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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 (α SMA), vimentin, and desmin. We examined the fibrogenic response of HIOs by treatment with transforming growth factor beta (TGF β) 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 (α SMA).

Results—Immunofluorescent staining of organoids identified a population of myofibroblasts within the HIO mesenchyme. TGF β stimulation of HIOs produced a dose-dependent pro-fibrotic response. Spironolactone treatment blocked the fibrogenic response of HIOs to TGF β .

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ESR- Acquisition, analysis, and interpretation of data; drafting of the manuscript.

LAJ- Analysis and interpretation of data, critical revision of the manuscript.

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JRS and PDRH – Study concept and design, obtained funding, critical revision of the manuscript.

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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.

Keywords

Organoid; Myofibroblasts; TGF β ; fibrosis model; drug screening

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. TGF β) and mechanical stress (e.g. matrix stiffness), as we and others have demonstrated (Hinz, 2010; Horowitz et al., 2012; Johnson et al., 2014; Johnson et al., 2013; Johnson et al., 2012c; 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 *S. 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; Chen et al., 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, 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).

Materials and Methods

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

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 TGF β treatment, HIOs were embedded in growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, catalog # 356231).

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 hour at 37 degrees C. An additional blocking step was performed using SFX signal enhancer (Invitrogen, Carlsbad, CA) for 30 minutes 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 hours. 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 α SMA was performed with 30 minute incubation using Cy3 conjugated mouse anti- α SMA (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 Olympus BX60 microscope and DP72 camera, with CellSens Standard imaging software, version 1.11 (Olympus America, Center Valley, PA). Images were merged using ImageJ software.

TGF β Fibrogenesis Model

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

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 manufacturer's protocol. The analysis of gene expression was determined by quantitative real-time PCR (QPCR) of collagen-1 A1

(COLA1), myosin light chain kinase (MYLK), fibronectin 1 (FN1), 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. α SMA (ACTA2) gene expression was determined with the SYBR Green assay using the following primers (ACTA2-F 5'-AATGCAGAAGGAGATCACGC-3', ACTA2-R 5'-TCCTGTTTGCTGATCCACATC-3') as previously described (Johnson et al., 2014). Cycling conditions were 95° C 10 minutes, followed by 40 cycles of 95° C 15 seconds and 62° C 60 seconds. Ct were calculated from GAPDH expression.

Preparation of Protein Lysates from HIO

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 hour in Cell Recovery Solution (Corning, Bedford, MA), followed by additional PBS washes, then were lysed in buffer containing 50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 1.5mM MgCl₂, 5mM EGTA, 1 % glycerol, plus Complete EDTA Free Protease and Phosphatase inhibitors (Roche, Indianapolis, IN), Sodium Pyrophosphate (30mM), Sodium Fluoride (50mM), and Sodium Orthovanadate (100uM). Lysates were cleared by centrifugation prior to loading 10–40 μ g total protein on SDS-PAGE gel.

Western Blot Analysis

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

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.

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 multilayer 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 (Figure 1A). We observed the organization of a population of intestinal myofibroblasts (white arrows) adjacent to the intestinal epithelium (Figure 1 A). These cells

were identified as α SMA⁺/vimentin⁺/desmin⁻ myofibroblasts (Figure 1 B and C, see high magnification inset).

Next, we determined whether HIOs would respond to TGF β treatment with an increase in α SMA-expressing cells, indicating fibrogenic activation. In previous studies (Johnson et al., 2014; Johnson et al., 2013), we demonstrated that isolated human colonic myofibroblasts respond to TGF β by inducing fibrogenic genes. To determine the effective dose of TGF β for HIOs, we first performed a 48-hour TGF β dose-response experiment (0.5 to 5 ng/mL TGF β) 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 the fibrogenic transcription factor MKL1. In contrast to isolated myofibroblasts, in HIOs, no induction of COL1A1, FN1, ACTA2, or MYLK was observed at 48 hours. However, MKL1 gene expression was induced (3-fold), and a trend towards induction of FN1 gene expression was also observed at 48 hours, with 2 ng/mL TGF β (Figure 1 D, E). Because myofibroblasts in HIOs are sequestered from their environment within layers of other cell populations (Figure 1 A), we postulated that a longer treatment would be necessary to induce a robust fibrogenic response in HIOs. Based on these results demonstrating a modest fibrogenic response starting at 2ng/mL, we treated HIOs with 2 ng/mL TGF β for 96 hours, and found marked induction of α SMA (red, Figure 1 G), compared to untreated HIOs (Figure 1 F).

To further validate the fibrogenic response observed in Figure 1F and 1G, we treated the HIOs with 2 ng/mL TGF β for 96 hours and then analyzed fibrogenic gene expression by QRT-PCR. Supporting the immunofluorescence data, significant induction of several fibrogenic genes in HIOs was observed at 96 hours, including COL1A1 (2.6-fold), FN1 (4.8-fold), ACTA2 (2.3-fold), MYLK (5.1-fold), and MKL1 (4.1-fold), as seen in Figure 2.

Next, to show proof-of-concept that HIOs can be used to test anti-fibrotic drugs, we determined whether the TGF β -mediated fibrogenic response of HIOs could be reversed by treatment with spironolactone. Spironolactone is a competitive aldosterone receptor antagonist that is commonly used as an anti-fibrotic medication in heart patients, and has proven protective in several rodent models of fibrosis (Ersoy et al., 2007; Gullulu et al., 2006; Nishimura et al., 2008; Rajagopalan and Pitt, 2003). Previously, our group determined that spironolactone was anti-fibrotic in isolated human colonic myofibroblasts and repressed TGF β -mediated induction of pro-fibrotic genes and proteins (Johnson et al., 2012a). To determine whether spironolactone could prevent TGF β -induced fibrogenic gene and protein expression in HIOs, we analyzed the expression of fibrogenic genes and proteins in HIOs by qPCR and Western blot after 96 hours of co-treatment of various concentrations of spironolactone with 2 ng/mL TGF β . Spironolactone (250 μ M) repressed TGF β -stimulated induction of COL1A1(3.9-fold), FN1 (3.4-fold), ACTA2 (3.9-fold), and MYLK (66-fold), as seen in Figure 3 A–D. The effect of spironolactone on α SMA protein expression was even more pronounced, reversing the TGF β -mediated induction of α SMA protein expression at a concentration of 100 μ M (Figure 3 E and F).

In conclusion, we have demonstrated that HIOs can be used to model intestinal fibrosis, and to test the effectiveness of anti-fibrotic drugs. Study of the pathology of fibrosis in

inflammatory bowel disease has, until recently, depended upon animal models that are complex, expensive, and possibly not relevant to the disease state (Brenna et al., 2013; Mestas and Hughes, 2004; Neurath et al., 2000), or 2D cell culture models that are reductionist and artificial. For example, the surface of a plastic culture dish is orders of magnitude stiffer than intestinal tissue (Mih et al., 2011), and induces an activated myofibroblast phenotype, characterized by increased number and organization of actin stress fibers and focal adhesions (Hinz, 2010). Because they contain multiple cell types and 3D architecture physiologically similar to the human intestine, our data suggest that HIOs are a viable new model to study fibrosis related to Crohn's disease.

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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
HIO	Human Intestinal Organoids
IF	Immunofluorescence
MKL1	megakaryoblastic leukemia (translocation) 1
MYLK	myosin light chain kinase
TGFβ	transforming growth factor beta

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Highlights

- Induction of intestinal fibrosis was modeled in human intestinal organoids (HIOs).
- HIOs recapitulate the fibrogenic response of isolated myofibroblasts.
- Spironolactone treatment blocked the fibrogenic response of HIOs to TGF β .
- HIOs are a promising model of intestinal fibrosis induction and treatment.

Figure 1.

HIOs contain myofibroblasts and are activated by TGF β . (A) Morphology (10 $\times$ magnification) of representative HIO illustrating the presence of a well-defined lumen (asterisk) and a population α SMA+ cells (red staining, white arrows). (B,C) α SMA + (red) cells were identified as resident myofibroblasts (α SMA +, vimentin+, desmin-) by staining with vimentin (green in B), and desmin (green in C), 20 $\times$ magnification. Individual representative vimentin+/desmin- myofibroblasts (circled) are shown in the insets. (D,E) Gene expression as determined by qPCR for pro-fibrotic genes FN1 (D) and MKL-1 (E) in

HIOs treated with increasing amounts of TGF β (0.5, 1, 2, 5 ng/mL) for 48 hrs. The expression of pro-fibrotic genes was normalized to GAPDH using the $\Delta\Delta$ Ct method. Results are from 3 independent experiments. Statistical comparisons are made between the untreated and TGF β -treated groups using the Paired Student's t-test. (F,G) Activation of myofibroblasts within the HIOs by TGF β . α SMA (red) and vimentin (green) was markedly induced in TGF β -treated HIO (G). Vimentin is stained green in Figure 1 F and G. The scale bar represents 50 μ M.

Figure 2.

Induction of fibrogenic gene expression in HIO by TGFβ. Matrigel-embedded HIOs were cultured for 96 hours in the presence or absence of TGFβ (2 ng/mL). (A) Expression of COL1A1, (B) Expression of FN1, (C) Expression of ACTA2, (D) Expression of MYLK, (E) Expression of MKL1. Gene expression was normalized to GAPDH expression. Statistical comparisons are made between untreated and TGFβ-treated organoids using the Paired Student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results are from 3 independent experiments performed in duplicate (total $n=6$).

Figure 3.

Inhibition of TGFβ-mediated induction of fibrogenic genes by spironolactone (SPIR) in HIOs. Organoids were embedded in Matrigel and cultured for 96 hours in the presence or absence of TGFβ (2 ng/mL) and 0, 25, 50, 100, and 250 μM spironolactone. Gene expression analysis: A–D. (A) Expression of COL1A1, (B) Expression of FN1, (C) Expression of ACTA2, (D) Expression of MYLK. The expression of pro-fibrotic genes was normalized to GAPDH using the Ct method. Results are from 3 independent experiments. (E). Representative αSMA Western blot of HIOs treated with TGFβ (2 ng/mL) and 100 or

250 μ M SPIR for 96 hours. (F) Quantitation of α SMA protein expression in response to co-treatment with TGF β and either 100 or 250 μ M SPIR. HIOs were treated for 96 hours with 2 ng/mL TGF β . Western data from 3 independent HIO experiments were quantified using ImageJ. α SMA band density was normalized to GAPDH band density, and within each experiment, treatment groups were normalized to untreated. Horizontal bars represent the geometric mean of each group. Statistical comparisons are made between untreated HIO and TGF β -treated or TGF β and SPIR treated HIOs using the Paired Student t test are denoted with asterisks (*p < 0.05, ** p < 0.01, ***p<0.001). Statistical comparisons between TGF β -treated or TGF β and SPIR treated HIOs are denoted with a pound sign (# p < 0.05, ## p < 0.01, ### p<0.001).