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☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

   Describe how sample size was determined.

   No statistical method was used to predetermine sample size. For in vitro experiments, sample size was established as at least 4 hydrogels per condition (20-30 spheroids/hydrogel or 2-4 HIOs or HLOs/hydrogel) with the premise that an outcome present in all hydrogels under a specific condition will reveal the population behavior submitted to this given condition. For in vivo experiments, sample size was established as 3 with the premise that an outcome present in 3 different animals under a specific condition will reveal the population behavior submitted to this given condition.

2. Data exclusions

   Describe any data exclusions.

   The exclusion criteria was to exclude data from mice euthanized because of sickness. No animal was excluded from studies.

3. Replication

   Describe whether the experimental findings were reliably reproduced.

   All attempts at replication were successful.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   For all experiments, one tube containing human intestinal spheroids, HIOs or HLOs was vortexed prior to mixing with different hydrogel precursor solutions, ensuring a random allocation of the biological tissue into the experimental groups. No randomization was used during inhibitor treatment or for animal experiments.

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Blinding was performed during outcome assessment. The researchers that performed the in vivo experiments were not the researchers that processed and analyzed the tissue resulting from these experiments.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Image acquisition of immunofluorescence experiments was performed using LAS X (Leica Microsystems, Inc.)
Quantification of spheroid/HIO viability and size parameters were performed using ImageJ 1.50b (National Institute of Health, USA).
RT-qPCR data analysis was performed using Microsoft Excel 2016 (Microsoft Corp.).
All statistical analyses were performed using GraphPad Prism 6.0.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The following antibodies were used according to the manufacturer's instructions. Primary antibody incubation was performed overnight at a 1:100 dilution, unless stated otherwise. Secondary antibody incubation was performed for 1 h at a 1:2000 dilution. Primary antibodies were validated for immunofluorescence labeling of frozen sections containing human or mouse tissue as specified in the manufacturer's website. Relevant citations can also be found in the manufacturer's website.
Primary Antibodies:
ZO-1 (33-9100, ThermoFisher Scientific)
β-CATENIN (610153, BD biosciences)
EZRIN (ab41672, Abcam) F(ab')2-Goat anti rabbit AlexaFluor 555 (A-21430, ThermoFisher Scientific)
KI67 (ab15580, Abcam)
CDX2 (MU392A-UC, Biogenex)
CHGA (sc-1488, Santa Cruz Biotechnology)
MUC2 (ab11197, Abcam)
DCAMKL-1 (DCLK1; ab31704, Abcam)
PDX1 (3470-1, Epitomics) Used at a 1:250 dilution
NUMA (MAB1281, EMD Millipore)
Human mitochondrial antigen, HUMIT (MAB1273, Millipore) Used at a 1:50 dilution
phalloidin-AlexaFluor 555 (A34055, ThermoFisher Scientific)
NKX2.1 (ab76013, Abcam)
P63 (sc-8344, Santa Cruz Biotechnology) Used at a 1:250 dilution.
E-cadherin, ECAD (610181, BD Biosciences) Used at a 1:500 dilution

Secondary Antibodies:
F(ab')2-Goat anti mouse AlexaFluor 488 (A-11017, ThermoFisher Scientific)
Goat anti rabbit AlexaFluor 488 (R37116, ThermoFisher Scientific)
Donkey anti rabbit Cy3 (711-165-152, Jackson Immuno)
Donkey anti-mouse AlexaFluor 488 (715-545-150, Jackson Immuno)

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Mycoplasma-free human ES cells (H9, NIH registry #0062).
iPS cells (line 20.1) were generated from normal human skin keratinocytes which were obtained from donors with informed consent (Cincinnati Children's Hospital Medical Center (CCHMC) Institutional Review Board protocol CR1_2008-0899) as described in Spence, 2011.
H2BmCherry-expressing human ES cell line was generated by infecting hESCs with a lentivirus containing PGK-H2BmCherry, which was a gift from Mark Mercola (Addgene plasmid # 21217).
HLOs were generated from human ES cells (UM63-1, NIH registry #0277).

b. Describe the method of cell line authentication used.

Stem cell lines are routinely monitored for chromosomal karyotype, at which time the number of chromosomes are confirmed, chromosomal abnormalities are ruled out and sex chromosomes of each line are confirmed. Functional and molecular authentication for pluripotency is assessed using a panel of antibody and qRT-PCR markers, and for the ability to undergo multi-lineage differentiation.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Male (8 weeks old) NOD-scid IL2Rg-null (NSG) mice (Jackson Laboratory) were used for all our experiments.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	The study did not involve human research participants.