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Intestinal organoids: A model of intestinal fibrosis for evaluating anti-fibrotic drugs

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ABSTRACT

Background & aims: Intestinal fibrosis is a critical complication of Crohn's disease (CD). Current *in vitro* models of intestinal fibrosis cannot model the complex intestinal architecture, while *in vivo* rodent models do not fully recapitulate human disease and have limited utility for large-scale screening. Here, we exploit recent advances in stem cell derived human intestinal organoids (HIOs) as a new human model of fibrosis in CD.
Methods: Human pluripotent stem cells were differentiated into HIOs. We identified myofibroblasts, the key effector cells of fibrosis, by immunofluorescence staining for alpha-smooth muscle actin (αSMA), vimentin, and desmin. We examined the fibrogenic response of HIOs by treatment with transforming growth factor beta (TGFβ) in the presence or absence of the anti-fibrotic drug spironolactone. Fibrotic response was assayed by expression of fibrogenic genes (COL1A1 (collagen, type I, alpha 1), ACTA2 (alpha smooth muscle actin), FN1 (fibronectin 1), MYLK (myosin light chain kinase), and MKL1 (megakaryoblastic leukemia (translocation) 1)) and proteins (αSMA).
Results: Immunofluorescent staining of organoids identified a population of myofibroblasts within the HIO mesenchyme. TGFβ stimulation of HIOs produced a dose-dependent pro-fibrotic response. Spironolactone treatment blocked the fibrogenic response of HIOs to TGFβ.
Conclusions: HIOs contain myofibroblasts and respond to a pro-fibrotic stimulus in a manner that is consistent with isolated human myofibroblasts. HIOs are a promising model system that might bridge the gap between current *in vitro* and *in vivo* models of intestinal fibrosis in IBD.

1. Introduction

Fibrosis is the final common pathway to organ failure in diseases of the heart, kidney, liver, lung, and intestine (Collard et al., 2012; Collins et al., 2010; Neff et al., 2011; O'Connell and Bristow, 1994; Rieder et al., 2007). Intestinal fibrosis, for which no pharmacological therapies exist, is the cause for intestinal obstruction and surgical resection in the majority of patients with Crohn's disease (CD) (Andres and Friedman, 1999; Sands et al., 2003).

Current *in vitro* and *in vivo* intestinal fibrosis models have both advantages and disadvantages. *In vitro* models of intestinal fibrosis have focused on the myofibroblast, the key effector cell of fibrosis, using fibrogenic cytokine stimulation (*e.g.* TGFβ) and mechanical stress (*e.g.* matrix stiffness), as we and others have demonstrated (Hinz, 2010; Horowitz et al., 2012; Johnson et al., 2012c, 2013, 2014; Liu et al., 2010). However, these culture models do not reflect 3D architecture and lack the multiple, specialized cell types of the intestinal structure; therefore they do not fully recapitulate *in vivo* physiology. Rodent models of intestinal fibrosis, including the rat 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Kim et al., 2008) and mouse *Salmonella typhimurium* (Grassl et al., 2008; Johnson et al., 2012b) models, have limited utility because they are not physiologically representative of human disease, and are expensive and inefficient, thus impractical for high-throughput screening.

Recent advances in the differentiation and development of intestinal tissue have resulted in the novel technique of generating human intestinal organoids (HIOs) in culture (McCracken et al., 2011). HIOs are generated from normal human pluripotent stem cells, recapitulating embryonic intestinal development and contain both epithelium and mesenchyme, including myofibroblasts (Spence et al., 2011). Moreover, they have proven to accurately represent *in vivo* physiology in a number of contexts (Chen et al., 2008, 2014; Leslie et al., 2014). Given that HIOs are the only organoid model that possesses both epithelium and mesenchyme, and that the mesenchymal layer is a major effector in fibrosis,

Abbreviations: ACTA2, alpha smooth muscle actin (gene); αSMA, alpha smooth muscle actin (protein); CD, Crohn's disease; COL1A1, collagen, type I, alpha 1; ECM, extracellular matrix; FN1, fibronectin 1; HIOs, human intestinal organoids; IF, immunofluorescence; MKL1, megakaryoblastic leukemia (translocation) 1; MYLK, myosin light chain kinase; TGFβ, transforming growth factor beta.

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we reasoned that HIOs could be used as a viable *in vitro* model of human intestinal fibrosis to bridge the gap between *in vitro* and *in vivo* models of intestinal fibrosis, enabling the study of multi-lineage human cells in a physiologically relevant environment, while being amenable to high-throughput drug screening (Astashkina and Grainger, 2014; Ranga et al., 2014). In this study we explore the potential for HIOs to be used as a model of intestinal fibrosis and as a tool for screening anti-fibrotic drugs, using spironolactone as our proof-of-concept drug because of its proven effectiveness in cardiac fibrosis (Pitt et al., 1999).

2. Materials and methods

Unless otherwise specified, all chemical reagents were obtained from Sigma Aldrich, St. Louis, MO.

2.1. HIO culture

All work was approved by the University of Michigan Human Pluripotent Stem Cell Research Oversight Committee (HPSCRO). Human embryonic stem cells (H9, Wicell Research Institute, Madison, WI) were differentiated into human intestinal organoids (HIOs) as described previously (McCracken et al., 2011; Spence et al., 2011). For the purpose of this study, organoids with high mesenchymal cell composition were chosen, as opposed to cyst-like, epithelial-high organoids. For TGFβ treatment, HIOs were embedded in growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, catalog # 356231).

2.2. Immunofluorescence staining and imaging

Cultured HIOs were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS, washed in PBS, and embedded in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA), then sliced on a cryotome and mounted on Fisherbrand Superfrost Plus slides. The tissue was then blocked and permeabilized in PBS containing 5% goat serum (Invitrogen, Carlsbad, CA) and 0.5% Triton X-100 (LabChem, Pittsburgh, PA) for 1 h at 37 °C. An additional blocking step was performed using an SFX signal enhancer (Invitrogen, Carlsbad, CA) for 30 min at room temperature, followed by incubation with primary antibodies to vimentin (Sigma, St. Louis, MO, catalog # SAB4503083) or to desmin (Abcam, Cambridge, MA, catalog # ab8976) at 1:100 for 2 h. Slides were washed with 0.05% Tween 20 (Bio-Rad, Hercules, CA) in PBS, followed by incubation with AlexaFluor 488-conjugated anti-mouse or anti-rabbit antibody (Invitrogen, Carlsbad, CA). Detection of αSMA was performed with 30 min incubation using Cy3 conjugated mouse anti-αSMA (Sigma, St. Louis, MO) at 1:200, co-stained with 4,6′ diamidino-2-phenylindole (DAPI), (Molecular Probes, Eugene, OR) at 1:1000, to visualize nuclei. Images were acquired using an Olympus BX60 microscope and a DP72 camera, with CellSens Standard imaging software, version 1.11 (Olympus America, Center Valley, PA). Images were merged using ImageJ software.

2.3. TGFβ fibrogenesis model

Recombinant human TGFβ was obtained from R&D Systems (Minneapolis, MN). TGFβ responsiveness was assayed by treatment of HIOs with increasing amounts of TGFβ (0.5 to 5 ng/mL) for 48 or 96 h. To determine the effect of spironolactone, HIOs were co-treated with 2 ng/mL TGFβ and 25 to 500 μM spironolactone prior to harvest for molecular analysis.

2.4. Real-time PCR analysis

RNA was extracted from HIOs using the RNeasy kit (Qiagen, Valencia, CA). RNAs were treated with RNase-free DNase prior to cDNA synthesis using the First Strand Synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The analysis of gene expression was determined by quantitative real-time PCR (qPCR) of collagen-1 A1 (COL1A1), myosin light chain kinase (MYLK), fibronectin 1 (FN1), and MKL1 (MRTF-A) genes and GAPDH was performed with the TaqMan gene expression assays (ABI, Foster City, CA). qPCR was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA) or iCycler (Bio-Rad, Hercules, CA) real-time PCR system. αSMA (ACTA2) gene expression was determined with the SYBR Green assay using the following primers (ACTA2-F 5′-AATGCAGAAGGAGATCACGC-3′, ACTA2-R 5′-TCCT GTTTGCTGATCCACATC-3′) as previously described (Johnson et al., 2014). Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 60 s. ΔΔCt was calculated from GAPDH expression.

2.5. Preparation of protein lysates from HIOs

HIOs were excised from Matrigel, pooled (9–12 organoids were pooled per sample), washed in ice-cold PBS containing protease inhibitors (Roche, Indianapolis, IN), washed for 1 h in Cell Recovery Solution (Corning, Bedford, MA), followed by additional PBS washes, then lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.5 Mm $MgCl_2$, 5 mM EGTA, 1% glycerol, plus cOmplete EDTA free protease and phosphatase inhibitors (Roche, Indianapolis, IN), sodium pyrophosphate (30 mM), sodium fluoride (50 mM), and sodium orthovanadate (100 μM). Lysates were cleared by centrifugation prior to loading 10–40 μg total protein on SDS-PAGE gel.

2.6. Western blot analysis

Total protein was separated by SDS-PAGE and probed for αSMA as previously described (Johnson et al., 2012c). αSMA primary antibody (Sigma, St. Louis, MO, catalog # A5228) was used at 1:400.

2.7. Statistical analysis

Each experiment was performed independently at least three times with similar results; findings from one representative experiment are presented. Comparisons across treatment groups were analyzed with analysis of variance (ANOVA), while pairwise comparisons between two groups were performed with Student's *t*-test.

All authors had access to the study data and had reviewed and approved the final manuscript.

3. Results and discussion

Myofibroblasts are the key effector cells of fibrosis (Powell et al., 1999). Recent advances in 3-dimensional culture have enabled the *in vitro* growth of organoids that recapitulate the multi-layer structure of the human intestine (McCracken et al., 2011). A previous study identified a population of myofibroblasts in HIOs (Spence et al., 2011). By immunofluorescence (IF) staining, we showed that HIOs from genetically normal human embryonic stem cells (hESCs, line H9) organize into complex structures comprised of a lumen (asterisk) surrounded by endodermally derived epithelium, and mesodermally derived mesenchymal layers (Fig. 1A). We observed the organization of a population of intestinal myofibroblasts (white arrows) adjacent to the intestinal epithelium (Fig. 1A). These cells were identified as αSMA^{+}/vimentin^{+}/desmin^{-} myofibroblasts (Fig. 1B and C, see high magnification inset).

Next, we determined whether HIOs would respond to TGFβ treatment with an increase in αSMA-expressing cells, indicating fibrogenic activation. In previous studies (Johnson et al., 2013, 2014), we demonstrated that isolated human colonic myofibroblasts respond to TGFβ by inducing fibrogenic genes. To determine the effective dose of TGFβ for HIOs, we first performed a 48-h TGFβ dose–response experiment (0.5 to 5 ng/mL TGFβ) and assayed for gene expression of the ECM components COL1A1 and FN1, the myofibroblast marker and component of actin stress fibers ACTA2, the actin contractile gene MYLK, and

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