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Supplemental Information

Nonadhesive Alginate Hydrogels Support Growth of Pluripotent Stem Cell-Derived Intestinal Organoids

Meghan M. Capeling, Michael Czerwinski, Sha Huang, Yu-Hwai Tsai, Angeline Wu, Melinda S. Nagy, Benjamin Juliar, Nambirajan Sundaram, Yang Song, Woojin M. Han, Shuichi Takayama, Eben Alsberg, Andres J. Garcia, Michael Helmrath, Andrew J. Putnam, and Jason R. Spence

Inventory of Supplemental Information:

Figure S1. Alginate supports HIO culture across multiple hPSC lines. This Figure is related to Figures 1 and 2 as we present evidence that HIOs derived from multiple hPSC lines can be cultured in alginate and present qRT-PCR analysis to compare expression levels of key epithelial genes between alginate and Matrigel-grown HIOs across multiple hPSC lines.

Figure S2. Alginate does not recapitulate intestinal epithelial niche in the absence of mesenchymal support. This Figure is related to Figures 1 and 2 as we demonstrate that organoid culture in alginate cannot be expanded to primary intestinal enteroids that lack a mesenchymal component, and provide preliminary evidence suggesting that mesenchymal support creates a niche to support intestinal epithelium in alginate.

Figure S3. Further characterization of alginate-grown HIOs. This Figure is related to Figure 1 as we demonstrate that HIO culture in alginate can be continued for up to 90 days.

Table S1. This Table is related to Figure 4 as we provide an Excel file listing specific genes that are significantly changed in all comparison groups in Figure 4.

Supplemental Experimental Procedures

- **Expanded methods**
- **qRT-PCR primer sequences**
- **Antibody information**

Supplemental References

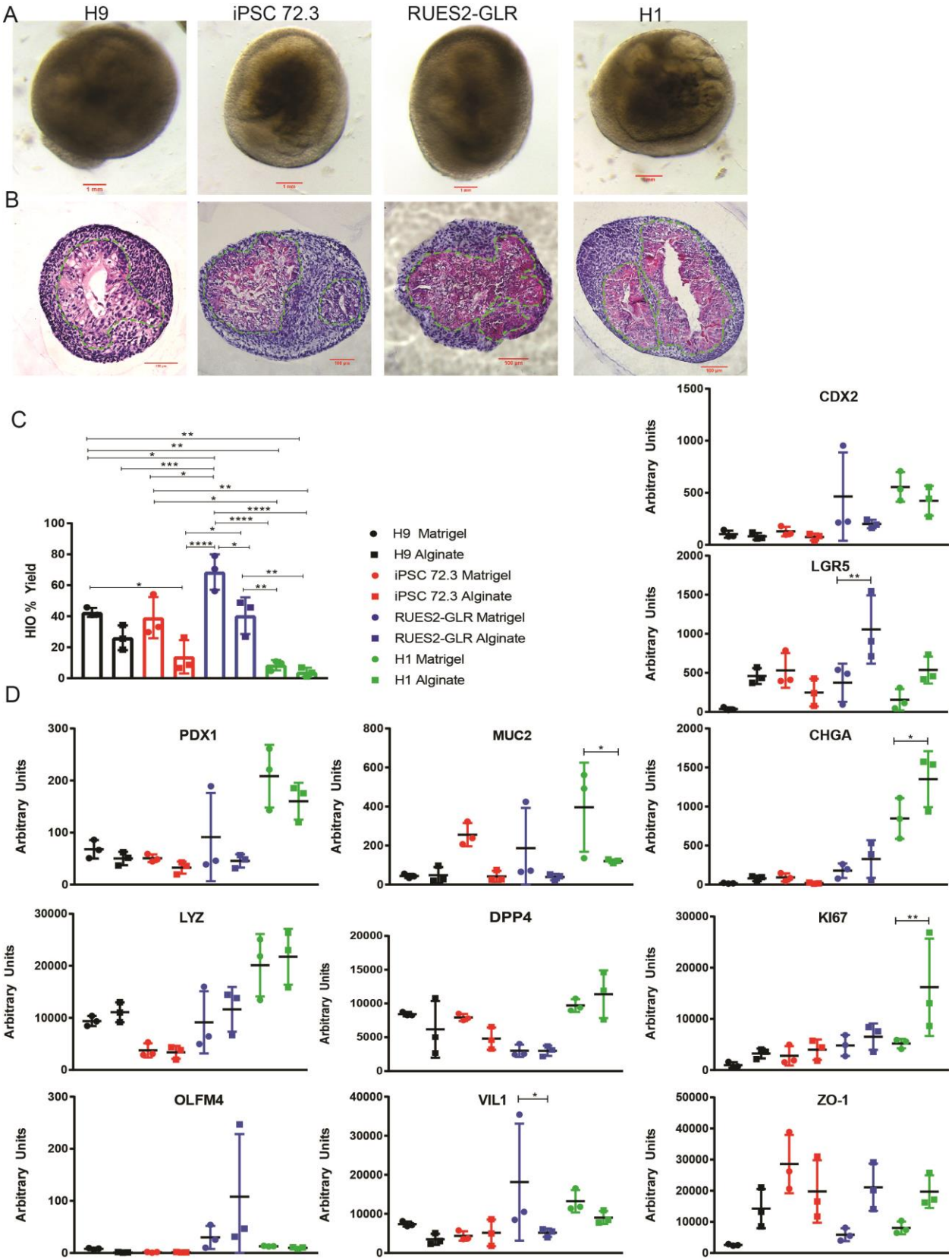
A
H9
iPSC 72.3
RUES2-GLR
H1
B
C
HIO % Yield
H9 Matrigel
H9 Alginate
iPSC 72.3 Matrigel
iPSC 72.3 Alginate
RUES2-GLR Matrigel
RUES2-GLR Alginate
H1 Matrigel
H1 Alginate
CDX2
LGR5
D
PDX1
MUC2
CHGA
LYZ
DPP4
KI67
OLFM4
VIL1
ZO-1
Arbitrary Units

Figure S1. Alginate supports HIO culture across multiple hPSC lines. Related to Figures 1 and 2. **(a)**: Brightfield images of HIOs derived from 4 independent hPSC lines cultured in 1% alginate for 28 days *in vitro*. Scale bar, 1 mm. **(b):** Hematoxylin and eosin (H&E) staining of HIOs derived from 4 independent hPSC lines cultured in 1% alginate for 28 days. Dashed lines outline the epithelium. Scale bar, 100μm. **(c)**: Quantification of HIO yield after 28 days in culture for HIOs derived from 4 independent hPSC lines. HIO yield was calculated as the percentage of spheroids which gave rise to HIOs. Data shown are the average yields from 3 independent experiments with n>100 spheroids per condition. Each point depicts overall yield from one experiment, while bars depict mean and SE. Significance was calculated with a one-way ANOVA and Tukey's multiple comparisons test. The strength of p values is reported according the following: $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. **(d)**: qRT-PCR analysis of CDX2, LGR5, PDX1, MUC2, CHGA, LYZ, DPP4, KI67, OLFM4, VIL1, ZO-1 expression in HIOs derived from 4 independent hPSC lines cultured in 1% alginate and Matrigel for 28 days *in vitro*. Expression levels are normalized to ECAD expression to account for varying amounts of epithelium in HIOs. Each point is representative of 6-10 HIOs pooled from the same batch. Data represent the mean ± SE. Significance was calculated with a one-way ANOVA and Tukey's multiple comparisons test where expression was compared between alginate and Matrigel-grown HIOs for each line. The strength of p values is reported according the following: $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

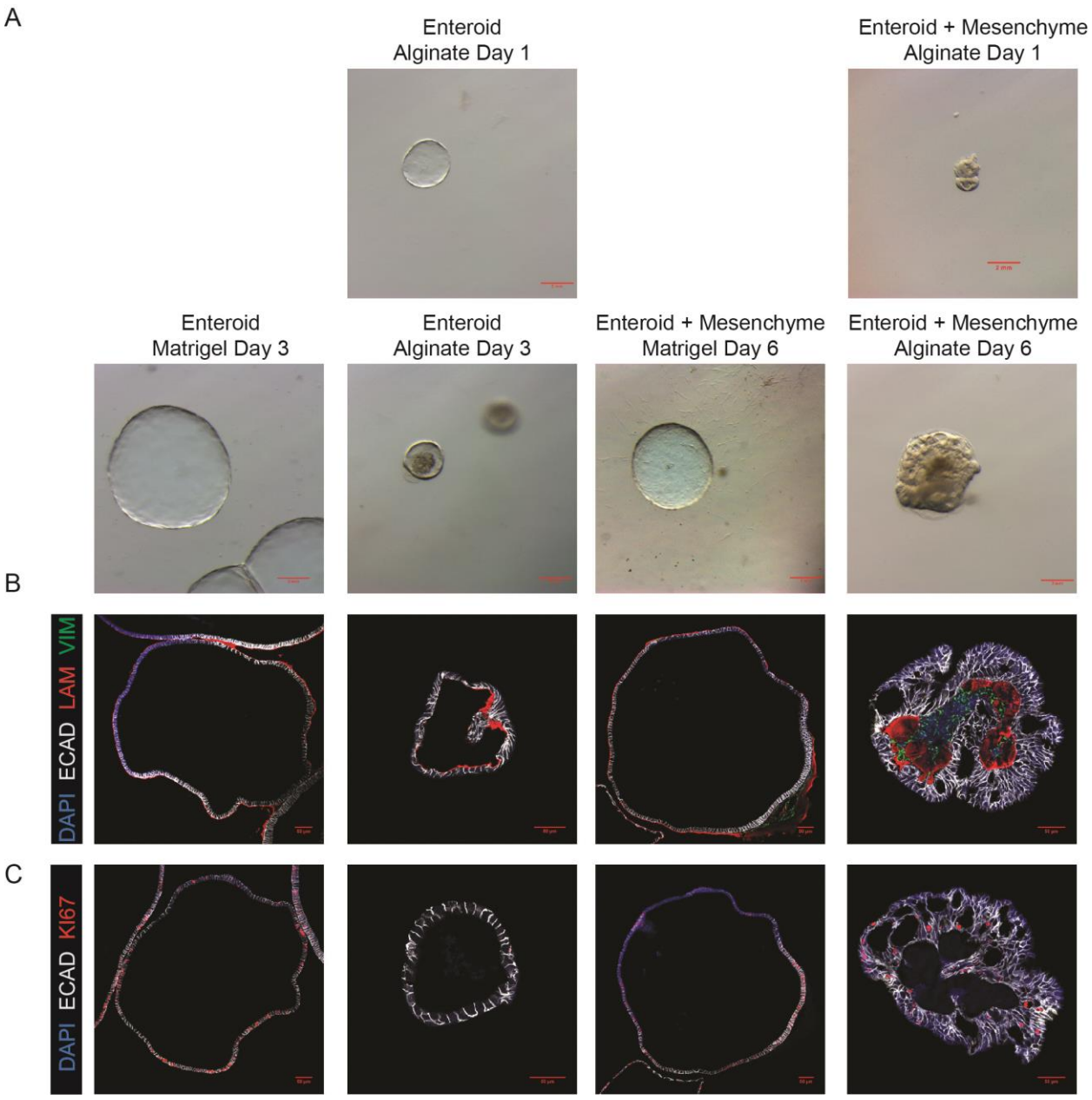

Figure S2. Alginate does not recapitulate intestinal epithelial niche in the absence of mesenchymal support. Related to Figures 1 and 2. **(a)**: Brightfield images of fetal intestinal enteroids cultured in Matrigel and re-embed in 1% alginate and Matrigel either alone or co-cultured with fetal intestinal mesenchymal cells. Scale bar, 1 mm. **(b)**: Representative images of intestinal enteroids embed in alginate and Matrigel with and without mesenchyme after 7 days stained for ECAD, LAM, and VIM. Scale bar, 50 µm. **(c)**: Representative images of intestinal enteroids embed in alginate and Matrigel with and without mesenchyme after 7 days stained for ECAD and KI67. Scale bar, 50 µm.

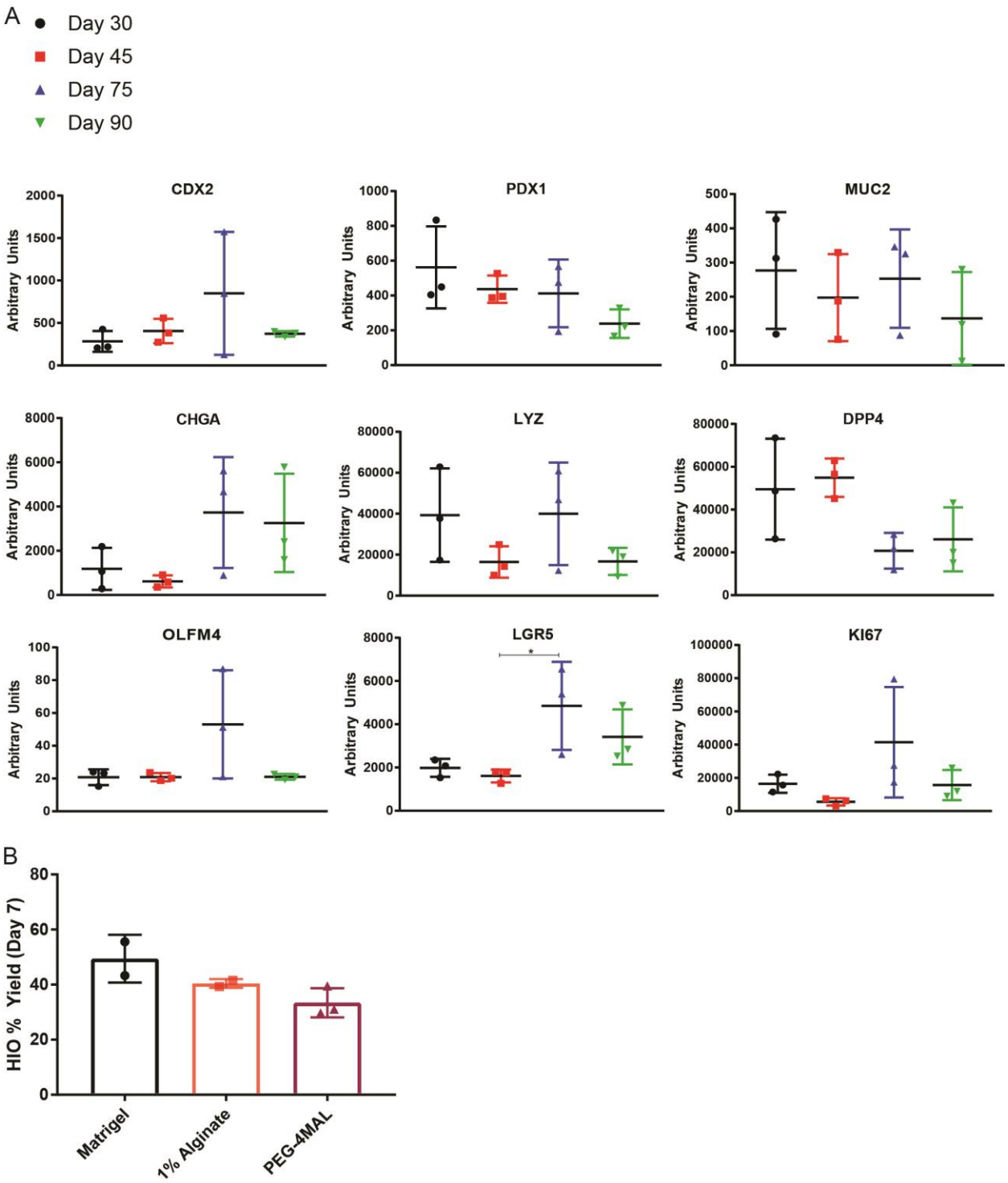

Figure S3. Further characterization of alginate-grown HIOs. Related to Figure 1. **(a)**: qRT-PCR analysis of CDX2, LGR5, PDX1, MUC2, CHGA, LYZ, DPP4, KI67, OLFM4, VIL1, and ZO-1 expression in HIOs cultured in 1% alginate over time for 90 days. Expression levels are normalized to ECAD to account for varying amounts of epithelium in HIOs. Each point is representative of 6-10 HIOs pooled from the same batch. Data represent the mean ± SE. Significance was calculated with a one-way ANOVA and Tukey's multiple comparisons test. The strength of p values is reported according the following: $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. **(b):** Quantification of the percentage of spheroids which gave rise to organoids after 7 days of culture in Matrigel, 1% alginate, and 4% PEG-4MAL. Data shown are the average yields from 2 (alginate and Matrigel) or 3 (PEG-4MAL)

independent experiments with n>100 spheroids per condition. Each point depicts overall yield from one experiment, while bars depict mean and SE. Significance was calculated with a one-way ANOVA and Tukey's multiple comparisons test. The strength of p values is reported according the following: $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

Supplemental Experimental Procedures

hESC/hIPSC Lines and Generation of hPSC-Derived Intestinal Organoids

hPSC lines were induced to differentiate into endoderm using Activin A (100ng/mL, R & D Systems) for 3 days in RPMI1640 media supplemented with 0%, 0.2%, 2% HyClone dFBS on subsequent days. Endoderm was induced to differentiate into the intestinal lineage by treating cells for 5-6 days with FGF4 (500ng/mL, generated as previously described (Leslie et al., 2015)) and CHIR99021 (2μM). Following differentiation, free-floating hindgut spheroids were collected from differentiated stem cell cultures after days 5 and 6 of hindgut specification and plated in Matrigel (diluted with Advanced DMEM/F12 to a final protein concentration of 8 mg/mL) or alginate droplets as described in Experimental Procedures on a 24-well tissue culture grade plate. Organoid growth media consisted of Advanced DMEM/F12 supplemented with 1X B27 (Thermo Fisher, Waltham, MA), GlutaMAX (Gibco, 1X), penicillin-streptomycin (Gibco, 100 U ml^{-1} penicillin; 100 μg ml^{-1} streptomycin), HEPES buffer (Gibco, 15 mM), epidermal growth factor (EGF) (R&D Systems; 100 ng/mL), Noggin-Fc (100ng/mL) (purified from conditioned media (Heijmans et al., 2013)), and R-Spondin2 (5% conditioned medium (Bell et al., 2008)). Media was changed every 5-7 days.

Maintenance of HIOs

For studies of up to 30 days, alginate and Matrigel-grown HIOs were not passaged. For the 90-day study of alginate-grown HIOs, organoids were passaged in a similar manner to previously described methods for hydrogel or Matrigel-grown HIOs (Cruz-Acuna et al., 2017; Spence et al. 2011). Briefly, HIOs were dislodged from the alginate hydrogel by pipetting up and down and transferred to a sterile Petri dish. Excess alginate was removed by cutting it away with a scalpel, and HIOs were manually cut in half with a scalpel. HIO halves were transferred to fresh alginate solutions and re-embed in a clean 24-well tissue culture grade plate upon gelation with calcium chloride.

Epithelial Isolation

HIOs and tHIOs were incubated in dispase (07923; STEMCELL Technologies) for 30 minutes on ice. Following incubation, dispase was removed and replaced with 100% fetal bovine serum for 15 minutes on ice. To mechanically separate the epithelium from mesenchyme, a volume of advanced Dulbecco's modified Eagle medium/F12 (12634010; Gibco) equal to the initial volume of fetal bovine serum was added to the tissue before vigorously pipetting the mixture several times. Epithelial fragments then settled to the bottom where they were collected manually on a stereoscope by pipet. The epithelium was washed with ice-cold advanced Dulbecco's modified Eagle medium/F12 and allowed to settle to the bottom of a 1.5-mL Eppendorf tube. The media was then withdrawn from the loose tissue pellet and replaced with Matrigel on ice. The Matrigel containing the isolated epithelium was gently mixed to evenly suspend the cells before being pipetted into individual 50 μL droplets in a 24-well plate. The plate containing the droplets was incubated at 37°C for 15 minutes to allow the Matrigel to solidify before adding LWRN growth media containing Thiazovivin (2.5 μmol/L), SB431542 (100 nmol/L), CHIR99021 (4 μmol/L), and Y27632 (10 μmol/L). LWRN growth media was produced as previously described (Tsai et al., 2018). In summary, conditioned media from L-WRN cells containing Wnt3a, Rspondin3, and Noggin was mixed at a ratio of 1:1 with 2× basal media comprised of 214 mL advanced Dulbecco's modified Eagle medium/F12, 5 mL GlutaMAX (Gibco, Japan) (100×, 200 mmol/L), 5 mL HEPES (100×, 1 mol/L), 5 mL N2 supplement (100×), 10 mL B27 supplement (50×), 5 mL penicillin/streptomycin (100×), 1 mL N-acetylcystine (500 mmol/L), and 5 mL nicotinamide (1 mol/L). After 24 hours, the media was replaced with LWRN growth media containing TZV (2.5 μmol/L), SB431542 (100 nmol/L), and CHIR99021 (4 μmol/L). After 3 days, cultures were maintained with LWRN growth media replaced every other day.

RNA-sequencing and Bioinformatics Analysis

RNA from each sample was isolated using MagMAX-96 Total RNA (AM1830; Applied Biosystems) RNA isolation kits and used as input for library generation with Takara SMARTer Stranded Total RNA Sample Prep Kit (634876; Takara Bio USA). Samples were sequenced for 50-bp single-end reads across 10 lanes on an Illumina HiSeq 2500 by the University of Michigan DNA Sequencing Core. All reads were quantified using Kallisto pseudo alignment to an index of transcripts from all human genes within the Ensembl GRCh38 database (Bray et al., 2016). Gene level data generated from Kallisto was used to create normalized data matrix of pseudoaligned sequences (Transcripts Per

Million, TPM) and differential expression was calculated using the Bioconductor package DEseq2. Estimated counts per transcript using the Bioconductor package tximport. Differential expression analysis was performed using the Bioconductor package DESeq2 using gene count data (Love et al., 2014). A gene was considered to be differentially expressed if it had a 2-fold or larger difference between groups and an adjusted *P* value of .01 or less. Principal component analysis and sample clustering were performed in R with log2 transformed and centered gene counts of gene level data on all genes that had a sum of at least 10 counts across all samples. Replicates for all samples were clustered by Euclidian distance, and pairwise Pearson correlation coefficients were plotted in R. All reads are deposited at the EMBL-EBI ArrayExpress archive under accession E-MTAB-7000.

RNA Extraction and quantitative RT-PCR Analysis

qRT-PCR experiments were carried out as previously described (Miller et al., 2018). RNA was extracted using the MagMAX-96 Total RNA Isolation System (Life Technologies). RNA quality and concentration was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Isolated RNA was used to generate a cDNA library using the SuperScript VILO cDNA master mix kit (Invitrogen) according to manufacturer's instructions. qRT-PCR analysis was conducted using Quantitect SYBR Green Master Mix (Qiagen) on a Step One Plus Real-Time PCR system (Life Technologies). Expression was calculated as a change relative to ECAD expression using arbitrary units, which were calculated by the following equation: [2^(ECAD Ct - Gene Ct)] x 10,000. Expression was normalized to ECAD as we analyzed epithelial-specific genes and there were variable levels of epithelium between samples. A Ct value of 40 or greater was considered not detectable. A list of primer sequences used can be found in the table below.

Primer Information

Gene Target	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	CTCTGCTCCTCCTGTTCGAC	TTAAAAGCAGCCCTGGTGAC
ECAD	TTGACGCCGAGAGCTACAC	GACCGGTGCAATCTTCAAA
CDX2	GGGCTCTCTGAGAGGCAGGT	GGTGACGGTGGGGTTTAGCA
LGR5	CAGCGTCTTCACCTCCTACC	TGGGAATGTATGTCAGAGCG
PDX1	CGTCCGCTTGTTCTCCTC	CCTTTCCCATGGATGAAGTC
MUC2	TGTAGGCATCGCTCTTCTCA	GACACCATCTACCTCACCCG
CHGA	CTGTCCTGGCTCTTCTGCTC	TGACCTCAACGATGCATTTC
LYZ	ACAAGCTACAGCATCAGCGA	GTAATGATGGCAAAACCCCA
DPP4	TCCCGGTGGGAGTACTATGA	CAGGGCTTTGGAGATCTGAG
KI67	CAGGGCTTTGGAGATCTGAG	TGACTTCCTTCCATTCTGAAGAC
OLFM4	ACCTTTCCCGTGGACAGAGT	TGGACATATTCCCTCACTTTGGA
VIL1	CCAAAGGCCTGAGTGAAATC	CCTGGAGCAGCTAGTGAACA
ZO-1	GGGAACAACATACAGTGACGC	CCCCACTCTGAAAATGAGGA

Note: All primer sequences were obtained from http://primerdepot.nci.nih.gov. All annealing temperatures are near 60°C.

Culture of Intestinal Epithelium and Intestinal Mesenchyme

Human fetal intestinal enteroids were generated by isolating the crypts from human fetal intestinal tissue and expanding them in Matrigel droplets as previously described (Tsai et al., 2018). Intestinal mesenchyme was isolated from human fetal duodenum that was separated from the epithelium and expanded in culture. For co-culture experiments, small enteroids were mechanically removed from Matrigel 3 days after passaging. Mesenchyme was passaged into single cells and counted using a hemocytometer. Enteroids were suspended in 10 µL droplets of LWRN medium on the bottom of a tissue culture dish with 1 enteroid per droplet. 2,000 mesenchymal cells were added to each droplet for a total droplet volume of no more than 20 µL. After placing the lid onto the plate, the plate was flipped upside down to establish hanging drop co-cultures. The plate containing hanging drops was floated in a larger tissue culture plate containing sterile PBS and left in a tissue culture incubator overnight for 16 hours to allow for aggregation of epithelium with mesenchyme. The plate containing the hanging drops was then quickly flipped to an upright

position. Co-culture aggregates were carefully removed by pipetting and suspended in 1% alginate and Matrigel solutions. Cultures were maintained for up to 10 days. LWRN medium was changed approximately every 3 days.

Antibody Information

Primary Antibody	Source	Catalog #	Dilution
Goat anti-E-cadherin	R&D Systems	af748	1:500
Mouse anti-E-cadherin	BD Transduction Laboratories	610181	1:500
Goat anti-Vimentin	R&D Systems	mab2105	1:500
Mouse anti-CDX2	BioGenex	MU392A-UC	1:300
Rabbit anti-PDX1	Epitomics, Inc	3470-1	1:300
Rabbit anti-ZO-1	Cell Signaling	13663	1:300
Rabbit anti-MUC2	Santa Cruz Biotechnology	sc-15334	1:300
Goat-anti CHGA	Santa Cruz Biotechnology	sc-1488	1:300
Goat-anti SOX9	R&D Systems	af3075	1:300
rabbit-anti KI67	Thermo Scientific	RM-9106-S1	1:300
Goat-anti DPP4	R&D Systems	af954	1:300
Goat-anti LYZ	Santa Cruz Biotechnology	sc-27958	1:300
Rabbit-anti DEFA5	Abcam	ab180515	1:300
Rabbit-anti OLFM4	Abcam	ab85046	1:300
Rabbit-anti LGR5	Abcam	ab75850	1:300
Goat-anti Villin	Santa Cruz	sc-7672	1:300

Supplemental References

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