Defining Matrix Composition to Promote *in vitro* Growth and Expansion of Intestinal Organoids

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Statement of Purpose: Current intestinal disease studies resort to cumbersome *in vivo* animal models or transformed cell lines due to lack of *in vitro* intestinal culture systems that appropriately mimic the physical and chemical properties of the intestinal extracellular matrix. In this study, Matrigel™, type-I collagen, and a novel Mixing Induced Two-Component Hydrogel (MITCH) [1] were investigated as model scaffolds to culture primary intestinal organoids. Unlike Matrigel™ and collagen, which are naturally derived materials, MITCH is a protein-based physical hydrogel fabricated from rational gene design and recombinant protein expression in *Escherichia Coli* that allows for independent tuning of matrix modulus and adhesion ligand density. Our purpose was to test the hypothesis that by varying physiological parameters such as scaffold modulus and cell-adhesion ligand density, via the use of these different materials, an optimal matrix could be inferred to help generate a well-defined three-dimensional (3D) protein-based scaffold for *in vitro* intestinal cultures, and potentially provide further understanding of the interplay between intestinal stem cells (ISCs) and their niche components.

Methods: Postnatal (days 0-21) C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used for isolating intestinal myofibroblasts (IMFs) and multi-cell type explants. IMFs have been suggested to play critical roles in supporting ISC growth and differentiation. In order to assess the best spatial configuration for IMF growth, IMFs were grown on 2D tissue-culture polystyrene (TCPS) and in 3D type-I collagen, and monitored daily over 7 days with a seeding density of 5,000 cells/well using CyQUANT® Cell Proliferation Assay (Invitrogen, Carlsbad, CA) in 96-well plates. Next, growth of intestinal explants within materials with different moduli was assessed. Minced intestinal tissues were embedded directly as explants into three different matrix materials, Matrigel™ (BD Biosciences, San Jose, CA), type-I collagen (Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan), and MITCH (10 wt %). All intestinal organoid cultures utilized an air-liquid interface that allows for direct air contact with the scaffold [2]. Cultures were grown at 37 ºC in 5% CO_2 with media containing 20% fetal bovine serum, 500 ng/ml Rspo1 and 50 µg/ml gentamicin. Time-lapse images were taken to compare IMF outgrowth from explants and epithelial expansion at 0, 1, 3, 5, and 7 days. Endpoint histological sections of explant cultures were stained by H&E for tissue morphology and by alcian blue for presence of epithelial mucin. Viscoelastic properties were characterized by parallel plate rheometry. The storage modulus (*G'*) was characterized at 37 ºC after overnight media immersion in the air-liquid interface setup.

Results: IMF proliferation rate on 2D TCPS was 1.5 times faster compared to within 3D collagen, with cell counts of $(15 \pm 5)x10^3$ and $(10 \pm 2)x10^3$ cells, respectively, at day 7. In Matrigel™, visual inspection and time-lapse imaging of the intestinal organoid cultures showed visible explant outgrowth of IMFs by day 1, and cyst formations by day 3 (Figure 1A), with gradual cyst enlargement through day 7. H&E of intestinal organoid cultures in Matrigel™ showed substantial epithelial development with alcian blue stain showing presence of mucin. In type-I collagen, explant outgrowth of IMFs was much more robust as IMFs visually expanded at a faster rate compared to growth on Matrigel™. Cyst formations in type-I collagen cultures were also visible by day 3 (Figure 1B), and cyst enlargement continued through day 7. H&E of intestinal organoid cultures in type-I collagen showed robust stromal layer development, but not as much epithelial expansion when compared to cultures in Matrigel™, although mucin as stained by alcian blue was present. In MITCH, there was initial robust IMF outgrowth, but cultures developed void zones surrounding the intestinal explants by day 3 (Figure 1C), and cyst formation was not observed through day 7. H&E of cultured intestinal organoids in MITCH showed limited IMF expansion and no substantial epithelial development, but were healthy since mucous layer was detectable. For all three materials, the mean storage modulus (*G'*) at 37 ºC were 16 ± 1 Pa (Matrigel™), 25 ± 3 Pa (type-I collagen), and ~50 Pa (MITCH) [1].

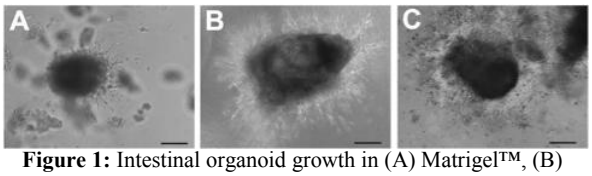

Figure 1: Intestinal organoid growth in (A) Matrigel™, (B) type-I collagen, and (C) MITCH at day 3. Bar = 200 µm.

Conclusions: IMF growth in 3D configuration was similarly substantial as compared to when grown on 2D surface. This lends support to inclusion of IMFs in 3D intestinal organoid cultures. More robust epithelial expansion was observed with Matrigel™, while more favorable IMF outgrowth was observed with type-I collagen. Neither robust epithelial expansion nor favorable IMF outgrowth was observed with MITCH, potentially a result of MITCH being less compliant and containing fewer cell-adhesion ligands. Lower modulus MITCH can be made by reducing the wt %; for example, 7.5 wt % gels have a modulus of ~20 Pa. Additional cell-adhesion ligands can be introduced into the MITCH polymer backbone. Future studies will include varying the MITCH modulus across 10-40 Pa, exploring other physiological parameters such as oxygen concentration, and quantifying these effects on ISCs that reside in intestinal organoids.

References: (1) Wong Po Foo CT, et al. Proc Natl Acad Sci. 2009; 106:22067-22072. (2) Ootani A, et al. Nat Med. 2009; 15:701-706.

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