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Improved differentiation of hESC-derived pancreatic progenitors by using human fetal pancreatic mesenchymal cells in a micro-scalable three-dimensional co-culture system

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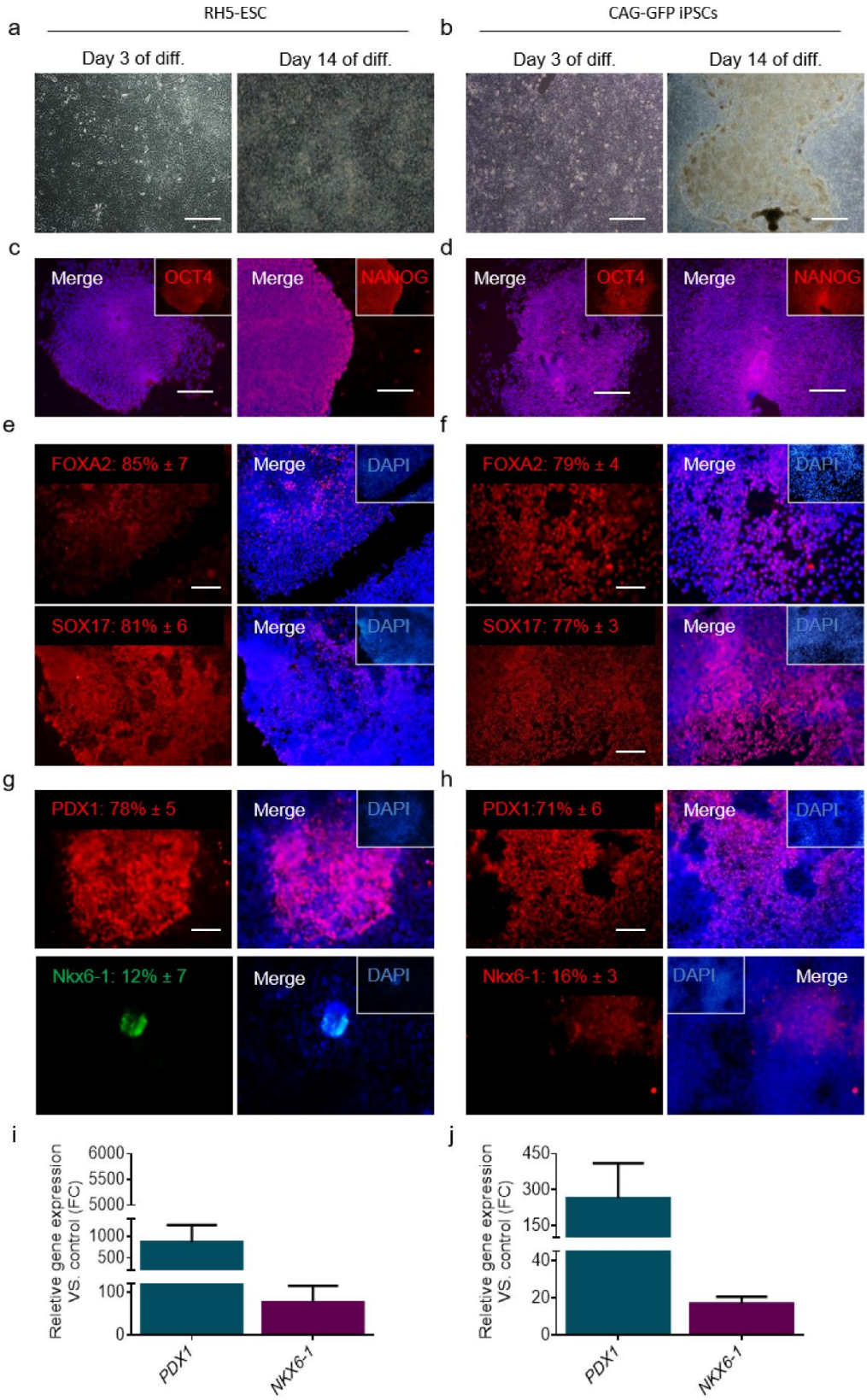
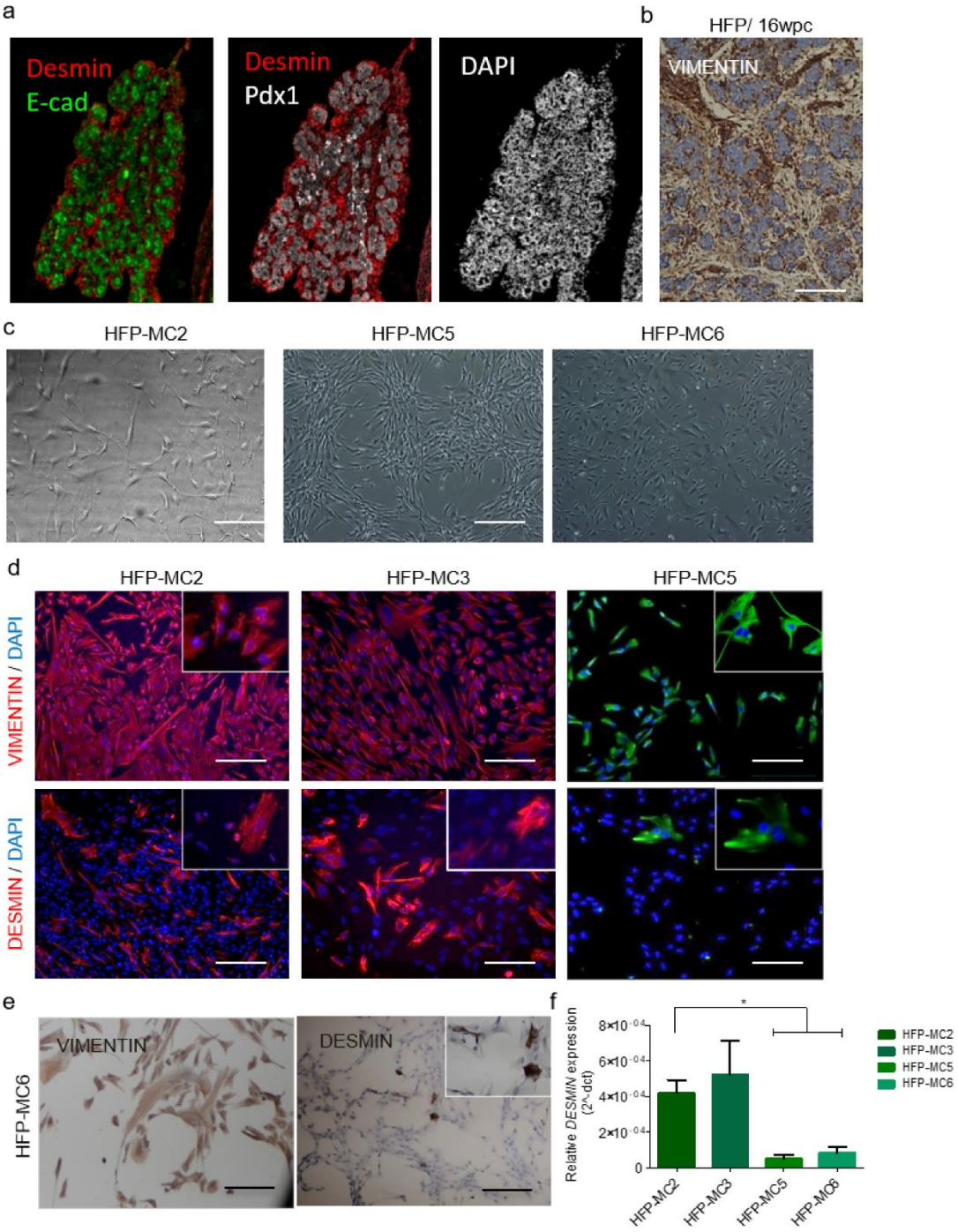

a
RH5-ESC
Day 3 of diff.
Day 14 of diff.
b
CAG-GFP iPSCs
Day 3 of diff.
Day 14 of diff.
c
Merge
OCT4
Merge
NANOG
d
Merge
OCT4
Merge
NANOG
e
FOXA2: 85% ± 7
Merge
DAPI
SOX17: 81% ± 6
Merge
DAPI
f
FOXA2: 79% ± 4
Merge
DAPI
SOX17: 77% ± 3
Merge
DAPI
g
PDX1: 78% ± 5
Merge
DAPI
Nkx6-1: 12% ± 7
Merge
DAPI
h
PDX1:71% ± 6
Merge
DAPI
Nkx6-1: 16% ± 3
DAPI
Merge
i
Reletive gene expression
VS. control (FC)
6000
5500
5000
1000
500
100
0
PDX1
NKX6-1
j
Reletive gene expression
VS. control (FC)
450
300
150
40
20
0
PDX1
NKX6-1

2

3

Supplementary Figure 1 (*related to figure 1*) Reproducibility of the pancreatic progenitors (PPs) differentiation protocol using different hPSC lines

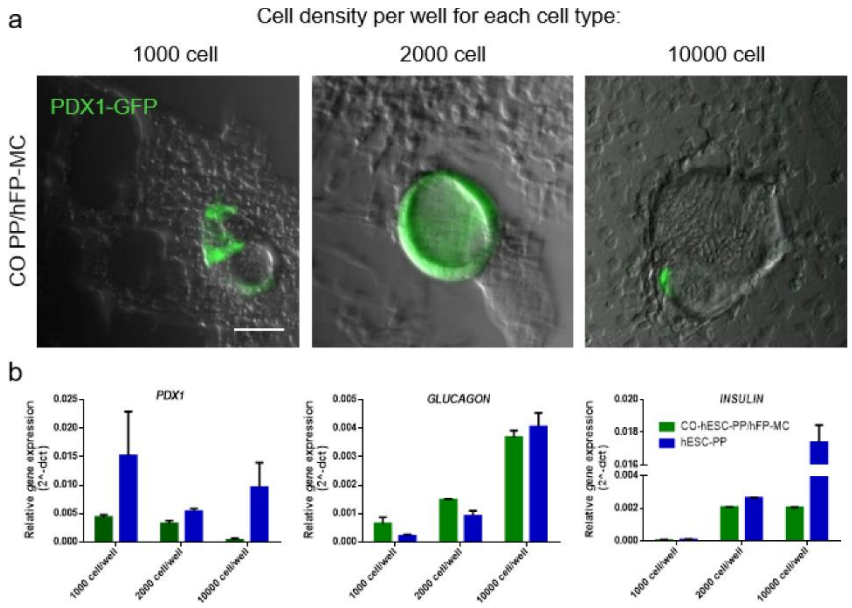
a) Bright-field images of days 3 and 14 of PP differentiation on RH5-embryonic stem cells (RH5-ESC). Scale bar: 100 µm. diff, differentiation. b) Bright-field images of days 3 and 14 of PP differentiation on CAG-human induced pluripotent stem cells (CAG-hiPSCs). Scale bars: 200 and 500 µm. c) Representative immunostaining of OCT4 and NANOG pluripotency markers of RH5-ESCs colony on Matrigel before starting differentiation. Scale bars: 500 and 200 µm, respectively. d) Representative immunostaining of OCT4 and NANOG markers of CAG-hiPSCs colony. Scale bars: 500 and 200 µm, respectively. e) Immunostaining was done for FOXA2 and SOX17 endodermal markers on day 3 of RH5-ESCs differentiation. Scale bar: 100 µm. f) Immunostaining was done on day 3 for FOXA2 and SOX17 markers during PP differentiation on CAG-hiPSCs. Scale bars: 50 µm (Upper panel) and 100 µm (Lower panel). g) Immunostaining was done on day 14 for PDX1 and NKX6-1 PP markers during PP differentiation on RH5-ESC. Scale bar: 100 µm. h) Immunostaining was done on day 14 for PDX1 and NKX6-1 markers during PP differentiation on CAG-hiPSCs. Scale bar: 100 µm. i) Quantitative RT-PCR (qRT-PCR) analysis of RH5-ESC line for *PDX1* and *NKX6-1* genes. j) qRT-PCR analysis of CAG-hiPSC line for *PDX1* and *NKX6-1* genes on day 14 of differentiation. Gene expressions were normalized against those of undifferentiated PSCs; n=4 biological samples, 2 technical repeats each. All error bars represent SEM. Nuclei are stained blue with DAPI. Bright-field and immunostaining pictures showed are representative of at least 3 independent experiments (n=3).



Supplementary Figure 2 (*related to figure 2*) Mesenchymal characteristics observed in mesenchymal cells derived from the different human fetal pancreas

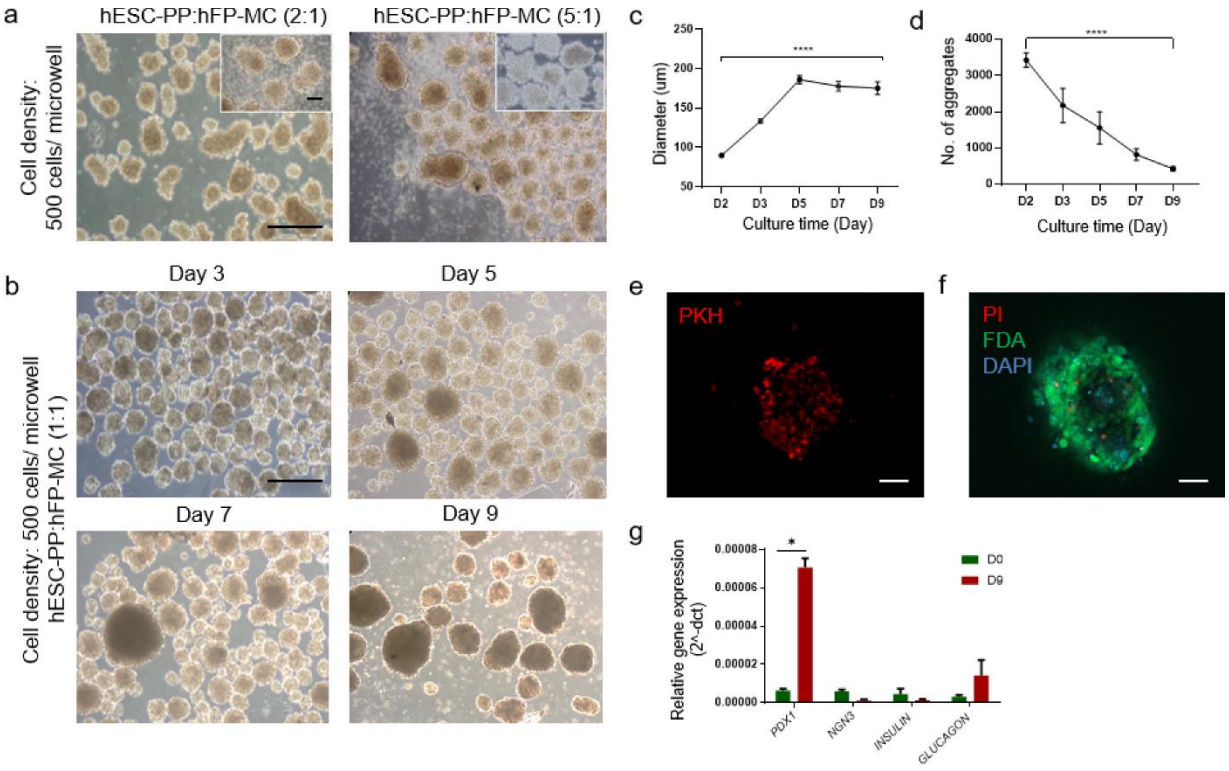
a) Immune staining on E14.5 mouse pancreas samples for Desmin, E-cadherin, and Pdx1 indicates that pancreatic mesenchymal cells express Desmin while pancreatic epithelium cells do not. b) Immunohistochemistry done on the human fetal pancreas of 16 weeks post-conception (WPC) demonstrates VIMENTIN-positive MCs surrounding

epithelium cells. Scale bar: 100 µm. c) Bright-field images of hFP-MC2, hFP-MC5, and hFP-MC6 in culture demonstrating the mesenchymal morphology. Scale bar: 500 µm. d and e) Immunostaining of VIMENTIN and DESMIN markers in hFP-MC2 (passage 1), hFP-MC3 (passage 2), hFP-MC5 (passage 4), and hFP-MC6 (passage 5) derived from different human fetuses. Although it seems that all cells express VIMENTIN, the expression of DESMIN differs among different fetal MCs. Scale bar: 200 µm. f) Relative gene expression (2^-Δct) of *DESMIN* gene in hFP-MCs from different sources compared to BM-MSC. n= 3 biological replicates. All bars represent a mean of 3 different passages (P2-8). All error bars represent SEM.



Supplementary Figure 3 (*related to figure 3*) Different cell densities of hESC-PP and hFP-MC towards the spheroid formation

a) Three different initial cell densities seeded in each well for CO PP/hFP-MC (1000, 2000, and 10000 cells for each cell type per well). The pancreatic progenitors (PPs) were differentiated from the huES4 PDX1-GFP cell line. GFP-PDX1 PPs are indicated in green. b) Relative gene expression (2^-Δct) of early PP gene, *PDX1*, and mature endocrine genes, *GLUCAGON* and *INSULIN* in hFP-MC and hESC-PP in three combinations of different initial cells densities. n= 2 biological replicates, each 2 technical repeats. All error bars represent SEM. CO PP/hFP-MC, hESC-PP co-cultured with human fetal pancreatic mesenchymal cell; and hESC-PP, human embryonic stem cell-derived pancreatic progenitor cultured solely in 3D system.



Supplementary Fig. 4 (*related to figure* 5) The effects of different cell ratios and the culture period in aggregates formation

a) Bright-field images of DC-PAs in suspension culture demonstrating the impact of different ratios of human embryonic stem cell-derived pancreatic progenitors (hESC-PPs) and human fetal pancreatic-derived mesenchymal cells (hFP-MCs) (2:1 and 5:1) for aggregate formation in 4 days' culture. Scale bar: 200 µm. White boxes indicate higher magnification. Scale bar: 100µm. b) Overview of DC-PAs morphology within 9 days of suspension culture at the cell density of 500 cells/microwell and cell ratio of 1:1. Scale bar: 200 µm. c) Quantification of DC-PAs mean diameter in 9 days of culture. An average number of 250 aggregates was measured in each group. Asterisks represent significant differences between groups (P <0.0001). d) Quantification of the average number of DC-PAs within 9 days of culture. N=4 biological replicates. Asterisks represent significant differences between groups (P <0.0001). e) PKH26-labeled hFP-MCs in DC-PAs after 9 days in culture. Scale bar: 50 µm. f) Confocal image of live/dead staining for DC-PAs on day 9 in culture. Scale bar: 50 µm. g) Relative expression (2^-Δct) of pancreatic progenitor (PP), endocrine progenitor, and endocrine genes in DC-PAs. N=2 biological replicates. All error bars represent SEM.

Supplementary Table 1. Primary and secondary antibodies that are used for immunostaining.

Antigen	Host	Company	Cat no.	Dilution
OCT4	Mouse	Santa Cruz	SC5279	1:200
NANOG	Mouse	Sigma-Aldrich	N3038	1:500
SOX17	Goat	R&D system	AF1924	1:200
FOXA2	Goat	Santa Cruz	SC6554	1:200
SOX9	Rabbit	Sigma-Aldrich	AB5535	1:500
PDX1	Goat	Abcam	ab47383	1:1000
NKX6.1	Mouse	DSHB	F55A10	1:1000
VIM	Mouse	Abcam	ab128507	1:200
DES	Mouse	Santa Cruz	SC-271677	1:100
INSULIN	Rabbit	Abcam	Ab115199	1:200
E-CADHERIN	Rat	Sigma-Aldrich	U3254	1:200
Anti-goat IgG-Alexa Fluor® 568	Donkey	Invitrogen	A11057	1:500
Anti-mouse IgG-Alexa Fluor® 568	Goat	Invitrogen	A21043	1:500
Anti-mouse IgG-Alexa Fluor® 488	Goat	Invitrogen	A21042	1:500
Anti-rat IgG-Alexa Fluor® 546		Invitrogen		1:500
Anti-rabbit IgG-Alexa Fluor® 488	Donkey	Invitrogen	A21206	1:500
Anti-mouse IgG-peroxidase		Invitrogen		1:500

Supplementary Table 2. Primers used in this study.

Gene	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	CTCATTTCCTGGTATGACAACGA	CTTCCTCTTGTGCTCTTGCT
Ki67	GTGCTCAACAACTTCATTTCCA	ACTGAAGAACACATTTCCTCCA
DESMIN	ATGTCTAAGCCAGACCTCAC	GTCGGTATTCCATCATCTCCT
VIMENTIN	GACGCCATCAACACCGAGTT	CTTTGTCGTTGGTTAGCTGGT
SOX17	CGGTATATTACTGCAACTATCCTG	GGATTTCCTTAGCTCCTCCA
PDX1	CCTTTCCCATGGATGAAGTC	GGAACTCCTTCTCCAGCTCT
NKX6.1	CTGGCCTGTACCCCTCATCA	CTTCCCGTCTTTGTCCAACAA
NGN3	GCTCATCGCTCTCTATTCTTTTGC	GGTTGAGGCGTCATCCTTTCT
GLUCAGON	AAGCATTTACTTTGTGGCTGGATT	TGATCTGGATTTCTCCTCTGTGTCT
INSULIN	AAGAGGCCATCAAGCAGATCA	CAGGAGGCGCATCCACA