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Supplemental information

Suspension culture in a rotating bioreactor for efficient generation of human intestinal organoids

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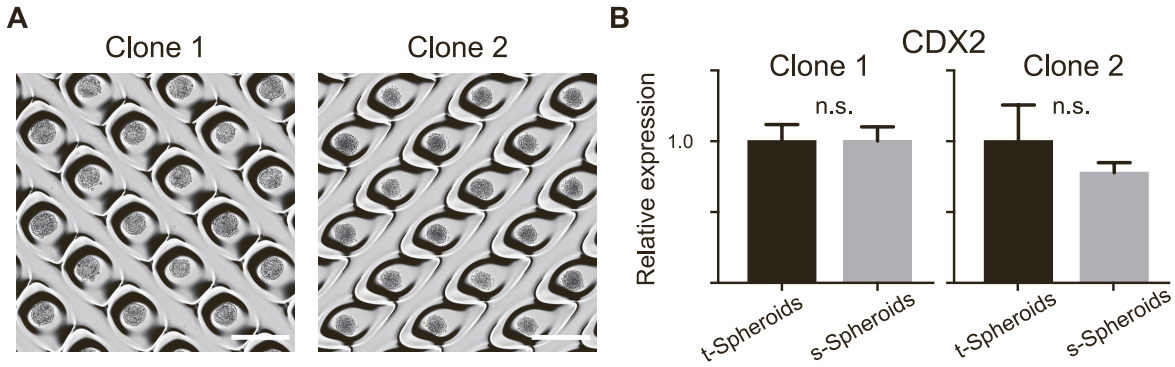

Figure S1 Generation of homogeneous suspension spheroids (s-Spheroids) from multiple iPSC lines. Related to Figure 1.

(A) Bright-field images of s-Spheroids differentiated from multiple iPSC lines using EZSPHERE 6-well plates. Scale bar, 500 μm. **(B)** *CDX2* expression in formed t-Spheroids and s-Spheroids induced from two independent iPSC clones was analyzed by qRT-PCR. Data are means ± s.d. for triplicate independent experiments. Unpaired, two-tailed *t*-test.

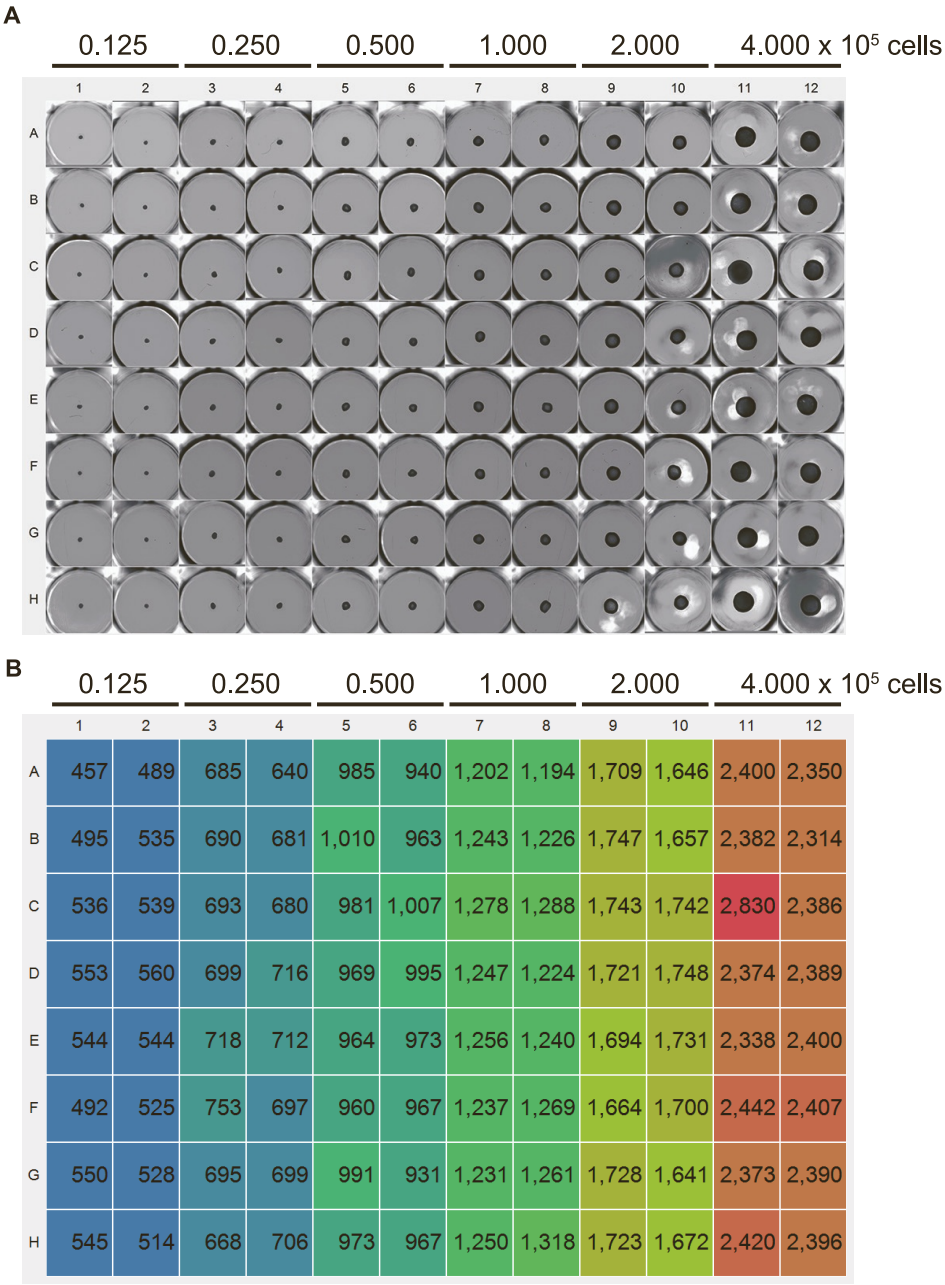

Figure S2 Effect of starting cell number on the size of suspension spheroids generated by seeding cells onto spheroid forming plate. Related to Figure 2.

A range of dissociated cells were seeded onto a 96-well spheroid forming plate (EZ-BindShut® SP 96-well plate) to create suspension spheroids. **(A)** Bright-field images of formed suspension hindgut spheroids on spheroid forming plate starting from 0.125 x 10^5 to 4 x 10^5 cells. Each cell number condition was performed in 16 independent wells. The diameter of spheroids after 24 hrs induction was analyzed by the bright-field imaging scanner, Cell^3 iMager duos. **(B)** Heat map of spheroid diameters (μm) in each condition was visualized with Cell^3 iMager duos Software Ver. 1.6 (SCREEN Holdings Co., Ltd., Kyoto, Japan).

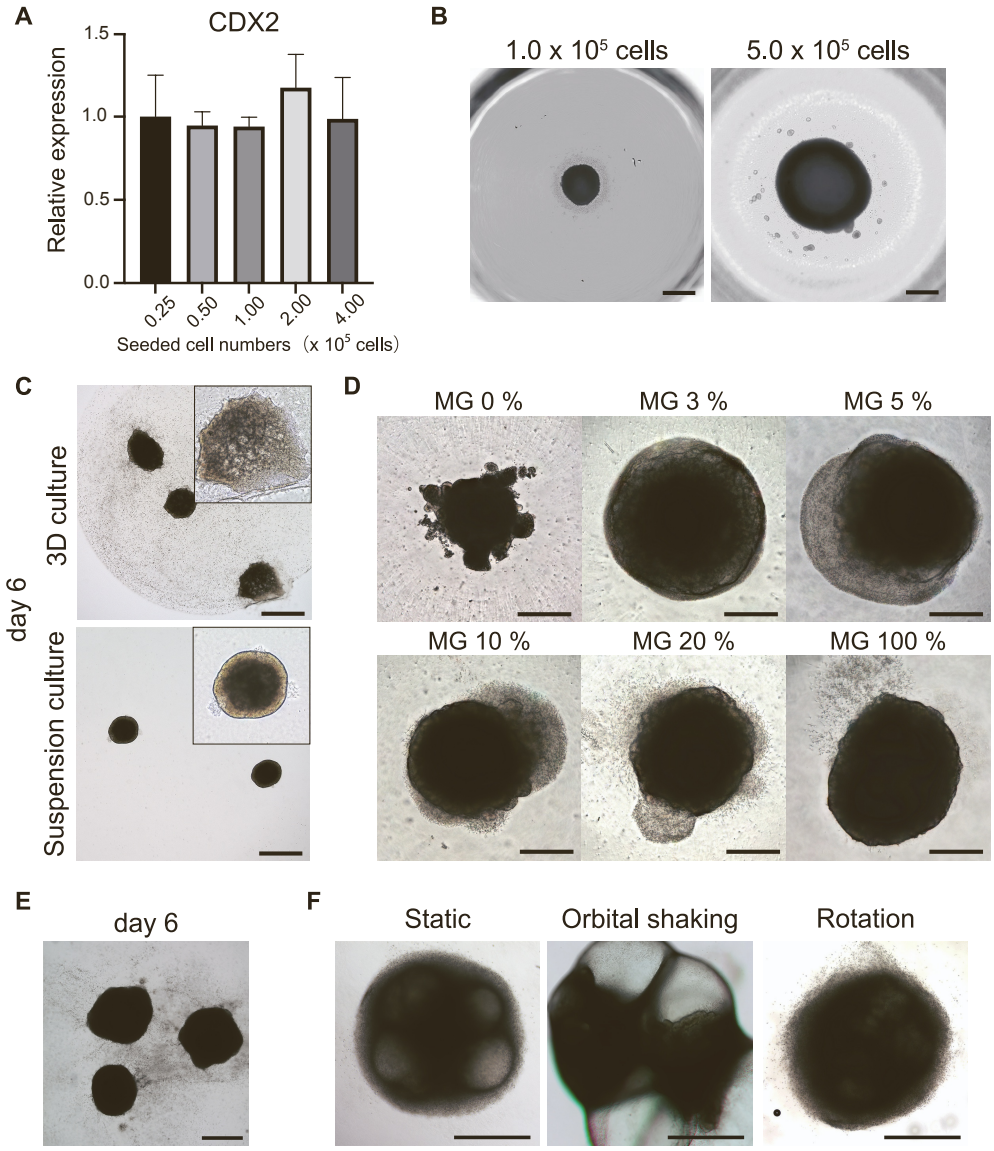

Figure S3 Optimized conditions for suspension spheroid expansion. Related to Figures 2 and 3.
(A) Expression of *CDX2* in s-Spheroids generated from different cell numbers was analyzed by qRT-PCR. Data are means ± s.d. for quadruplicate independent experiments. Unpaired, two-tailed *t*-test. **(B)** s-Spheroids generated from 1 x 10^5 cells are spherical, whereas s-Spheroids generated from 5 x 10^5 cells are biconcave-disk-like as the lighter colored center illustrate thinner structure compared to the rim. Scale bar, 1000 µm. **(C)** Bright-field images of s-Spheroids cultivated in the traditional three-dimensional culture (upper panel) and the suspension culture (lower panel). s-Spheroids easily attached to the bottom of the plate in three-dimensional culture. Scale bar, 1000 µm. **(D)** Single spheroids were formed on the EZ-BindShut and cultured in suspension with the spheroid culture medium adding various concentrations of Matrigel (0 %, 3 %, 5 %, 10 %, 20 %, and 100 %) for 14 days. Light microscopy images of spheroids showed at least 3 % of Matrigel was needed to maintain suspension spheroids. Budding feature was observed in spheroids cultured in 10 % - 20 % Matrigel conditions. Scale bar, 500 µm. **(E)** A bright-field image of dead large spheroids generated from 1 x 10^6 cells in a static suspension culture. Scale bar, 1000 µm. **(F)** Bright-field images of s-Spheroids cultured in suspension in a static (left), an orbital shaking (middle), and rotational bioreactor (right) condition. s-Spheroids cultured in an orbital shaker gather in the center and fuse. Scale bar, 1000 µm.

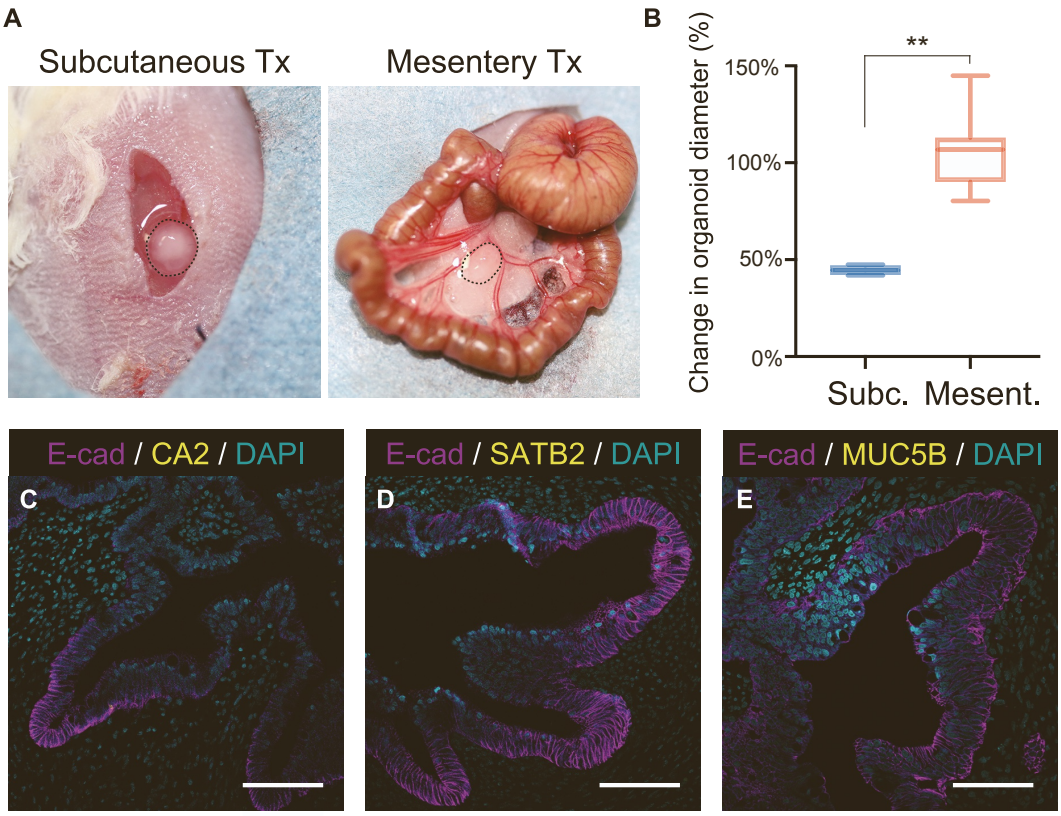

Figure S4 Engrafted various-size s-HIOs show *in vivo* growth into small intestinal tissue. Related to Figure 4.

Cultured s-HIOs matured from middle-sized s-spheroids were transplanted into the mesentery and subcutaneous lesion of NSG mice. **(A)** after 4 weeks of *in vivo* growth, the s-HIOs have engrafted into the mesentery (Mesentery Tx) and subcutaneous lesion (Subcutaneous Tx). The grafts are outlined in black. **(B)** s-HIOs engrafted into mesentery (Mesent.) grew larger than those engrafted into the subcutaneous region (Subc.) at the time of harvest (change in size = 44.58 % ± 2.514 vs 106.3 % ± 20.77, $P < .0001$). **(C)-(E)** Immunostaining of engrafted intestinal HIOs revealing lack of colonic markers; carbonic anhydrase II (CA2), Special AT-rich sequence-binding protein 2 (SATB2), colonic type goblet cell marker MUC5B. Scale bar, 100 μm.

Table S1 List of growth factors. Related to Figure 2.

Factor	Final concentration	Epithelial growth	Mesenchymal growth
Wnt3a	200 ng/ml	↑	↓
CHIR99021	3 μM	↑	↓
FGF-2	50 ng/ml	↑	↑
FGF-4	50 ng/ml	↑	↑
IGF-1	100 ng/ml	→	→
SB202190	10 μM	→	→
A83-01	500 nM	↑	→
Nicotinamide	10 mM	→	→
Forskolin	20 μM	↑	↓