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Bioengineering functional human sphincteric and non-sphincteric gastrointestinal smooth muscle constructs

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ABSTRACT

Digestion and motility of luminal content through the gastrointestinal (GI) tract are achieved by cooperation between distinct cell types. Much of the 3 dimensional (3D) *in vitro* modeling used to study the GI physiology and disease focus solely on epithelial cells and not smooth muscle cells (SMCs). SMCs of the gut function either to propel and mix luminal contents (phasic; non-sphincteric) or to act as barriers to prevent the movement of luminal materials (tonic; sphincteric). Motility disorders including pyloric stenosis and chronic intestinal pseudoobstruction (CIPO) affect sphincteric and non-sphincteric SMCs, respectively. Bioengineering offers a useful tool to develop functional GI tissue mimics that possess similar characteristics to native tissue. The objective of this study was to bioengineer 3D human pyloric sphincter and small intestinal (SI) constructs *in vitro* that recapitulate the contractile phenotypes of sphincteric and non-sphincteric human GI SMCs. Bioengineered 3D human pylorus and circular SI SMC constructs were developed and displayed a contractile phenotype. Constructs composed of human pylorus SMCs displayed tonic SMC characteristics, including generation of basal tone, at higher levels than SI SMC constructs which is similar to what is seen in native tissue. Both constructs contracted in response to potassium chloride (KCl) and acetylcholine (ACh) and relaxed in response to vasoactive intestinal peptide (VIP). These studies provide the first bioengineered human pylorus constructs that maintain a sphincteric phenotype. These bioengineered constructs provide appropriate models to study motility disorders of the gut or replacement tissues for various GI organs.

1. Introduction

The gastrointestinal (GI) tract is a tubular hollow organ composed of an array of cell types, including smooth muscle cells (SMCs), neurons, interstitial cells of Cajal (ICC) and epithelial cells, which cooperate to support peristalsis, secretion, digestion, and absorption [1–4]. The majority of current *in vitro* 3D models study GI development, physiology and disease of only the epithelium (mucosa), which covers the lumen of the gut and has primary roles in secretion of digestive enzymes and absorption of nutrients [5–12]. Motility of luminal contents is carried out by SMCs that surround the mucosa, which receives input from neurons and ICCs [13–15]. SMCs are considered as the basic functional units that perform contraction and relaxation. Numerous GI diseases including pyloric stenosis [16,17] and chronic intestinal pseudoobstruction (CIPO) [18,19] affect SMCs of the gut leading to dysmotility. There are few 3D functional models that accurately recapitulate the phenotypic and functional characteristics of SMCs within the gut. Of the studies that have investigated functional enteric SMCs *in vitro* the use of decellularized scaffolds [20–23] or collagen sponges [24–27] have been the most successful, highlighting the importance of the extracellular matrix in generating functional 3D models.

Abbreviations: GI, gastrointestinal; SMCs, smooth muscle cells; CIPO, chronic intestinal pseudoobstruction; SI, small intestine; KCl, potassium chloride; ACh, acetylcholine; VIP, vasoactive intestinal peptide; SMA, α smooth muscle actin; LES, lower esophageal sphincter; IAS, internal anal sphincter; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; EGF, Epidermal Growth Factor; bFGF, basic Fibroblast Growth Factor; HBSS, Hank's Balanced Salt Solution; RIPA, radioimmunoprecipitation assay; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline-0.1% Tween 20; HRP, horseradish peroxidase; IHC, immunohistochemical; H&E, hematoxylin and eosin; SEM, standard error of the mean.

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GI SMCs can be isolated from the gut, expanded and cultured *in vitro*, however, over time SMCs will lose their characteristic contractile phenotype and acquire a synthetic phenotype in culture [28–30]. A contractile SMC phenotype is characterized by expression of SMC markers including smoothelin, α smooth muscle actin (SMA), caldesmon and calponin along with decreased migration and proliferation of cells [29,31]. SMCs that have acquired a synthetic phenotype will down regulate SMC markers and begin to migrate and proliferate [29,32]. This makes *in vitro* studies of SMC physiology and dysfunction more challenging. In this study, we provide a bioengineering approach to construct smooth muscle constructs using primary isolated human SMCs providing a platform to better understand the phenotype of SMCs *in vitro*.

*SMCs of the GI tract display sub-phenotypes that can be either tonic (sphincteric) or phasic (non-sphincteric). Sphincters, such as the lower esophageal sphincter (LES), pylorus and internal anal sphincter (IAS), are tonically contracted and ensure unidirectional movement of luminal content through the GI tract. Proteins involved in tonic contraction of SMCs include C-kinase potentiated protein phosphatase-1 inhibitor (CPI-17), Ras homolog gene family member A (RhoA) and Rho-associated protein kinase II (ROCKII) [13,33,34]. When appropriate signals are received, the sphincters relax and allow passage of the luminal content. Phasic SMCs, such as those found in the lower part of the esophagus, stomach, small intestine (SI) and colon, mediate peristalsis through rhythmic segmentation of the gut wall to propel materials in an aboral direction. SMCs also facilitate mixing of the luminal contents [32,35–38]. Currently, there are no *in vitro* systems that recapitulate and compare the characteristics of both smooth muscle sub-phenotypes.

The objective of this study was to utilize bioengineering techniques to develop 3D *in vitro* models that appropriately recapitulate the contractile phenotypes of sphincteric and non-sphincteric human GI SMCs. In this study, human pylorus-derived SMCs were used to bioengineer 3D sphincteric constructs and SI-derived SMCs were used to bioengineer 3D non-sphincteric constructs and these constructs were compared using biochemical and physiological assays.

2. Material and methods

2.1. Reagents

Culture media reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. Growth media for SMCs contained Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1X antibiotics–antimycotics, and L-glutamine. Growth media for neurospheres contained Neurobasal (Life Technologies, Grand Island, NY), 1X N2 supplement (Life Technologies), 20 ng/mL recombinant human Epidermal Growth Factor (EGF, Stemgent, San Diego, CA), 20 ng/mL recombinant basic Fibroblast Growth Factor (bFGF, Stemgent, San Diego, CA) and 1X antibiotics. Differentiation media contained Neurobasal-A (Life Technologies), 1X B27 supplement (Life Technologies), 2% FBS and 1X antibiotics [39]. Rat tail collagen Type I was purchased from BD Biosciences (Bedford, MA), dispase and DNAse from Roche Applied Science (Indianapolis, IN), collagenase from Worthington Biochemicals (Lakewood, NY) and Hank's Balanced Salt Solution (HBSS) from Thermo Scientific HyClone (Logan, UT).

2.2. Isolation of smooth muscle cells from human pyloric sphincters and small intestines

Human pylorus and intestinal tissues were obtained through Carolina Donor Services and Wake Forest Baptist Medical Center (IRB#: IRB00007586) in accordance with The Code of Ethics of the World Medical Association. Sphincteric smooth muscle cells were obtained from the pyloric sphincter and SI smooth muscle cells were consistently obtained from the duodenum. SMCs were isolated from human GI tissues as described previously [40,41]. Briefly, pylorus and SI tissues were removed by sharp dissections and manually cleaned by removing fat and mucosa with a surgical blade. Tissue were washed in ice-cold HBSS solution containing 2X antibiotics–antimycotics 5 times, minced in sterile conditions and again washed with sterile HBSS. Tissues were digested twice in HBSS with 0.1% collagenase type II (Worthington Biochemical, Lakewood, NJ) at 37 °C with agitation for 1 h. After the second digest, samples were centrifuged at 600*g* for 10 min, and the supernatant was discarded. The cells were washed, resuspended in SMC growth media, placed in tissue culture dishes and incubated at 37 °C with 5% CO_2.

2.3. Bioengineered circular SMC constructs

Bioengineering pylorus ($n = 3$) and SI ($n = 3$) SI SMC constructs were generated utilizing similar methods described previously to generate IAS constructs [41,42]. Three replicates were performed for each experiment on the bioengineered constructs generated from different patients. Human SMCs cultured for less than 4 passages were used in constructs. Sylgard coated 35-mm culture dishes were used with an 8 mm diameter central Sylgard post. SMCs were enzymatically detached with trypsin and counted using a hemacytometer. 500,000 SMCs (either pylorus or SI) were re-suspended in 1 mL of 0.8 mg/mL Collagen I solution in 1X DMEM with 10% FBS and 1X antibiotics–antimycotics. This collagen I SMC solution was placed on Sylgard coated plates with central Sylgard posts and allowed to gel for 1 h. 1 mL of SMC differentiation media was added to constructs which were incubated at 37 °C and 5% CO_2. By day 3 SMCs contracted around central Sylgard posts forming circular tissues with an internal diameter of 8 mm. Every other day media was aspirated and 1 mL of fresh differentiation media added.

2.4. Immunoblot analyses

Confluent monolayer cultures (2D) or days 10–12 bioengineered constructs (3D) were harvested for analysis. Lysates were collected in radioimmunoprecipitation assay (RIPA) buffer and immunodetection performed as described earlier [43,44]. Briefly, 30 μg of total protein were loaded on 8% polyacrylamide gels and separated with SDS–PAGE. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes and even loading determined by ponceau S (0.1%, SigmaAldrich, St Louis, MO, USA) staining. Membranes were incubated with 1X Tris-buffered saline-0.1% Tween 20 (TBS-T) containing 5% nonfat milk, then incubated with antibodies specific for smoothelin (abcam, Cambridge, MA, USA), caldesmon, SMA, β-actin (SigmaAldrich, St Louis, MO, USA) CPI-17, phosphorylated CPI-17 (pCPI-17), RhoA or ROCKII (EMD Millipore, Billerica, Massachusetts, USA). Unbound antibodies were removed by washing, then membranes were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody. Antibody-bound proteins were detected using HyGLO chemiluminescent HRP reagent (Denville Scientific Inc, South Plainfield, NJ, USA) and pictures taken with a chemiluminescent imaging system (FujiFilm LAS-3000).

2.5. Immunohistochemical characterization of smooth muscle cells within bioengineered constructs

Immunohistochemical (IHC) staining was performed following previously established protocols for staining bioengineered tissues [41,43]. Bioengineered constructs at days 10–12 were fixed in 3.7%