sign of dysmotility. Longitudinal smooth muscle function in the piebald mouse improved following implantation of an ENS derived cell line (64). Intraperitoneal injection of enteric neuronal stem cells into lethal spotting rats (ls/ls) demonstrated engraftment from the stomach to the cecum, with an associated expression of markers indicating neuronal differentiation (65). Enteric neuronal stem cells carrying the GFP transgene and transplanted directly into recipient mouse colons were able to migrate, differentiate into functional neurons and glial cells, and form ganglion like structures (66).

Embryonic mouse and neonatal human enteric neuronal progenitor cells differentiated into mature neuronal phenotypes following transplantation into embryonic mouse hindgut organotypic cultures (59). ENS-derived neural stem cells isolated from human or rodent bowels have been successfully transplanted into recipient embryonic aganglionic bowel

explants (54, 59). These cells survived, migrated and differentiated and were able to impart neuronally-dependent contraction and calcium mobilization (59).

4.1.2 Central nervous system as a source for stem cells—Neural stem cells derived from the central nervous system have been also tested for their ability to treat gastrointestinal disorders (67). Micci et al. transplanted CNS-derived neuronal stem cells isolated from embryonic mouse brain into mouse pylorus. Neuronal stem cells differentiated upon transplantation, and rescued gastric function in the nNOS^{-/-} mice (68). Dong et al. transplanted CNS-derived neuronal progenitor cells into aganglionic rodent rectum. These cells demonstrated differentiation into glial and neuronal (nitrergic and cholinergic) subtypes, and restored rectoanal inhibitory reflex in the rat (69). Additionally, ex vivo studies have been conducted. CNS-derived neuronal stem cells can acquire an enteric-like neuronal phenotype when co-cultured with mouse longitudinal muscle-myenteric plexus preparations (60, 70, 71).

4.2 Neo-innervation of smooth muscle

Our group has shown the ability to neo-innervate bioengineered smooth muscle tissue constructs. An intrinsically innervated smooth muscle tissue construct was bioengineered using smooth muscle cells and neural progenitor cells isolated from full thickness adult rabbit colons. The intrinsically innervated smooth muscle construct was placed around chitosan scaffold next to a bioengineered smooth muscle construct that initially lacked innervation. The latter became neo-innervated after a period of 14 days in culture. Both constructs responded to electrical field stimulation and stained positive for β -III tubulin. This study provides a methodology whereby a combination of biomaterials and cells are employed to reinstate innervation in neuromuscular diseases of the gut (72).

Currently, regenerative medicine is directed at restoring innervation of gut smooth muscle with the goal of differentiating stem cells into functional subtypes of neurons. Providing chemical cues to the enteric neural progenitor cells can modulate their fate in vitro. The benefit of directing the differentiation of neural progenitor cells into specific neuronal subtypes is to obtain an enriched population of certain types of neurons. This is promising when it comes to treating GI disorders that are caused by the lack of specific population of neurons (73–75). Gastric emptying and gastric neuromuscular dysfunctions are characterized by the absence of intrinsic inhibitory neurons. Achalasia is a condition in which the sphincter (lower esophageal sphincter LES or internal anal sphincter IAS) is in a tonic contractile state and is unable to relax. In all cases, an increased population of nitrergic neurons is beneficial in restoring motility, facilitating gastric emptying and reducing sphincteric tone.

We isolated enteric neural progenitor cells from full-thickness adult rabbit guts following a previously described protocol (76). The enteric neural progenitor cells were positive for p75 (neural-crest lineage) and for sox2 and Nestin (progenitor status). The extracellular matrix is an important factor in determining lineage fate of stem cells. We evaluated in vitro differentiation capacity of enteric neural progenitor cells on different extracellular matrix components known to be abundant in the myenteric plexus (77). The extent of neuronal and

glial differentiation varied among different substrates. Composite collagen mixtures (Collagen IV, laminin and heparan sulfate) improved neuronal differentiation, however glial differentiation was reduced.

In a recent study, we bioengineered intrinsically innervated human IAS constructs. We isolated human IAS smooth muscle cells and human enteric neural progenitor cells from the adult human small intestine. Cells were co-cultured in collagen hydrogel and formed three-dimensional tissue constructs. Immunostaining studies demonstrated the presence of contractile smooth muscle, glial cells and mature neuronal subtypes. The mature neurons were able to produce excitatory (choline acetyltransferase ChAT) or inhibitory (vasoactive intestinal peptide VIP and neuronal nitric oxide synthase nNOS) neurotransmitters. Additionally, the constructs responded to appropriate physiological stimuli using neurotransmitters that are normally present in the gut. Neuronal integrity was also demonstrated by the response to electric field stimulation. We were the first to show the ability to innervate bioengineered human smooth muscle tissues using gut-derived enteric neural progenitor cells (78).

Enteric glia are distributed in all layers of the GI tract wall and play a regulatory role at different levels. During the regeneration process, a glial population could be beneficial in providing trophic and structural support for neurons. They would ensure neuronal survival, protection and differentiation. Additionally, glial cells are also involved in enteric neurotransmission by regulating neurotransmitters synthesis. At the mucosal level, glial cells play a role in regulating the integrity of the epithelial barrier. Although there aren't established GI disorders that specifically affect enteric glia, it is beneficial to identify, in future studies, factors that selectively induce glial differentiation (79, 80).

5 In vivo studies – current status

Studies involving implantation offer a basis for clinical translation. Several studies reported different attempts to reconstruct segments of the GI tract in animal models. Neo-vascularization and neo-innervation are among the most challenging factors to be considered following implantation. It is of paramount importance that the implant integrates with the host tissue, receives adequate blood supply for survival of cells and becomes innervated for proper functionality. Many studies were conducted in rodents and resulted in promising outcomes; however, larger animal models are pre-requisites for approaching clinical settings.

5.1 Tubular organs reconstruction

In an attempt to scale up graft size, large animal studies have been conducted. A synthetic bioabsorbable polymer patch was used to reconstruct the esophageal wall in pigs (81). Up to 12 weeks of follow-up after implantation revealed regeneration of the esophageal muscular layer with alignment similar to native tissue. Other studies have also used decellularized matrices (urinary bladder and small intestine submucosa SIS) as an attempt to reconstruct the esophagus in dog models (82, 83). Gastric patches, in addition to polymeric scaffolds, represent potential methods for reconstructing the stomach. In vivo studies resulted in regeneration of the epithelial layers but failed to regenerate the muscularis layer in terms of

architecture and function (34, 84). In regenerating the small intestine, synthetic scaffolds seeded with organoid units were successful in regenerating the epithelial layer after implantation (85–87). The challenge remains in recapitulating the organization of both circular and longitudinal muscle layers. In another study, SIS seeded with smooth muscle cells resulted in partial epithelialization following implantation but was not successful in regenerating the muscularis layer (88).

5.2 Sphincter reconstruction

Sphincters are composed of tonic circular smooth muscle and exhibit high pressures due to the contracture state of the muscle. Sphincteric deficiencies caused by damage of the smooth muscle, lead to different disorders that can have overwhelming impacts on patients' lives. These disorders include gastroesophageal reflux disease (GERD) which affects the LES, fecal incontinence (FI) which affects the IAS, achalasia (LES and IAS) and stenosis (Pylorus). First attempts to repair defects in sphincters used cell-based therapies which involved injection of skeletal muscle-derived stem cells (89, 90). Functional contractile smooth muscle was not reported. Tissue engineering offers a different approach to repair sphincteric degeneration.

Our group has well-established protocols for engineering sphincteric smooth muscle. We have bioengineered intrinsically innervated internal anal sphincter smooth muscle tissue constructs. The constructs were bioengineered using human IAS smooth muscle cells co-cultured with immortalized fetal enteric neurons (91). The presence of excitatory and inhibitory neurons in the constructs was demonstrated by RT-PCR and immunostaining studies. The constructs were subcutaneously implanted in mice. After 4 weeks, the harvested constructs were neo-vascularised and preserved the neuronal network as depicted by immunostaining. The harvested constructs maintained physiological functionality (myogenic and neuronal components) in response to Ach, VIP and electrical field stimulation. Bioengineered human IAS constructs, by us and others, have demonstrated basal tone values ranging from 0.4mN to 0.7mN, measured in organ bath studies (92–94). Forces measured in organ bath studies are not directly comparable to clinical data obtained for basal tone from anorectal manometry. The discrepancy when this type of direct comparison is made to human IAS is thought to arise from not having an adequate number of cells within the engineered constructs. Furthermore, several neurohumoral mechanisms and additional muscle groups are responsible for the generation of basal tone in the human IAS, which aren't captured while measuring isometric force from bioengineered smooth muscle constructs (95). Although the magnitude of the forces were substantially reduced when compared to forces that would be produced in an intact human IAS tissue, bioengineered constructs retained key aspects of IAS physiology by generating spontaneous elevated basal tone. While considering these constructs for clinical replacement of IAS, rigorous quality standards have to be established and met, which are discussed in later sections. However, orthotopic transplantation to augment sphincteric function in place of repeated injections of inert bulking agents like Deflux® and botulinum toxin into the IAS appears to be an inviting option.

In a separate study, we studied the effect of different angiogenic factors on the viability and functionality of bioengineered smooth muscle tissue constructs (96). The bioengineered IAS constructs were implanted subcutaneously along with osmotic pumps in the back of mice. The pumps were delivering one of the 3 different factors; FGF, VEGF or PDGF. All growth factors promoted neo-vascularization, survival and maintenance of contractile smooth muscle phenotype in the constructs when compared to control (implanted constructs without delivery of growth factors). However, PDGF and VEGF resulted in superior basal muscle tone generated by the implanted constructs.

6 Future perspectives

Advances in biomaterials and tissue engineering have brought regenerative medicine of the neuro-musculature of the GI tract to fruition. Over the decades, biomaterial characteristics have been refined to imitate near native architecture and promote optimal interaction with several cell types. Several material and bioreactor based methods have been reported to facilitate smooth muscle cell alignment, recapitulating native muscle organization similar to the GI tract. From a biological point of view, however, the cell types within the native GI tract are diverse to a point where isolation and characterization of individual cell types must have defined standards. A standard set of markers to identify cell types have to be established, in order to standardize cell sourcing for transplantation or tissue replacements. Of specific interest, given the notoriety of smooth muscle cells to dedifferentiate into synthetic phenotypes during proliferation, care must be taken to ensure that replacement tissue constructs have constituent smooth muscle cells that express specific markers of the contractile phenotype. Reinstatement of GI function must compulsorily include cells other than smooth muscle and neurons. As the role of the interstitial cells of Cajal in GI function emerges, these cells may be critical in reinstatement of the pacemaking activity.

6.1 Quality control of smooth muscle phenotype

Several distinct smooth muscle phenotypes have been identified during development of the intestinal smooth musculature, including myoblasts, immature myocytes and mature smooth muscle myocytes (97, 98). This continuum of smooth muscle phenotypes result from hierarchical inductive events of specific gene products including isoactins, calponin and a myosin heavy chain isoform, among several others (99, 100). Mature smooth muscle cells retain the notorious ability to de-differentiate into synthetic, proliferative and possibly migratory phenotypes when they are isolated from their native in vivo milieu and placed in culture. However, these cells are also documented to recapture their myogenic program in vitro. The challenge in deriving sufficient numbers of smooth muscle cells for GI tissue engineering is that the induction of smooth muscle proliferation in low density cultures with serum supplemented medium is an automatic result of smooth muscle de-differentiation. Although this developmental flexibility permits a high proliferative index, de-differentiated smooth muscle cells demonstrate reduced contractility and acetylcholine responsiveness (101, 102). The recovery of the “contractile” smooth muscle phenotype is paramount to its function in generating forces required for the various motility patterns of the GI tract. Although research has shown that high density cultures of smooth muscle cells with cellular syncytium and high cell-cell contact result in a more contractile phenotype (103),

establishing reproducible standards for analyzing the “quality” of the constituent cells is important. Researchers often report the expression of α -smooth muscle actin as confirming smooth muscle phenotype. However, in some cases, populations of cultured fibroblasts and myofibroblasts express this marker, making it less reliable in identifying smooth muscle phenotype in itself (104, 105). Alternatively, the smooth muscle specific heavy isoform of caldesmon, calponin and smoothelin are all induced and observed in “contractile” smooth muscle cells or fully mature smooth muscle cells (100, 106, 107). These markers may be more reliable while ensuring the contractile phenotype of the smooth muscle cells. Several studies now document differential transcriptional activities and gene expression profiles of genes encoding both contractile proteins as well as those associated with the extracellular matrix in the various phenotypes of smooth muscle (108–113). While aiming for translational ability, GI tissue engineering must take a cue from vascular tissue engineering in establishing rigorous quality control based on cDNA screening, to ensure contractile smooth muscle phenotypes.

6.2 Integration of neo-innervation

The principal roadblock in clinical translation remains adequate and appropriate reinstatement of innervation of the ENS. Autologous sources of stem cells derived from the GI tract have been identified and isolated in sufficient numbers to demonstrate the feasibility of this cell source for therapy. Current therapies focus on injecting various types of stem and progenitor cells, in the hope that environmental cues will drive them to differentiate into phenotypes appropriate for the location. Trophic factor regimens that drive subtype differentiation and maintain phenotype need to be identified in vitro, for incorporation in the next generation of bioactive scaffolds. Of particular interest are extracellular matrix based microenvironmental cues that have the capability of modulating both trophic and morphogenetic signaling to the encapsulated neuronal stem and progenitor cells. The roadmap to neo-innervation must include neuronal network formation and connectivity, failing which discordance between neo-innervation and existing complex reflex pathways of the gut may persist. More fundamental research studying the interactions and communication of enteric neurons with peripheral neurons and amongst themselves is required, in order to be able to establish standards for neural connectivity. Overall, in view of clinical translation, basic questions pertaining to phenotypes of isolated cell types (smooth muscle, neurons, stem/progenitor cells, interstitial cells) have to be investigated. Several studies outlined within this review show promise for the identification of some of these factors, driving regenerative medicine of the GI neuro-musculature to be a reality in the coming decades.

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References

1. Furness JB. The enteric nervous system and neurogastroenterology. *Nature Reviews Gastroenterology and Hepatology*. 2012; 9(5):286–94.
2. Sanders KM, Koh SD, Ro S, Ward SM. Regulation of gastrointestinal motility—insights from smooth muscle biology. *Nature Reviews Gastroenterology and Hepatology*. 2012; 9(11):633–45.

3. Chumpitazi B, Nurko S. Pediatric gastrointestinal motility disorders: challenges and a clinical update. *Gastroenterology & hepatology*. 2008; 4(2):140. [PubMed: 21904491]
4. Smith GT, Moran TH, Coyle JT, Kuhar MJ, O'Donahue TL, McHugh PR. Anatomic localization of cholecystokinin receptors to the pyloric sphincter. *The American journal of physiology*. 1984; 246(1 Pt 2):R127–30. [PubMed: 6320669]
5. Szymanski PT, Chacko TK, Rovner AS, Goyal RK. Differences in contractile protein content and isoforms in phasic and tonic smooth muscles. *The American journal of physiology*. 1998; 275(3 Pt 1):C684–92. [PubMed: 9730952]
6. Patel CA, Rattan S. Spontaneously tonic smooth muscle has characteristically higher levels of RhoA/ROK compared with the phasic smooth muscle. *American journal of physiology Gastrointestinal and liver physiology*. 2006; 291(5):G830–7.10.1152/ajpgi.00130.2006. [PubMed: 16763289]
7. Pott J, Hornef M. Innate immune signalling at the intestinal epithelium in homeostasis and disease. *EMBO reports*. 2012; 13(8):684–98. [PubMed: 22801555]
8. Martin-Belmonte F, Perez-Moreno M. Epithelial cell polarity, stem cells and cancer. *Nature reviews Cancer*. 2012; 12(1):23–38.10.1038/nrc3169.
9. Karlsson J, Putsep K, Chu H, Kays RJ, Bevins CL, Andersson M. Regional variations in Paneth cell antimicrobial peptide expression along the mouse intestinal tract. *BMC immunology*. 2008; 9:37.10.1186/1471-2172-9-37 [PubMed: 18637162]
10. Marchiando AM, Shen L, Graham WV, Edelblum KL, Duckworth CA, Guan Y, et al. The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding. *Gastroenterology*. 2011; 140(4):1208–18. e1–2.10.1053/j.gastro.2011.01.004 [PubMed: 21237166]
11. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *The Journal of nutrition*. 2011; 141(5):769–76.10.3945/jn.110.135657. [PubMed: 21430248]
12. Sternini C. Structural and chemical organization of the myenteric plexus. *Annual review of physiology*. 1988; 50:81–93.10.1146/annurev.ph.50.030188.000501.
13. Furness JB. Types of neurons in the enteric nervous system. *Journal of the autonomic nervous system*. 2000; 81(1–3):87–96. [PubMed: 10869706]
14. Wedel T, Roblick U, Gleiss J, Schiedeck T, Bruch HP, Kuhnel W, et al. Organization of the enteric nervous system in the human colon demonstrated by wholemount immunohistochemistry with special reference to the submucous plexus. *Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft*. 1999; 181(4):327–37.10.1016/S0940-9602(99)80122-8. [PubMed: 10427369]
15. Costa M, Brookes SJ, Hennig GW. Anatomy and physiology of the enteric nervous system. *Gut*. 2000; 47(Suppl 4):iv15-9. discussion iv26. [PubMed: 11076898]
16. Vanner S, Surprenant A. Neural reflexes controlling intestinal microcirculation. *The American journal of physiology*. 1996; 271(2 Pt 1):G223–30. [PubMed: 8770037]
17. Timmermans JP, Hens J, Adriaensen D. Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. *The Anatomical record*. 2001; 262(1):71–8. [PubMed: 11146430]
18. Vanner S, Macnaughton WK. Submucosal secretomotor and vasodilator reflexes. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2004; 16 (Suppl 1):39–43.10.1111/j.1743-3150.2004.00473.x. [PubMed: 15066003]
19. Konturek SJ, Konturek JW, Pawlik T, Brzozowski T. Brain-gut axis and its role in the control of food intake. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society*. 2004; 55(1 Pt 2):137–54. [PubMed: 15082874]
20. Ward SM, Sanders KM, Hirst GD. Role of interstitial cells of Cajal in neural control of gastrointestinal smooth muscles. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2004; 16 (Suppl 1):112–7.10.1111/j.1743-3150.2004.00485.x. [PubMed: 15066015]
21. Sanders KM, Koh SD, Ward SM. Interstitial cells of Cajal as pacemakers in the gastrointestinal tract. *Annu Rev Physiol*. 2006; 68:307–43. [PubMed: 16460275]

22. Sanders KM. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology*. 1996; 111(2):492–515. [PubMed: 8690216]
23. Thomsen L, Robinson TL, Lee JC, Faraway LA, Hughes MJ, Andrews DW, et al. Interstitial cells of Cajal generate a rhythmic pacemaker current. *Nature medicine*. 1998; 4(7):848–51.
24. Epperson A, Hatton WJ, Callaghan B, Doherty P, Walker RL, Sanders KM, et al. Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal. *American journal of physiology Cell physiology*. 2000; 279(2):C529–39. [PubMed: 10913020]
25. Langton P, Ward SM, Carl A, Norell MA, Sanders KM. Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86(18):7280–4. [PubMed: 2550938]
26. Lovett M, Lee K, Edwards A, Kaplan DL. Vascularization strategies for tissue engineering. *Tissue engineering Part B, Reviews*. 2009; 15(3):353–70.10.1089/ten.TEB.2009.0085 [PubMed: 19496677]
27. Rouwkema J, Rivron NC, van Blitterswijk CA. Vascularization in tissue engineering. *Trends in biotechnology*. 2008; 26(8):434–41.10.1016/j.tibtech.2008.04.009. [PubMed: 18585808]
28. Yang S, Leong K-F, Du Z, Chua C-K. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. *Tissue engineering*. 2001; 7(6):679–89. [PubMed: 11749726]
29. Hori Y, Nakamura T, Kimura D, Kaino K, Kurokawa Y, Satomi S, et al. Experimental study on tissue engineering of the small intestine by mesenchymal stem cell seeding. *The Journal of surgical research*. 2002; 102(2):156–60.10.1006/jsre.2001.6294. [PubMed: 11796013]
30. Lee M, Wu BM, Stelzner M, Reichardt HM, Dunn JC. Intestinal smooth muscle cell maintenance by basic fibroblast growth factor. *Tissue engineering Part A*. 2008; 14(8):1395–402.10.1089/ten.tea.2007.0232. [PubMed: 18680389]
31. Hori Y, Nakamura T, Kimura D, Kaino K, Kurokawa Y, Satomi S, et al. Functional analysis of the tissue-engineered stomach wall. *Artificial organs*. 2002; 26(10):868–72. [PubMed: 12296927]
32. Araki M, Tao H, Sato T, Nakajima N, Hyon SH, Nagayasu T, et al. Development of a new tissue-engineered sheet for reconstruction of the stomach. *Artificial organs*. 2009; 33(10):818–26.10.1111/j.1525-1594.2009.00808.x. [PubMed: 19839991]
33. Nakase Y, Hagiwara A, Nakamura T, Kin S, Nakashima S, Yoshikawa T, et al. Tissue engineering of small intestinal tissue using collagen sponge scaffolds seeded with smooth muscle cells. *Tissue engineering*. 2006; 12(2):403–12. [PubMed: 16548698]
34. Speer AL, Sala FG, Matthews JA, Grikscheit TC. Murine tissue-engineered stomach demonstrates epithelial differentiation. *The Journal of surgical research*. 2011; 171(1):6–14.10.1016/j.jss.2011.03.062. [PubMed: 21571313]
35. Madhally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. *Biomaterials*. 1999; 20(12):1133–42. [PubMed: 10382829]
36. Kim IY, Seo SJ, Moon HS, Yoo MK, Park IY, Kim BC, et al. Chitosan and its derivatives for tissue engineering applications. *Biotechnology advances*. 2008; 26(1):1–21.10.1016/j.biotechadv.2007.07.009. [PubMed: 17884325]
37. Zakhem E, Raghavan S, Gilmont RR, Bitar KN. Chitosan-based scaffolds for the support of smooth muscle constructs in intestinal tissue engineering. *Biomaterials*. 2012; 33(19):4810–7.10.1016/j.biomaterials.2012.03.051 [PubMed: 22483012]
38. Somara S, Gilmont RR, Dennis RG, Bitar KN. Bioengineered internal anal sphincter derived from isolated human internal anal sphincter smooth muscle cells. *Gastroenterology*. 2009; 137(1):53–61. [PubMed: 19328796]
39. de Toledo OM, Marquezini MV, Jia KB, de Pinheiro MC, Mora OA. Biochemical and cytochemical characterization of extracellular proteoglycans in the inner circular smooth muscle layer of dog small intestine. *IUBMB life*. 2002; 54(1):19–25.10.1080/15216540213824. [PubMed: 12387571]
40. Saxena AK, Kofler K, Ainodhofer H, Hollwarth ME. Esophagus tissue engineering: hybrid approach with esophageal epithelium and unidirectional smooth muscle tissue component generation in vitro. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract*. 2009; 13(6):1037–43.10.1007/s11605-009-0836-4. [PubMed: 19277795]

41. Totonelli G, Maghsoudlou P, Garriboli M, Riegler J, Orlando G, Burns AJ, et al. A rat decellularized small bowel scaffold that preserves villus-crypt architecture for intestinal regeneration. *Biomaterials*. 2012; 33(12):3401–10. [PubMed: 22305104]
42. Raghavan S, Lam MT, Foster LL, Gilmont RR, Somara S, Takayama S, et al. Bioengineered three-dimensional physiological model of colonic longitudinal smooth muscle in vitro. *Tissue Engineering Part C: Methods*. 2010; 16(5):999–1009. [PubMed: 20001822]
43. Zhu Y, Ong WF. Epithelium regeneration on collagen (IV) grafted polycaprolactone for esophageal tissue engineering. *Materials Science and Engineering: C*. 2009; 29(3):1046–50.
44. Zhu Y, Leong MF, Ong WF, Chan-Park MB, Chian KS. Esophageal epithelium regeneration on fibronectin grafted poly(L-lactide-co-caprolactone) (PLLC) nanofiber scaffold. *Biomaterials*. 2007; 28(5):861–8.10.1016/j.biomaterials.2006.09.051. [PubMed: 17081604]
45. Levin DE, Barthel ER, Speer AL, Sala FG, Hou X, Torashima Y, et al. Human tissue-engineered small intestine forms from postnatal progenitor cells. *Journal of pediatric surgery*. 2013; 48(1): 129–37. [PubMed: 23331805]
46. Seliktar D, Black RA, Vito RP, Nerem RM. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. *Annals of biomedical engineering*. 2000; 28(4):351–62. [PubMed: 10870892]
47. Konig G, McAllister TN, Dusserre N, Garrido SA, Iyican C, Marini A, et al. Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery. *Biomaterials*. 2009; 30(8):1542–50.10.1016/j.biomaterials.2008.11.011 [PubMed: 19111338]
48. L'Heureux N, Dusserre N, Konig G, Victor B, Keire P, Wight TN, et al. Human tissue-engineered blood vessels for adult arterial revascularization. *Nature medicine*. 2006; 12(3):361–5.10.1038/nm1364
49. Watters DA, Smith AN, Eastwood MA, Anderson KC, Elton RA. Mechanical properties of the rat colon: the effect of age, sex and different conditions of storage. *Quarterly journal of experimental physiology*. 1985; 70(1):151–62. [PubMed: 4011826]
50. Egorov VI, Schastlivtsev IV, Prut EV, Baranov AO, Turusov RA. Mechanical properties of the human gastrointestinal tract. *Journal of biomechanics*. 2002; 35(10):1417–25. [PubMed: 12231288]
51. Goyal RK, Biancani P, Phillips A, Spiro HM. Mechanical properties of the esophageal wall. *The Journal of clinical investigation*. 1971; 50(7):1456–65.10.1172/JCI106630 [PubMed: 5090061]
52. Watters DA, Smith AN, Eastwood MA, Anderson KC, Elton RA, Mugerwa JW. Mechanical properties of the colon: comparison of the features of the African and European colon in vitro. *Gut*. 1985; 26(4):384–92. [PubMed: 3920126]
53. Kwiatek MA, Hirano I, Kahrilas PJ, Rothe J, Luger D, Pandolfino JE. Mechanical properties of the esophagus in eosinophilic esophagitis. *Gastroenterology*. 2011; 140(1):82–90.10.1053/j.gastro.2010.09.037 [PubMed: 20858491]
54. Almond S, Lindley RM, Kenny SE, Connell MG, Edgar DH. Characterisation and transplantation of enteric nervous system progenitor cells. *Gut*. 2007; 56(4):489–96.10.1136/gut.2006.094565 [PubMed: 16973717]
55. Suárez-Rodríguez R, Belkind-Gerson J. Cultured Nestin-Positive Cells from Postnatal Mouse Small Bowel Differentiate Ex Vivo into Neurons, Glia, and Smooth Muscle. *Stem Cells*. 2004; 22(7):1373–85. [PubMed: 15579654]
56. Schafer KH, Hagl CI, Rauch U. Differentiation of neurospheres from the enteric nervous system. *Pediatric surgery international*. 2003; 19(5):340–4.10.1007/s00383-003-1007-4. [PubMed: 12845455]
57. Belkind-Gerson J, Carreon-Rodriguez A, Benedict LA, Steiger C, Pieretti A, Nagy N, et al. Nestin-expressing cells in the gut give rise to enteric neurons and glial cells. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2013; 25(1):61–9. e7.10.1111/nmo.12015 [PubMed: 22998406]
58. Silva AT, Wardhaugh T, Dolatshad NF, Jones S, Saffrey MJ. Neural progenitors from isolated postnatal rat myenteric ganglia: Expansion as neurospheres and differentiation in vitro. *Brain research*. 2008; 1218:47–53. [PubMed: 18514173]

59. Lindley RM, Hawcutt DB, Connell MG, Almond SL, Vannucchi MG, Fausone-Pellegrini MS, et al. Human and mouse enteric nervous system neurosphere transplants regulate the function of aganglionic embryonic distal colon. *Gastroenterology*. 2008; 135(1):205–16. e6.10.1053/j.gastro.2008.03.035. [PubMed: 18515088]
60. Metzger M, Caldwell C, Barlow AJ, Burns AJ, Thapar N. Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. *Gastroenterology*. 2009; 136(7):2214–25. e1–3.10.1053/j.gastro.2009.02.048. [PubMed: 19505425]
61. Rauch U, Hänsen A, Hagl C, Holland-Cunz S, Schäfer K-H. Isolation and cultivation of neuronal precursor cells from the developing human enteric nervous system as a tool for cell therapy in dysganglionosis. *International journal of colorectal disease*. 2006; 21(6):554–9. [PubMed: 16267668]
62. Pan WK, Zheng BJ, Gao Y, Qin H, Liu Y. Transplantation of neonatal gut neural crest progenitors reconstructs ganglionic function in benzalkonium chloride-treated homogenic rat colon. *Journal of Surgical Research*. 2011; 167(2):e221–e30. [PubMed: 21392806]
63. Hotta R, Stamp LA, Foong JP, McConnell SN, Bergner AJ, Anderson RB, et al. Transplanted progenitors generate functional enteric neurons in the postnatal colon. *The Journal of clinical investigation*. 2013; 123(3):1182. [PubMed: 23454768]
64. Anitha M, Joseph I, Ding X, Torre ER, Sawchuk MA, Mwangi S, et al. Characterization of fetal and postnatal enteric neuronal cell lines with improvement in intestinal neural function. *Gastroenterology*. 2008; 134(5):1424–35. Epub 2008/05/13. 10.1053/j.gastro.2008.02.018 [PubMed: 18471518]
65. Tsai YH, Murakami N, Garipey CE. Postnatal intestinal engraftment of prospectively selected enteric neural crest stem cells in a rat model of Hirschsprung disease. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2011; 23(4):362–9.10.1111/j.1365-2982.2010.01656.x [PubMed: 21199176]
66. Hotta R, Stamp LA, Foong JP, McConnell SN, Bergner AJ, Anderson RB, et al. Transplanted progenitors generate functional enteric neurons in the postnatal colon. *J Clin Invest*. 2013; 123(3):1182–91. Epub 2013/03/05. 10.1172/JCI65963 [PubMed: 23454768]
67. Kulkarni S, Zou B, Hanson J, Micci M-A, Tiwari G, Becker L, et al. Gut-derived factors promote neurogenesis of CNS-neural stem cells and nudge their differentiation to an enteric-like neuronal phenotype. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2011; 301(4):G644–G55. [PubMed: 21817062]
68. Micci MA, Kahrig KM, Simmons RS, Sarna SK, Espejo-Navarro MR, Pasricha PJ. Neural stem cell transplantation in the stomach rescues gastric function in neuronal nitric oxide synthase-deficient mice. *Gastroenterology*. 2005; 129(6):1817–24.10.1053/j.gastro.2005.08.055. [PubMed: 16344050]
69. Dong YL, Liu W, Gao YM, Wu RD, Zhang YH, Wang HF, et al. Neural stem cell transplantation rescues rectum function in the aganglionic rat. *Transplantation proceedings*. 2008; 40(10):3646–52.10.1016/j.transproceed.2008.06.107. [PubMed: 19100458]
70. Kulkarni S, Zou B, Hanson J, Micci MA, Tiwari G, Becker L, et al. Gut-derived factors promote neurogenesis of CNS-neural stem cells and nudge their differentiation to an enteric-like neuronal phenotype. *American journal of physiology Gastrointestinal and liver physiology*. 2011; 301(4):G644–55.10.1152/ajpgi.00123.2011 [PubMed: 21817062]
71. Metzger M, Bareiss PM, Danker T, Wagner S, Hennenlotter J, Guenther E, et al. Expansion and differentiation of neural progenitors derived from the human adult enteric nervous system. *Gastroenterology*. 2009; 137(6):2063–73. e4. Epub 2009/06/25 S0016-5085(09)00931-7 [pii]. 10.1053/j.gastro.2009.06.038. [PubMed: 19549531]
72. Zakhem E, Raghavan S, Bitar KN. Neo-innervation of a bioengineered intestinal smooth muscle construct around chitosan scaffold. *Biomaterials*. 2013
73. Holloway RH, Dodds WJ, Helm JF, Hogan WJ, Dent J, Arndorfer RC. Integrity of cholinergic innervation to the lower esophageal sphincter in achalasia. *Gastroenterology*. 1986; 90(4):924–9. Epub 1986/04/01. S0016508586001233 [pii]. [PubMed: 3949120]
74. De Giorgio R, Di Simone MP, Stanghellini V, Barbara G, Tonini M, Salvioli B, et al. Esophageal and gastric nitric oxide synthesizing innervation in primary achalasia. *Am J Gastroenterol*. 1999;

- 94(9):2357–62. Epub 1999/09/14 S000292709900413X [pii]. 10.1111/j.1572-0241.1999.01357.x. [PubMed: 10483991]
75. Hirakawa H, Kobayashi H, O'Briain DS, Puri P. Absence of NADPH-diaphorase activity in internal anal sphincter (IAS) achalasia. *J Pediatr Gastroenterol Nutr.* 1995; 20(1):54–8. Epub 1995/01/01. [PubMed: 7533833]
 76. Almond S, Lindley RM, Kenny SE, Connell MG, Edgar DH. Characterisation and transplantation of enteric nervous system progenitor cells. *Gut.* 2007; 56(4):489–96. [PubMed: 16973717]
 77. Raghavan S, Gilmont RR, Bitar KN. Neuroglial differentiation of adult enteric neuronal progenitor cells as a function of extracellular matrix composition. *Biomaterials.* 2013
 78. Gilmont RR, Raghavan S, Miyasaka EA, Varadarajan S, Somara S, Teitelbaum DH, et al. *In Situ* Implanted Bioengineered Human Internal Anal Sphincter Innervated With Human Enteric Neuronal Progenitor Cells Maintain Myogenic and Neurogenic Physiological Functionality. *Gastroenterology.* 2012; 142(5):S-17.
 79. Ruhl A. Glial cells in the gut. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society.* 2005; 17(6):777–90. 10.1111/j.1365-2982.2005.00687.x. [PubMed: 16336493]
 80. Cabarrocas J, Savidge TC, Liblau RS. Role of enteric glial cells in inflammatory bowel disease. *Glia.* 2003; 41(1):81–93. 10.1002/glia.10169. [PubMed: 12465048]
 81. Aikawa M, Miyazawa M, Okamoto K, Okada K, Akimoto N, Sato H, et al. A bioabsorbable polymer patch for the treatment of esophageal defect in a porcine model. *Journal of gastroenterology.* 2012:1–8.
 82. Badylak SF, Vorp DA, Spievack AR, Simmons-Byrd A, Hanke J, Freytes DO, et al. Esophageal reconstruction with ECM and muscle tissue in a dog model. *The Journal of surgical research.* 2005; 128(1):87–97. 10.1016/j.jss.2005.03.002. [PubMed: 15922361]
 83. Tan B, Wei RQ, Tan MY, Luo JC, Deng L, Chen XH, et al. Tissue engineered esophagus by mesenchymal stem cell seeding for esophageal repair in a canine model. *The Journal of surgical research.* 2013; 182(1):40–8. 10.1016/j.jss.2012.07.054. [PubMed: 22925499]
 84. Sala FG, Kunisaki SM, Ochoa ER, Vacanti J, Grikscheit TC. Tissue-engineered small intestine and stomach form from autologous tissue in a preclinical large animal model. *The Journal of surgical research.* 2009; 156(2):205–12. 10.1016/j.jss.2009.03.062. [PubMed: 19665143]
 85. Grikscheit TC, Siddique A, Ochoa ER, Srinivasan A, Alsberg E, Hodin RA, et al. Tissue-engineered small intestine improves recovery after massive small bowel resection. *Annals of surgery.* 2004; 240(5):748–54. [PubMed: 15492554]
 86. Grikscheit TC, Ochoa ER, Ramsanahie A, Alsberg E, Mooney D, Whang EE, et al. Tissue-engineered large intestine resembles native colon with appropriate in vitro physiology and architecture. *Annals of surgery.* 2003; 238(1):35–41. 10.1097/01.SLA.0000074964.77367.4a [PubMed: 12832963]
 87. Sala FG, Matthews JA, Speer AL, Torashima Y, Barthel ER, Grikscheit TC. A multicellular approach forms a significant amount of tissue-engineered small intestine in the mouse. *Tissue engineering Part A.* 2011; 17(13–14):1841–50. 10.1089/ten.TEA.2010.0564 [PubMed: 21395443]
 88. Qin HH, Dunn JC. Small intestinal submucosa seeded with intestinal smooth muscle cells in a rodent jejunal interposition model. *The Journal of surgical research.* 2011; 171(1):e21–6. 10.1016/j.jss.2011.08.001 [PubMed: 21937060]
 89. Pasricha PJ, Ahmed I, Jankowski RJ, Micci MA. Endoscopic injection of skeletal muscle-derived cells augments gut smooth muscle sphincter function: implications for a novel therapeutic approach. *Gastrointestinal endoscopy.* 2009; 70(6):1231–7. 10.1016/j.gie.2009.05.014. [PubMed: 19647239]
 90. Sung-Bum Kang M, Lee HN, Lee JY, Park J-S, Lee HS, Lee JY. Sphincter contractility after muscle-derived stem cells autograft into the cryoinjured anal sphincters of rats. *Diseases of the Colon & Rectum.* 2008; 51(9):1367–73. [PubMed: 18536965]
 91. Raghavan S, Gilmont RR, Miyasaka EA, Somara S, Srinivasan S, Teitelbaum DH, et al. Successful implantation of bioengineered, intrinsically innervated, human internal anal sphincter. *Gastroenterology.* 2011; 141(1):310–9. 10.1053/j.gastro.2011.03.056 [PubMed: 21463628]

92. Gilmont RR, Raghavan S, Somara S, Bitar KN. Bioengineering of Physiologically Functional Intrinsically Innervated Human Internal Anal Sphincter Constructs. *Tissue engineering Part A*. 2014;10.1089/ten.TEA.2013.0422
93. Singh J, Rattan S. Bioengineered human IAS reconstructs with functional and molecular properties similar to intact IAS. *American journal of physiology Gastrointestinal and liver physiology*. 2012; 303(6):G713–22.10.1152/ajpgi.00112.2012. [PubMed: 22790596]
94. Raghavan S, Miyasaka EA, Gilmont RR, Somara S, Teitelbaum DH, Bitar KN. Perianal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells. *Surgery*. 2013;10.1016/j.surg.2013.12.023
95. Rattan S, Puri RN, Fan YP. Involvement of rho and rho-associated kinase in sphincteric smooth muscle contraction by angiotensin II. *Experimental biology and medicine*. 2003; 228(8):972–81. [PubMed: 12968070]
96. Miyasaka EA, Raghavan S, Gilmont RR, Mittal K, Somara S, Bitar KN, et al. In vivo growth of a bioengineered internal anal sphincter: comparison of growth factors for optimization of growth and survival. *Pediatric surgery international*. 2011; 27(2):137–43.10.1007/s00383-010-2786-z [PubMed: 21046117]
97. Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. *Physiological reviews*. 1979; 59(1):1–61. [PubMed: 108688]
98. Halayko AJ, Solway J. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. *Journal of applied physiology*. 2001; 90(1):358–68. [PubMed: 11133929]
99. Brittingham J, Phiel C, Trzyna WC, Gabbeta V, McHugh KM. Identification of distinct molecular phenotypes in cultured gastrointestinal smooth muscle cells. *Gastroenterology*. 1998; 115(3):605–17. [PubMed: 9721158]
100. Frid MG, Shekhonin BV, Koteliensky VE, Glukhova MA. Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Developmental biology*. 1992; 153(2):185–93. [PubMed: 1397676]
101. Halayko AJ, Camoretti-Mercado B, Forsythe SM, Vieira JE, Mitchell RW, Wylam ME, et al. Divergent differentiation paths in airway smooth muscle culture: induction of functionally contractile myocytes. *The American journal of physiology*. 1999; 276(1 Pt 1):L197–206. [PubMed: 9887072]
102. McHugh KM. Molecular analysis of gastrointestinal smooth muscle development. *J Pediatr Gastroenterol Nutr*. 1996; 23(4):379–94. [PubMed: 8956171]
103. Chamley-Campbell JH, Campbell GR. What controls smooth muscle phenotype? *Atherosclerosis*. 1981; 40(3–4):347–57. [PubMed: 7332614]
104. Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Laboratory investigation; a journal of technical methods and pathology*. 1990; 63(1):21–9.
105. Desmoulière A, Rubbia-Brandt L, Abdiu A, Walz T, Macieira-Coelho A, Gabbiani G. α -Smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by γ -interferon. *Experimental cell research*. 1992; 201(1):64–73. [PubMed: 1612128]
106. van der Loop FT, Gabbiani G, Kohnen G, Ramaekers FC, van Eys GJ. Differentiation of smooth muscle cells in human blood vessels as defined by smoothelin, a novel marker for the contractile phenotype. *Arteriosclerosis, thrombosis, and vascular biology*. 1997; 17(4):665–71.
107. Niessen P, Rensen S, van Deursen J, De Man J, De Laet A, Vanderwinden JM, et al. Smoothelin-a is essential for functional intestinal smooth muscle contractility in mice. *Gastroenterology*. 2005; 129(5):1592–601.10.1053/j.gastro.2005.08.018. [PubMed: 16285958]
108. Shanahan CM, Weissberg PL. Smooth muscle cell heterogeneity: patterns of gene expression in vascular smooth muscle cells in vitro and in vivo. *Arteriosclerosis, thrombosis, and vascular biology*. 1998; 18(3):333–8.
109. Liddell RA, Syms M, McHugh KM. Heterogeneous isoactin gene expression in the adult rat gastrointestinal tract. *Gastroenterology*. 1993; 105(2):347–56. [PubMed: 8335189]
110. Sobue K, Hayashi K, Nishida W. Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. *Molecular and cellular biochemistry*. 1999; 190(1–2): 105–18. [PubMed: 10098977]

111. Shanahan CM, Weissberg PL, Metcalfe JC. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. *Circulation research*. 1993; 73(1):193–204. [PubMed: 8508530]
112. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. *The Journal of biological chemistry*. 2009; 284(6):3728–38.10.1074/jbc.M808788200 [PubMed: 19088079]
113. Bourgier C, Haydont V, Milliat F, Francois A, Holler V, Lasser P, et al. Inhibition of Rho kinase modulates radiation induced fibrogenic phenotype in intestinal smooth muscle cells through alteration of the cytoskeleton and connective tissue growth factor expression. *Gut*. 2005; 54(3): 336–43.10.1136/gut.2004.051169 [PubMed: 15710979]