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Supplemental Information

Long-Term Culture of Self-renewing Pancreatic Progenitors Derived from Human Pluripotent Stem Cells

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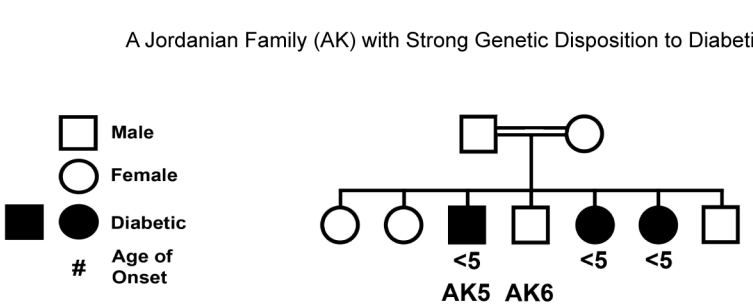
A


A Jordanian Family (AK) with Strong Genetic Disposition to Diabetic Beta Cell Failure
Male
Female
Diabetic
Age of Onset
<5
<5
<5
AK5
AK6

B


AK5 Clone 11 (AK5-11)
AK6 Clone 13 (AK6-13)
OCT4+
92.5
OCT4+
96.6
Count
OCT4

C

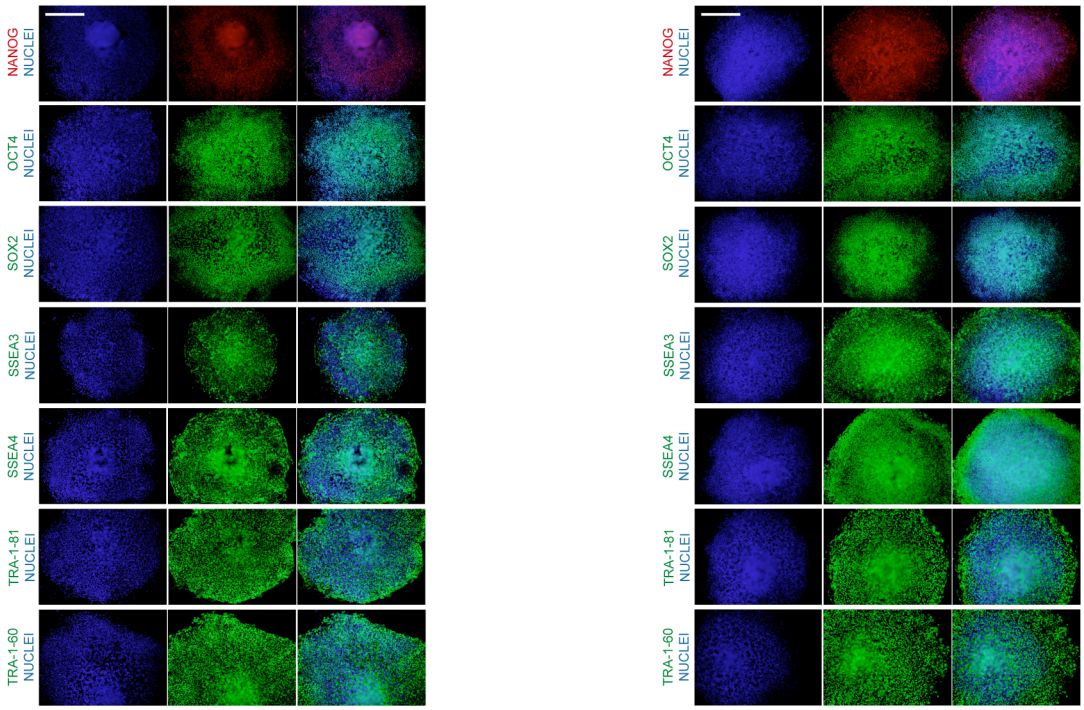

NANOG NUCLEI
OCT4 NUCLEI
SOX2 NUCLEI
SSEA3 NUCLEI
SSEA4 NUCLEI
TRA-1-81 NUCLEI
TRA-1-60 NUCLEI

Figure S1: Generation of hiPSC lines from diabetic and healthy sibling fibroblasts.

A) Pedigree of a consanguineous Jordanian family with several diabetic siblings. All diabetic siblings developed the disease before 5 years of age. Skin biopsies taken from individuals AK5 and AK6 were used to generate fibroblasts from which hiPSC were derived.

B) Intracellular flow cytometric analysis of OCT4 expression and C) Immunostaining for established markers of pluripotency for hiPSC clones AK5-11, AK6-13 and AK6-8 (not shown). Scale bar, 100 μm.

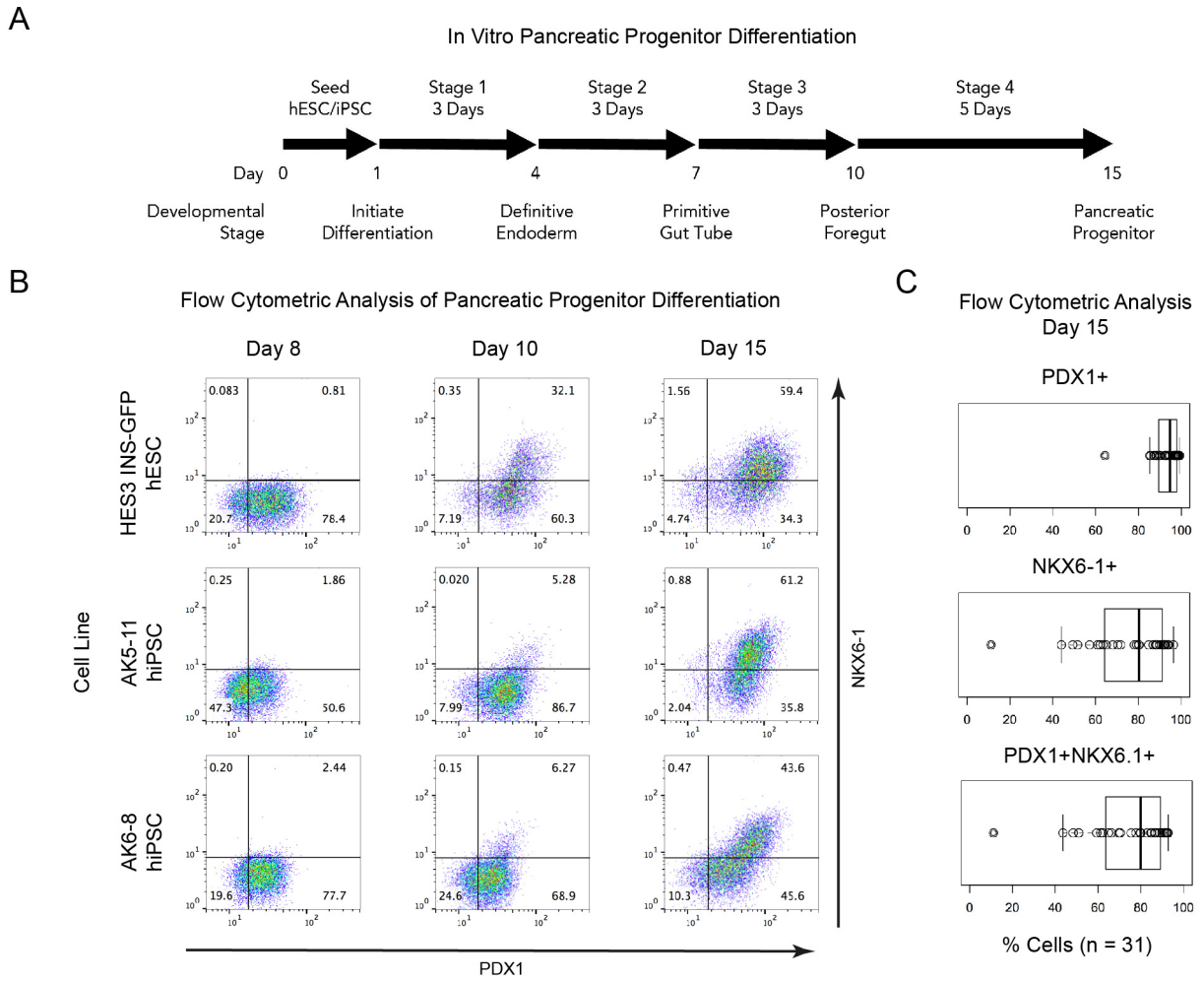

A
In Vitro Pancreatic Progenitor Differentiation
Seed hESC/iPSC
Stage 1 3 Days
Stage 2 3 Days
Stage 3 3 Days
Stage 4 5 Days
Day 0 1 4 7 10 15
Developmental Stage
Initiate Differentiation
Definitive Endoderm
Primitive Gut Tube
Posterior Foregut
Pancreatic Progenitor
B
Flow Cytometric Analysis of Pancreatic Progenitor Differentiation
Day 8
Day 10
Day 15
HES3 INS-GFP hESC
AK5-11 hiPSC
AK6-8 hiPSC
Cell Line
NKX6-1
PDX1
C
Flow Cytometric Analysis Day 15
PDX1+
NKX6-1+
PDX1+NKX6.1+
% Cells (n = 31)
D
Established cPP Cell Lines Generated from Genetically Diverse iPSC and hESC (passage 6)
PDX1
FOXA2
GATA6
SOX9
HNF4A
RFX6
Log2 Normalised Expression
H9 #1
H9 #2
HES3
AK5-11 #1
AK5-11 #2
AK6-8
AK6-13

Figure S2: Directed differentiation of pancreatic progenitor cells and generation of cPP cells from diverse human pluripotent stem cell lines

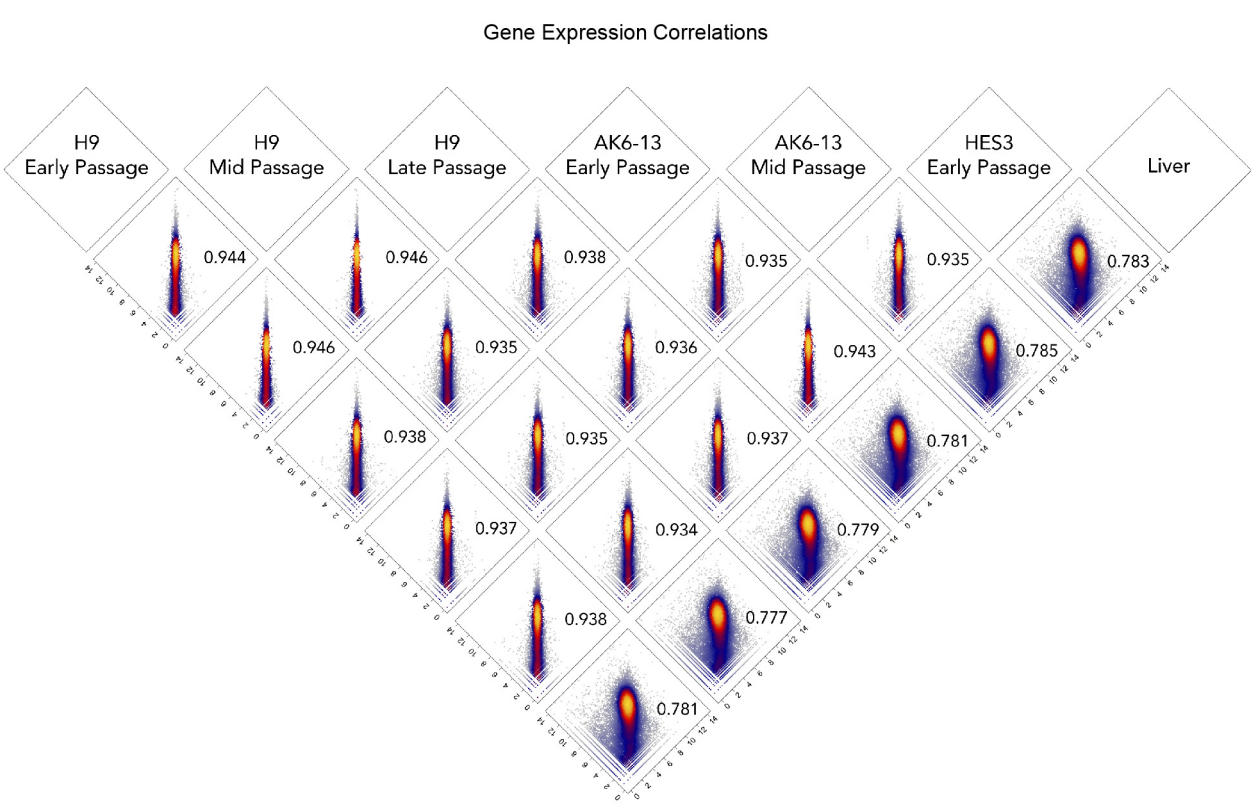
A) Time-course of pancreatic progenitor differentiation protocol. In our experiments, stage 1 was extended to last 3 days, rather than 2 as per the manufacturer's instructions, by repeating the final day's treatment.

B) Intracellular flow cytometric analysis of PDX1 and NKX6-1 at days 8, 10 and 15 of differentiation using hES3 INS-GFP reporter hESC (Micallef et al., 2011; Titmarsh et al., 2016) and the in-house hiPSC lines AK5-11 and AK6-8. PDX1 is detected before NKX6-1 in all cases, although individual lines exhibit variable differentiation kinetics. Gates are based on cells stained with isotype control antibodies.

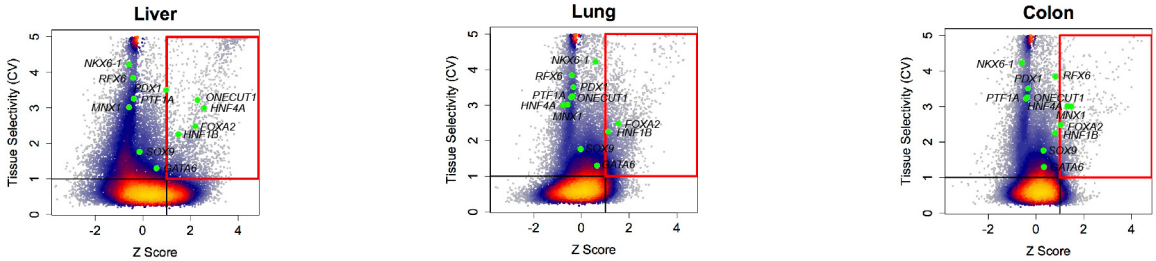
C) Percentage PDX1+ and/or NKX6-1+ at day 15 of differentiation. Each circle represents one of 31 independent experiments encompassing 2 hESC lines and 6 hiPSC lines. The vertical black bar shows the median percentage of cells that are PDX1+ (95%), NKX6-1+ (80%) or PDX1+NKX6-1+ (80%).

D) Gene expression measured by qRT-PCR using samples harvested from cPP cell lines at passage 6. We analyzed cPP cells derived from the following pluripotent cell lines: H9 and HES3 hESC, and AK5-11, AK6-8 and AK6-13 hiPSC. Two independent pedigrees were derived from H9 and AK5-11 cell lines. Expression levels are shown normalized to those of H9 hESC and are plotted on a $\log_2$ scale. Error bars represent the standard error of three technical replicates.

A


Gene Expression Correlations
H9 Early Passage
H9 Mid Passage
H9 Late Passage
AK6-13 Early Passage
AK6-13 Mid Passage
HES3 Early Passage
Liver
0.944
0.946
0.938
0.935
0.935
0.783
0.946
0.935
0.936
0.943
0.785
0.938
0.935
0.937
0.781
0.937
0.934
0.779
0.938
0.777
0.781

B


Specifically Expressed Genes
Liver
Lung
Colon
Tissue Selectivity (CV)
Z Score
NKX6-1
RFX6
PDX1
PTF1A
HNF4A
MNX1
ONECUT1
FOXA2
HNF1B
SOX9
GATA6

C

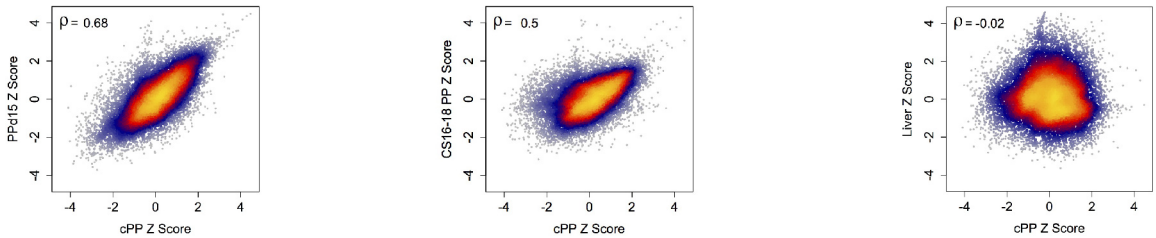

Z-Scores Correlations
ρ = 0.68
PPd15 Z Score
cPP Z Score
ρ = 0.5
CS16-18 PP Z Score
cPP Z Score
ρ = -0.02
Liver Z Score
cPP Z Score

Figure S3: Transcriptome analysis of cPP cells by RNA-seq.

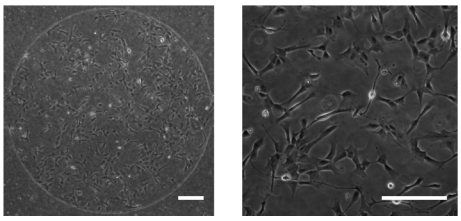
A) Correlations between gene expression levels for cPP cells from three different genetic backgrounds (H9, AK6-13 and HES3) at early (6-8), mid (11-13) and late (18) passages. Log_2-transformed gene counts measured by RNA-seq were plotted for each gene. Gene counts in cPP samples are compared to liver for comparison. The Spearman correlation coefficient for each pair of samples is shown on the corresponding plot. Heat colors denote the number of transcripts. Gene counts are strongly correlated between cPP samples regardless of genetic background or passage number, but not with liver.

B) Identification of specifically expressed genes in liver, lung and colon samples. Genes associated with early pancreatic development are not typically found to be specifically expressed by these tissues.

C) Z-score correlations for cPP, PPd15, CS16-18 PP and liver samples. Z-scores are strongly correlated between in vitro and in vivo pancreatic progenitor samples but not between these samples and liver.

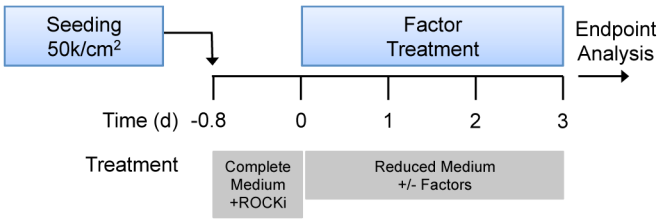
A

cPP Cells Plated in MBA Chamber



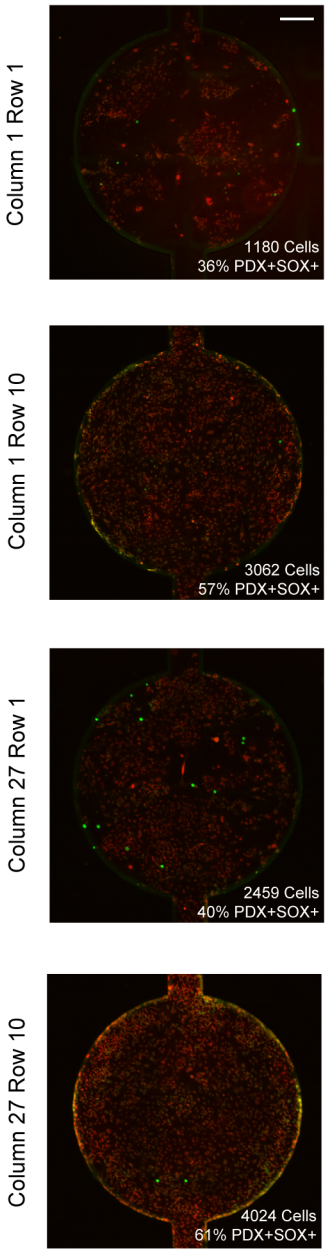
B

MBA Screening Protocol



C

PDX1 SOX9



D

Processed Data Metrics for MBA Screen Extracted from Fluorescence Image

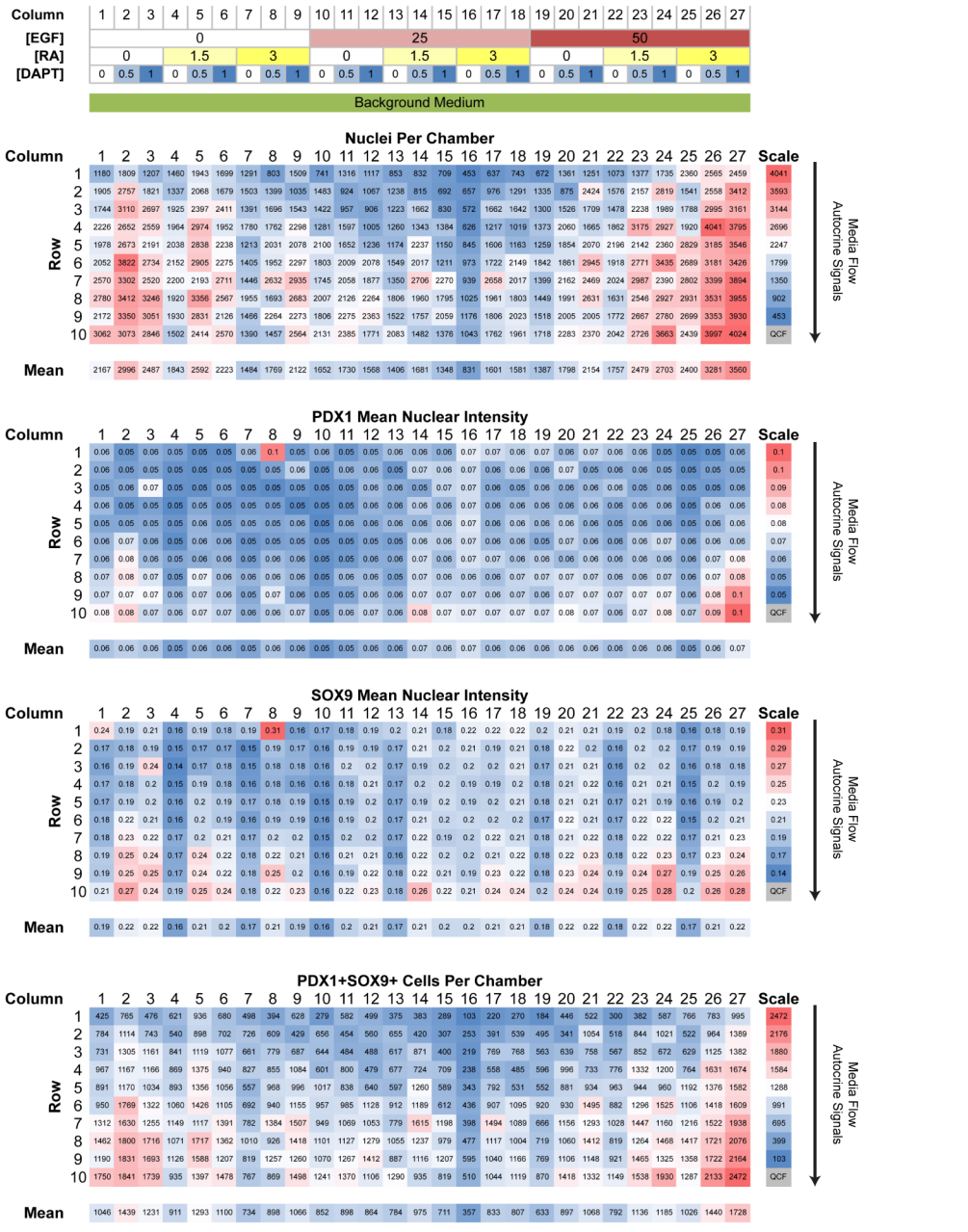


Figure S4: Microbioreactor Array (MBA) Screening of Factors Required to Propagate cPP Cells

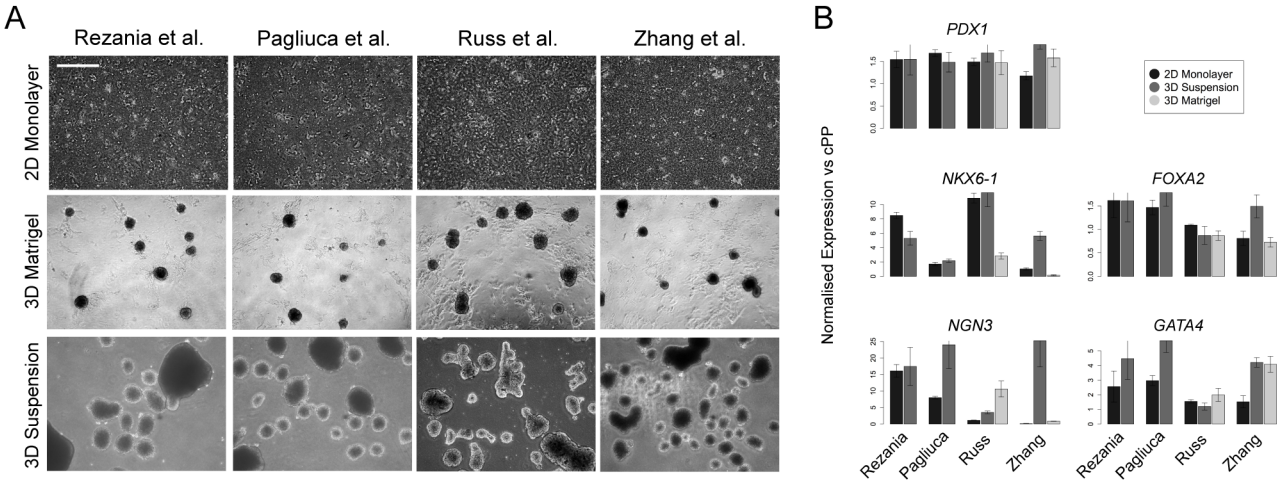
A) Phase contrast images of $PDX1^{+}SOX9^{+}$ cPP cells seeded into Matrigel-coated MBAs and allowed to attach for 20 h with periodic feeding. Each MBA device has 270 chambers arranged as shown in S4D. Scale bar, 100 μm.

B) Protocol used for MBA screening.

C) Individual chambers of MBA device (270 culture chambers) stained with anti-PDX1 (green) and anti-SOX9 (red) antibodies. Hoechst 33342 (not shown) was used for nuclei identification. The chambers were selected to show the range of proliferation rates and protein expression observed across different signaling environments. Scale bar, 100 μm.

D) Endpoint measurements for each chamber in the MBA. Schematic above shows compositions of media applied to each column of the MBA (EGF, ng/mL; RA, μM; DAPT, μM). Cell culture media flow was from top (Row 1) to bottom (Row 10) down a column, thereby concentrating autocrine factors towards the bottom of the column. Mean measurements for each column are given below. QCF: data flagged for quality control issue during image processing. Values were extracted from images such as those in S4C using an image segmentation algorithm as described previously (Titmarsh et al., 2016).

Comparison of Published Beta Cell Differentiation Protocols - NKX6-1 Induction Step



Optimization of NKX6-1 Induction Step of Russ et al. Beta Cell Differentiation Protocol

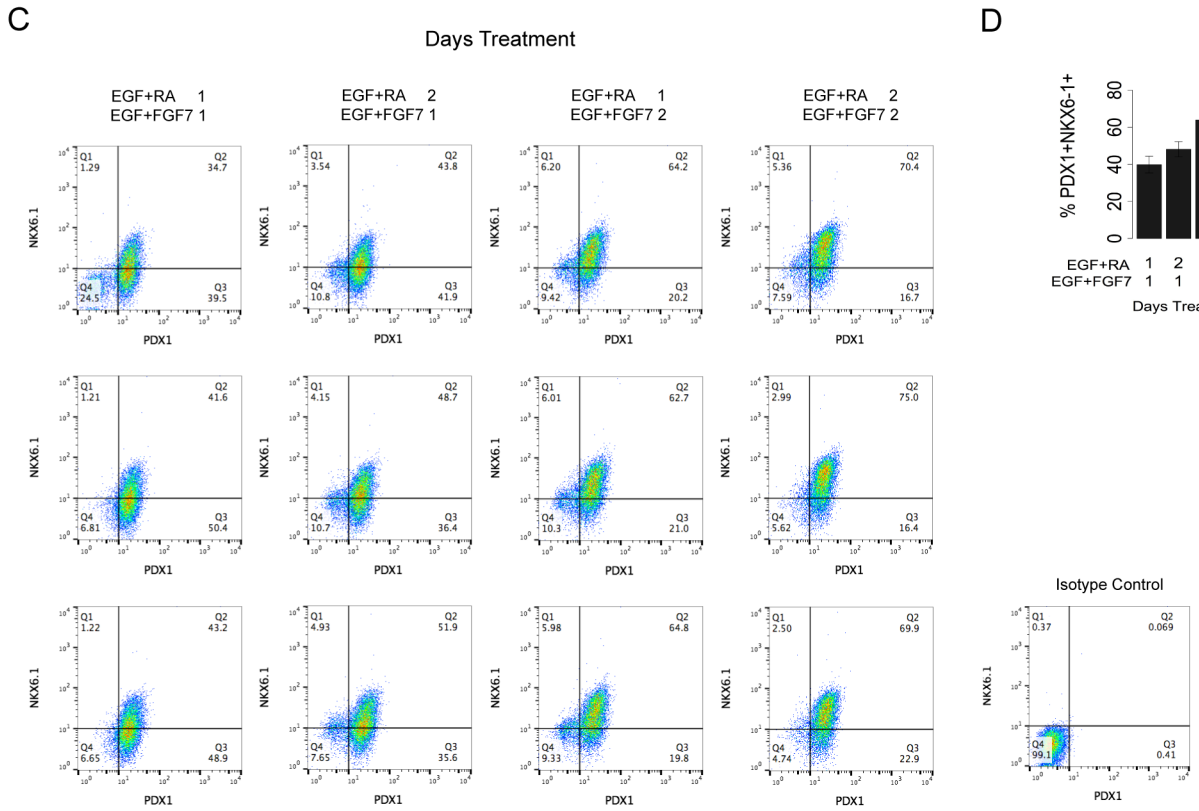


Figure S5: Optimization of cPP Beta Cell Differentiation

A) Application of NKX6-1 induction step of published beta cell differentiation protocols to cPP cells. We established 2D-monolayer, 3D-matrigel and 3D-suspension cultures in complete cPP media before exposing cells to growth factor regimes based on published beta cell differentiation protocols (see Supplementary Experimental Procedures). Phase contrast images were taken at the end of each treatment. Scale bar, 100 μm.

B) Gene expression measured by qRT-PCR using samples harvested in (A). When cells were exposed to the Rezania and Pagliuca differentiation regimes using the 3D matrigel platform, we were unable to recover sufficient material to carry out qRT-PCR analysis. Error bars represent the standard error of three technical replicates.

C) Optimization of the NKX6-1 induction step of the Russ et al. differentiation regime. Differentiations were carried out using the 3D-suspension platform. The lengths of the two growth factor treatments were varied to maximize the percentage of cells that reactivate NKX6-1 expression. PDX1 and NKX6-1 were measured by intracellular flow cytometric analysis. Three independent experiments are shown for each condition.

D) Percentage PDX1+NKX6-1+ cells generated in C.

Supplementary Table 1

Samples used for RNA-seq analysis.

Supplementary Table 2

Normalized gene counts for samples listed in supplementary table 1. The values for cPP and PPd15 cells are the mean values across the various samples listed in supplementary table 1. Genes (rows) are ordered according to the product of the coefficient of variance (column AI) and cPP Z-score (column AH). Raw RNA-seq read files are available for download at ArrayExpress under accession number E-MTAB-5731.

Supplementary Experimental Procedures

Human Pluripotent Stem Cell lines

The following hESC lines were used in this study: H9 (WA09) were purchased from WiCell, HES3 (ES03) were provided by ES Cell International Pte. Ltd., and the HES-3 *INS*$^{GFP/w}$ reporter line was a gift from the Stanley lab (Micallef et al., 2011). The hiPSC lines used in this study were derived in-house from human fibroblasts and are designated AK5-11, AK6-8 and AK6-13 (Figure S1).

Generation of hiPSC

Fibroblasts were obtained by punch skin biopsy and reprogrammed to generate hiPSC. Fibroblasts were reprogrammed using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific, A16517) in accordance with the manufacturer's instructions. Cells were passaged and plated onto irradiated mouse embryonic feeders 7 days after viral transfection. Thereafter, hiPSC colonies were picked between days 17-28 and maintained in DMEM/F12 (Sigma, D6421) supplemented with 20% Knock Out Serum Replacement (Thermo Fisher Scientific, 10828-028), 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific, 21985-023), 2 mM L-glutamine (Thermo Fisher Scientific, 25030), 0.2 mM NEAA (Thermo Fisher Scientific, 11140-050) and 5 ng/mL bFGF (Peprotech, 100-18B). Staining with the following antibodies was used to confirm pluripotency (Figure S1): NANOG (R&D Systems, AF1997, 1:200), OCT4 (Santa Cruz, 111351, 1:200), SOX2 (R&D Systems MAB2018, 1:200), SSEA3 (Millipore, MAB4303, 1:50), SSEA4 (Millipore, MAB4304, 1:200), TRA-1-81 (Millipore, MAB4381, 1:200), TRA-1-60 (Millipore, MAB4360, 1:200). Primary antibodies were recognized by Alexa-fluorophore conjugated secondary antibodies raised in Donkey (Thermo Fisher Scientific, 1:500). The study protocol was approved by the National University of Singapore Institutional Review Board (NUS IRB 10-051). The study was conducted in accordance with the Declaration of Helsinki and written informed consent was obtained from the participants.

Expansion of 3T3-J2 feeders

3T3-J2 feeder cells (passage 9, gift from Dr. Yann Barrandon) were expanded on tissue culture plastic (coated with 0.1% gelatin (Sigma, G2625) for 30 min) in 3T3-J2 culture media and passaged as single cells by treating with 0.25% Trypsin for 5 min (Thermo Fisher Scientific, 25200056). 3T3-J2 culture

media is composed of the following: DMEM high glucose (Thermo Fisher Scientific, 11960), 10% Fetal Bovine Serum (FBS, ES cell qualified, Thermo Fisher Scientific, 16141079), 2 mM L-glutamine (Thermo Fisher Scientific, 25030), and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific, 15140122). Feeder cells were mitotically inactivated by gamma irradiation (20 grays for 30 min) then frozen in culture media + DMSO. Individual batches of FBS are selected to enable 3T3-J2 cells to maintain cPP cultures, whilst 3T3-J2 cells are never cultured beyond passage 12 and should be seeded at 3.5-5x10^3 cells/cm^2 and not allowed to exceed 1.3x10^4 cells/cm^2.

Preparation of 3T3-J2 feeder-coated culture vessels

Thawed 3T3-J2 cells were seeded at 0.5-1x10^6 cells/cm^2 onto tissue culture plates coated with 0.1% gelatin (Sigma, G2625) for 30 min and maintained in 3T3-J2 culture media for up to 3 days until required. The optimal plating density must be determined empirically for each batch of feeders and is assessed based on the ability to maintain colony morphology without significantly hindering growth, since increasing feeder density improves colony morphology and blocks differentiation, but results in reduced proliferation rates. Tissue culture vessels containing feeders were washed once with DMEM to remove residual FBS prior to addition of cPP culture media.

Metaphase spread preparation, chromosome counting and M-FISH Karyotyping

Cells grown to ~75-80% confluency were treated with 100 ng/ml Colcemid solution (Gibco, 15212012) for 6 h, trypsinized and centrifuged at 1000 rpm for 10 min. Cell pellets were resuspended in 75 mM KCl and incubated for 15 min in a 37°C waterbath. 1/10 volume of 3:1 methanol/acetic acid was added to cells followed by centrifugation at 1000 rpm for 15 min. Cells were then fixed by resuspension in 3:1 methanol/acetic acid solution, incubated for 30 min at room temperature, centrifuged at 1200 rpm for 5 min and finally washed once more with fixative. Cells were resuspended in a small volume of fixative, dropped onto clean glass slides and left to air dry. Multicolor FISH (M-FISH) was performed according to manufacturer's instructions (MetaSystems). Automated acquisition of chromosome spreads was performed using Metafer imaging platform (MetaSystem). Ikaros and Fiji software were used to determine the chromosome number per spread and analyze M-FISH images.

RNA-seq read alignment, gene count calculation and normlization

Raw fastq files were downloaded with the fastq-dump function of the SRA-toolkit (v 2.8.0). We mapped reads with STAR (v2.5.1a) (Dobin et al., 2013) using an index based on the soft masked primary assembly of reference genome GRCh38 and corresponding gene annotation gtf file (GRCh38.83). Both were obtained from the Ensembl FTP site. Read overhang was set to 99 bp for index generation. Default mapping parameters were retained with the following exceptions: "--outFilterType BySJout" to reduce the number of spurious junctions, "--alignSJoverhangMin 10" minimum read overhang for unannotated junctions, "--alignSJDBoverhangMin 1" minimum overhang for annotated junctions, "--outFilterMatchNminOverLread 0.95" to allow up to 5% mismatched bases (per pair) if no better alignment can be found, "--alignIntronMin 20" to allow short introns, "--alignIntronMax 2000000" to set an upper limit on intron length, "--outMultimapperOrder Random" to randomize the choice of the primary alignment from the highest scoring alignments, "--outFilterIntronMotifs RemoveNoncanonicalUnannotated" to bias mapping towards known transcripts and "--chimSegmentMin 0" to suppress any chimeric mapping output.

The mapped reads of all samples were then jointly processed with featureCounts (Dobin et al., 2013; Liao et al., 2014) as implemented in the package "Rsubread" (v1.16.1) in R (v3.1.2). Default settings were used with the following exceptions: "annot.ext=GTFfile, isGTFAnnotationFile=TRUE, GTF.featureType='exon' " to use the same gtf annotation file as in STAR index, "useMetaFeatures=TRUE, GTF.attrType='gene' " to summarize counts to the gene level, "allowMultiOverlap=TRUE" to allow counting in overlapping genes, "isPairedEnd" was set as appropriate for the respective samples, "strandSpecific=0" because not all libraries were strand-specific and finally "countMultiMappingReads=TRUE". The resulting count table was normalized to account for sequencing depth and count distribution with the TMM method (Robinson and Oshlack, 2010) as implemented in edgeR (v3.8.6) using default settings.

Bioinformatics Analysis

RNA-seq gene expression analysis was carried out using normalized counts for each gene in each tissue type (Supplementary Table 2). Where technical replicates are available for samples described in other studies, we aligned these reads and determined gene counts separately, then calculated average

gene counts. Furthermore, unless otherwise stated, gene counts for cPP and PPd15 cells are the mean of three independent samples harvested from cells derived from H9 and HES3 hESC, and AK6-13 hiPSC. For global comparisons of gene expression profiles, we compared 60,675 ENSEMBL genes or (where stated) 19,875 ENSEMBL protein-coding genes expressed at >5 normalized counts in at least one sample. All of the following analysis was carried out in R, using base packages unless stated otherwise.

Hierarchical Clustering of RNA-Seq Transcriptomes (Figure 3A)

Euclidian distances between pairs of $\log_2$-transformed global gene counts were calculated using the R function *dist()* and the distances plotted as a Dendrogram using the *hclust()* function.

Heatmaps (Figures 3B, 3E and 3F)

Heatmaps were plotted using the function *heatmap.2()*.

Specifically Expressed Genes (Figure 3C)

Specifically expressed genes are defined as those with CV > 1 (Coefficient of Variance) and Z-score > 1. CV is defined as the mean divided by the standard deviation across all samples, in this case the aforementioned 23 published tissue datasets plus the cPP and PPd15 gene counts described here. Z-score is defined as the difference between expression in the sample of interest and the mean for all samples, divided by the standard deviation across all samples. When calculating the Z-score for pancreatic samples other pancreatic samples are excluded.

Gene Ontogeny Analysis (Figure 3D)

The web-based gene set analysis tool kit at http://www.webgestalt.org/ was used to analyze Gene Ontogeny (GO) terms associated with genes specifically expressed by cPP cells. Protein-coding genes were ordered according to the product of the coefficient of variance and Z-score for cPP cells (see above) and the top 250 genes selected for enrichment analysis. The Over Representations Analysis (ORA) tool was used to calculate fold-enrichment for biological process GO terms across these 250 genes, using all protein coding genes as the reference set, and the corresponding p-value adjusted by the Benjamini-Hochberg multiple test adjustment. GO terms were ordered according to fold-

enrichment and those associated with < 5 genes and/or an adjusted p-value > 0.01 were eliminated from the enriched set.

In vitro differentiation

Establishing differentiation cultures

Initially, cPP cells were cultured to confluency to eliminate feeder cells then treated with gentle cell dissociation reagent to generate single cells. Single cells were resuspended in cPP culture media + 10 μM Y27632 and seeded according to differentiation platform. To establish 3D sphere cultures, 2 x 10^6 cells were seeded into each well of an ultra low adhesion 6 well plate (Corning, 3471) in 2 mL media and placed on a nutator overnight. Compact spheres typically form after 24 hours. To establish 3D matrigel cultures, AggreWell 400 plates (Stemcell Technologies, 27840) were used to generate spheres of ~200 cells according to the manufacturers instructions. After 24 hours ~1200 spheres were resuspended in 500 μL 1:5-diluted hESC-qualified matrigel (Corning, 354277) and deposited into each well of a 24 well plate. Plates were incubated at 37°C for 60 min to allow matrigel to solidify before addition of media. To establish 2D monolayer cultures, cells were seeded at 6.65 x 10^5 cells/cm^2 on tissue culture plastic coated with matrigel diluted 1:50.

NKX6-1 induction tests

Differentiation cultures were treated with the following signaling regimes, based upon several recently published protocols, with minor alterations (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015; Zhang et al., 2009). Differentiation media 1 consists of MCDB 131 media (Thermo Fisher Scientific, 10372-01), 2.5 g/L sodium bicarbonate (Lonza, 17-613E), 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 10 mM glucose (VWR International, 101174Y), and 2% bovine serum albumin (Sigma, A9418). Differentiation media 2 consists of DMEM high glucose, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin. Media based on PP2 induction media described by Pagliuca et al. consists of differentiation media 1 supplemented with 50 ng/mL FGF7 (R&D Systems, 251-KG), 0.25 mM ascorbic acid (Sigma, A4544), 100 nM RA, 0.25 μM SANT-1 (Sigma, S4572), and 0.5% ITS-X (Thermo Fisher Scientific, 51500056). Media was completely replenished daily for 5 days. Media based on stage 4 media described by Rezania et al. was additionally supplemented with 300 nM Indolactam-V (Stemcell Technologies, 72312) and 200 nM LDN-193189 (Stemcell Technologies,

72142), and was completely replenished daily for 5 days. Media based on day 13-20 media described by Zhang et al. consists of differentiation media 2 supplemented with 10 ng/mL bFGF, 10 mM nicotinamide (Sigma, 24,020-6), 50 ng/mL exendin-4 (Sigma, E7144), 10 ng/mL BMP4 (R&D Systems, 314-BP), and 1% ITS-X. Media was completely replenished daily for 5 days. Media based on day 7-9 media described by Russ et al. consists of differentiation media 2 supplemented with 1X B27 supplement, 50 ng/mL EGF, 1 μM RA (first 24 hours), and 50 ng/mL FGF7 (second 24 hours). Media was completely replenished daily for 2 days.

Quantitative RT-PCR

RNA was isolated from samples using an RNeasy mini kit (Qiagen, cat # 74104) and reverse transcribed to generate cDNA using a high-capacity reverse transcription kit and random hexamer primers (Applied Biosystems, 4368814, 1 μg RNA per 20 μL reaction). Quantitative RT-PCR was carried out using SYBR Select Mastermix (Applied Biosystems, 4472908). Data were analyzed using the ΔΔCT method, and normalized to expression of the housekeeping gene TBP in each sample. The primers used for qRT-PCR are shown in Supplementary Table 3.

Immunofluorescence staining

The following primary antibodies were used for immunofluorescence staining: mouse monoclonal anti-PDX1 (R&D Systems, MAB2419, 1:50), rabbit anti-SOX9 (Sigma, HPA001758, 1:2000), rabbit anti-HNF6 (ONECUT1) (Santa Cruz, SC13050, 1:100), goat anti-FOXA2 (R&D Systems, AF2400, 1:200), rabbit anti-GATA6 (Cell Signaling Technologies, 5851, 1:1600), sheep anti-NGN3 (R&D Systems, AF3444, 1:200), mouse anti-NKX6-1 (developmental studies hybridoma bank, F55A12, 1:80), mouse monoclonal anti-NKX2-2 (BD biosciences, 564731, 1:400), mouse monoclonal anti-pro-Insulin c-peptide (Millipore, 05-1109, 1:100), rabbit monoclonal anti-glucagon (Cell Signaling Technologies, 8233, 1:400), rat monoclonal anti-KRT19 (developmental studies hybridoma bank, TROMA-III-s, 1:10), sheep anti-trypsin (pan-specific) (R&D Systems, AF3586, 1:13). Primary antibodies were recognized by Alexa-fluorophore conjugated secondary antibodies raised in Donkey (Thermo Fisher Scientific, 1:500). Images were acquired using an Olympus FV1000 inverted confocal microscope.

Immunofluorescence staining transplanted kidneys

Mouse kidneys were dissected, cleaned, longitudinally sectioned, embedded in Jung freezing medium (Leica, 020108926), and cryopreserved in liquid nitrogen. Sections (6µm) were mounted on APES-coated glass slides, dried and fixed in 4% paraformaldehyde for 10 min at room temperature. After washing 3X with PBS for 15 min, samples were permeabilised with PBS containing 0.3% Triton X-100 for 10 min, then blocked for 1 hour each in Rodent block M (Biocare medical, RBM961H) and blocking buffer (PBS + 20% normal donkey serum + 1% BSA + 0.3% Triton X-100). After washing 3X with wash buffer (PBS + 0.1% Tween-20 + 0.1% BSA) for 15 min, samples were incubated overnight at 4^{o}C with primary antibodies diluted in blocking buffer. After washing 3X with wash buffer for 15 min, samples were incubated at room temperature for 1 hour with secondary antibodies diluted 1:500 in blocking buffer. All subsequent steps were carried out in the dark. After washing 1X with wash buffer, samples were incubated at room temperature for 20 min with 2µg/mL Hoechst-33342 (Thermo Fisher Scientific, 62249) diluted in PBS. Finally, after washing 3X with wash buffer for 15 min, samples were covered with Vecashield hard set mounting medium (Vector Laboratories, H-1400), covered with a coverslip and sealed.

Immunofluorescence staining cultured cells

Adherent cells were washed 2X with PBS then fixed in 4% paraformaldehyde for 20 min at room temperature. After washing 3X with wash buffer (PBS + 0.1% BSA), samples were incubated with blocking buffer (PBS + 20% normal donkey serum + 0.1% BSA + 0.3% Triton X-100) for 1 hour at room temperature. Samples were then incubated overnight at 4^{o}C with primary antibodies diluted in blocking buffer. After washing 3X with wash buffer for 15 min, samples were incubated at room temperature for 1 hour with secondary antibodies diluted 1:500 in blocking buffer. All subsequent steps were carried out in the dark. After washing 3X with wash buffer for 15 min, samples were incubated at room temperature for 15 min with 2µg/mL Hoechst-33342 (Thermo Fisher Scientific, 62249) diluted in PBS. Finally, samples were washed 2X with PBS for 15 min and imaged.

Immunofluorescence staining differentiation spheres

Differentiation spheres were washed 1X with PBS + 2% serum then fixed in 4% paraformaldehyde for 30 min at room temperature. After washing 1X for 15 min with wash buffer (PBS + 0.1% BSA + 0.1% Tween-20), samples were blocked for 6 hours in blocking buffer (PBS + 20% normal donkey serum + 1% BSA + 0.3% Triton X-100). Samples were then incubated overnight at 4^{o}C with primary antibodies diluted in blocking buffer. After washing 2X with wash buffer for 15 min, samples were incubated at 4^{o}C for 6 hours with secondary antibodies diluted 1:500 in blocking buffer. All subsequent steps were carried out in the dark. After washing 1X with wash buffer for 15 min, samples were incubated at room temperature for 1 hour with 2μg/mL Hoechst-33342 (Thermo Fisher Scientific, 62249) diluted in PBS. Finally, spheres were washed 2X with PBS for 30 min, resuspended in Vectashield hard set mounting medium (Vector Laboratories, H-1400), mounted on glass slides, covered with a coverslip and sealed. All washing and incubation steps are carried out in 1.5mL Eppendorf tubes.

Flow cytometry

Single cells were generated using accutase (Thermo Fisher Scientific, 14190), washed 1X with PBS + 1% serum, then fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were washed 1X with wash/permeabilization buffer (BD, 554723), then up to 10^6 cells were incubated with primary or isotype control antibody diluted in 250μL wash/permeabilization buffer for the required length of time (see below for antibody dilutions and incubation times). For unconjugated antibodies, cells were washed 1X with wash/permeabilization buffer then incubated for 15 min with secondary antibody diluted in wash/permeabilization buffer. If staining for a second antigen, cells were washed 1X with wash/permeabilization buffer then subject to the aforementioned incubation step(s). After washing 1X with wash/permeabilization buffer, cells were resuspend cells in PBS + 1% serum and analyzed using a BD FACSCalibur flow cytometer. All steps were carried out at room temperature and cells were pelleted by centrifugation at 6000 rpm for 5 min in a microcentrifuge.

The following antibodies were used: mouse monoclonal anti-PDX1 PE-conjugate (BD biosciences, 562161, 1:50, 45 min), mouse IgG1 PE-conjugate (BD biosciences, 556650, 1:50, 45 min), mouse monoclonal anti-NKX6.1 (developmental studies hybridoma bank, F55A12, 1:25, 45 min), goat anti-mouse IgG APC-conjugate (BD biosciences, 550828, 1:400, 15 min), mouse monoclonal anti-Oct3/4

Alexa Fluor 488-conjugate (BD biosciences, 560253, 1:5, 60 min), mouse monoclonal anti-pro-Insulin c-peptide (Millipore, 05-1109, 1:100, 60 min), anti-mouse IgG Alexa Fluor 488-conjugate (Thermo Fisher Scientific, A21202, 1:300, 30 min). All flow cytometry experiments were gated using cells stained only with fluorophore-conjugated isotype control (in the case of directly conjugated primary antibodies) or fluorophore-conjugated secondary antibodies.

Microbioreactor Array (MBA) Screening of cPP Maintenance and Proliferation

Microbioreactor arrays (previously described, (Titmarsh et al., 2012)) were used to screen the effects of combinations of exogenous signaling molecules on cPP cells. MBAs provide combinatorial mixing of input factors, combined with continuous flow of culture media over culture chambers. MBAs were autoclaved and filled with sterile PBS, then coated (2-4 h, room temperature) with a single 1 mL injection of hESC-qualified matrigel at the manufacturer's recommended concentration. cPP cells in suspension in complete medium at 5 $\times 10^6$/mL were then seeded in the MBA, giving a surface density of 50×10^6cells/cm^2. Cells were allowed to attach for a total of 20 h, with a media exchange performed every 6 h. Subsequently, factor provision was commenced with an initial filling step of 300μL, followed by constant perfusion of factors at 36 μL/h, for a total culture time of 3 days. At the endpoint, cells were rinsed with PBS, fixed with 2% PFA/PBS solution for 30 min, then rinsed with PBS and blocked/permeabilised with PBS + 20% normal donkey serum + 0.1% BSA + 0.3% Triton X-100 for 30 min. Then, cells were labeled with primary antibodies against PDX1 (R&D Systems, MAB2419, 1:25), and SOX9 (Sigma, HPA001758, 1:1000) diluted in blocking buffer, overnight at 4°C. Cells were then washed with 0.1% BSA/PBS and labeled with Alexa-fluorophore conjugated secondary antibodies (Thermo Fisher Scientific, 1:500 dilution) and Hoechst 33342 (2 μg/mL) for 1 hour. Finally, cells were rinsed with PBS, and the MBA inlets and outlets plugged closed. The MBA was then mounted in a microplate adapter and imaged. Nuclear segmentation and quantification of nuclear intensities of PDX1 and SOX9 then proceeded similarly as previously described (Titmarsh et al., 2016).

Supplementary References

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