METHODS

Maintenance of PSCs.

Human embryonic stem cells and induced pluripotent stem cells were maintained on Matrigel (BD Biosciences) in mTesR1 media [32,33]. Cells were passaged approximately every 4 days, depending on colony density. To passage PSCs, they were washed with DMEM/F12 media (no serum)(Invitrogen) and incubated in DMEM/F12 with 1mg/mL dispase (Invitrogen) until colony edges started to detach from the dish. The dish was then washed 3 times with DMEM/F12 media. After the final wash, DMEM/F12 was replaced with mTesR1. Colonies were scraped off of the dish with a cell scraper and gently triturated into small clumps and passaged onto fresh Matrigel-coated plates.

Differentiation of PSCs into Definitive Endoderm (DE).

Differentiation into Definitive Endoderm was carried out as previously described [11]. Briefly, a 3 day ActivinA (R&D systems) differentiation protocol was used. Cells were treated with ActivinA (100ng/mL) for three consecutive days in RPMI 1640 media (Invitrogen) with increasing concentrations of 0%, 0.2%, 2% HyClone defined FBS (dFBS) (Thermo Scientific).

Differentiation of Definitive Endoderm in permissive media (Protocol for figure 1).

After differentiation into definitive endoderm, cells were incubated in DMEM/F12 plus 2% defined fetal bovine serum (dFBS) with either 0, 50, or 500ng/ml FGF4 and/or 0, 50, or 500ng/ml Wnt3a (R&D Systems) for 6, 48, or 96 hours. Cultures were then grown in permissive media consisting of DMEM plus 10% fetal bovine serum (FBS) for an additional 7 days.

Directed differentiation into hindgut and intestinal organoids.

After differentiation into definitive endoderm, cells were incubated in 2% dFBS-DMEM/F12 with either 50 or 500ng/ml FGF4 and/or 50 or 500ng/ml Wnt3a (R&D Systems) for 2-4 days. After 2 days with treatment of growth factors, 3-dimensional floating spheroids were present in the culture. 3-dimensional spheroids were transferred into an *in vitro* system previously described to support intestinal growth and differentiation [15,16]. Briefly, spheroids were embedded in Matrigel (BD Bioscience # 356237) containing 500ng/mL R-Spondin1 (R&D Systems), 100ng/mL Noggin (R&D Systems) and 50ng/mL EGF (R&D Systems). After the Matrigel solidified, media (Advanced DMEM/F12 (Invitrogen) supplemented with L-Glutamine, 10 μM Hepes, N2 supplement (R&D Systems), B27 supplement (Invitrogen), and Pen/Strep containing growth factors was overlaid and replaced every 4 days.

Generation and characterization of induced pluripotent stem cell lines

Normal human skin keratinocytes (NHSK) were obtained from donors with informed consent (CCHMC IRB protocol CR1_2008-0899). NHSKs were isolated from punch biopsies following trypsinization and subsequent culture on irradiated NIH3T3 feeder cells in F media [34]. For iPSC generation, NHSKs were transduced on two consecutive days with a 1:1:1:1 mix of recombinant RD114-pseudotyped retroviruses expressing Oct4, Sox2, Klf4 and cMyc [35,36] in the presence of 8μg/mL polybrene. Twenty-four hours after the second transduction the virus mix was replaced with fresh F media and cells were incubated for an additional three days. Cells were then trypsinized and seeded into 6 well dishes containing $1.875x10^5$ irradiated mouse fibroblasts per well and Epilife medium. On the following day, media was replaced with DMEM/F12 50:50 media supplemented with 20% knockout serum replacement, 1mM L-glutamine, 0.1mM β-mercaptoethanol, 1x non-essential amino acids, 4ng/mL basic fibroblast growth factor, and 0.5mM valproic acid. Morphologically identifiable iPSC colonies arose after 2-3 weeks and were picked manually, expanded and analyzed for expression of human pluripotent stem cell markers Nanog, DNMT3b, Tra1-60 and Tra1-81 [37,38]. Early passage iPSC lines were adapted to feeder-free culture conditions consisting of maintenance in mTeSR1 (Stem Cell Technologies) in culture dishes coated with matrigel (BD Biosciences) and lines were karyotyped.

Microarray analysis of human ESCs, iPSCs and DE cultures.

For microarray analysis, RNA was isolated from undifferentiated and 3-day activin treated HESC and iPSC cultures and used create target DNA for hybridization to Affymetrix Human 1.0 Gene ST Arrays

using standard procedures (Affymetrix, Santa Clara, CA). Independent biological triplicates were performed for each cell line and condition. Affymetrix microarray Cel files were subjected to RMA normalization in GeneSpring 10.1. Probe sets were first filtered for those that are over expressed or underexpressed and then subjected to statistical analysis for differential expression by 2 fold or more between undifferentiated and differentiated cultures with $p < 0.05$ using the Students T-test. Log2 gene expression ratios were then subjected to hierarchical clustering using the standard correlation distance metric as implemented in GeneSpring.

Adenoviral-mediated expression of NEUROG3. Adenoviral plasmids were obtained from Addgene and particles were generated as previously described [31]. Transduction was done on 28 day organoids that were removed from Matrigel, manually bisected then incubated in Ad-GFP or Ad-Neurog3 viral supernatant and media at a 1:1 ratio for approximately 4 hours. Organoids were then re-embedded in Matrigel and incubated overnight with viral supernatant and media at a 1:1 ratio. The next day, fresh organoid media was placed on the cultures and was changed as described until the end of the experiment.

shRNA knockdown hESC lines.

GipZ shRNA lentiviral vectors were obtained from Open Biosystems (GipZ-Neurog3 Open biosystems clone # v2lhs_309089; v2lhs_309091; v2lhs_309093; v2lhs_309092 and GipZ-Control; Openbiosystems clone # RHS4346). The Cincinnati Children's Hospital Medical Center Viral Vector Core produced high titer lentiviral particles for each plasmid. Low passage H9 hESCs were dissociated into a single cell suspension using Accutase, were spun down and resuspended in mTesR1containing 10 uM Y-27632. Cells were plated at low density and incubated with lentivirus for 24 hours. Neurog3 shRNA knockdown line, particles from all four vectors were used. mTesR1 was replaced daily, and after 72 hours selection for puromycin (2-4 ug/mL) resistant hESCs was carried out. Puromycin resistant colonies were routinely maintained and passaged in mTesR1+Puromycin (4 ug/mL).

ß-Ala-Lys-AMCA Uptake.

ß-Ala-Lys-AMCA was purchased from BioTrend Chemicals U.S.A. and was resuspended in water. Intestinal organoids were cut in half using a scalpel and were incubated for four hours in Advanced DMEM/F12 plus 24 uM ß-Ala-Lys-AMCA. Following incubation, organoids were washed several times in PBS, embedded in O.C.T. freezing medium and were frozen at -70C. 10 micron cryosections were cut and processed for standart immunohistochemistry.

Tissue processing, immunohistochemistry and microscopy.

Tissues were fixed for 1 hour to overnight in 4% paraformaldehyde or 3% glutaraldehyde for transmission electron microscopy (TEM). Cultured PSCs and DE cells were stained directly. Hindgut and intestinal organoids were, embedded in paraffin, epoxy resin LX-112 (Ladd Research, Burlington, VT), or frozen in OCT. Sections were cut 6-10 micrometers for standard microscopy and 0.1 micrometers for TEM. TEM sections were stained with uranyl acetate. Paraffin sections were deparaffinized, subjected to antigen retrieval, blocked in the appropriate serum (5% serum in 1x PBS + 0.5% triton-X) for 30 minutes, and incubated with primary antibody overnight at 4 degrees Celsius. Slides were washed and incubated in secondary antibody in blocking buffer for 2 hours at room temperature. For a list of antibodies used and dilutions, see Supplementary table 3. Slides were washed and mounted using Fluormount-G. Confocal images were captured on a Zeiss LSM510 and Z-stacks were analyzed and assembled using AxioVision software. An Hitachi H7600 transmission electron microscope was used to capture images.

RNA isolation, Reverse Transcription an qPCR

RNA was isolated using the Nucleospin II RNA isolation kit (Clonetech). Reverse Transcription was carried out using the SuperScriptIII Supermix (Invitrogen) according to manufacturers protocol. Finally, qPCR was carried out using Quantitect SybrGreen MasterMix (Qiagen) on a Chromo4 Real-Time PCR (BioRad). PCR primers sequences were typically obtained from qPrimerDepot (http://primerdepot.nci.nih.gov/). Primer sequences are available upon request.

Methods references

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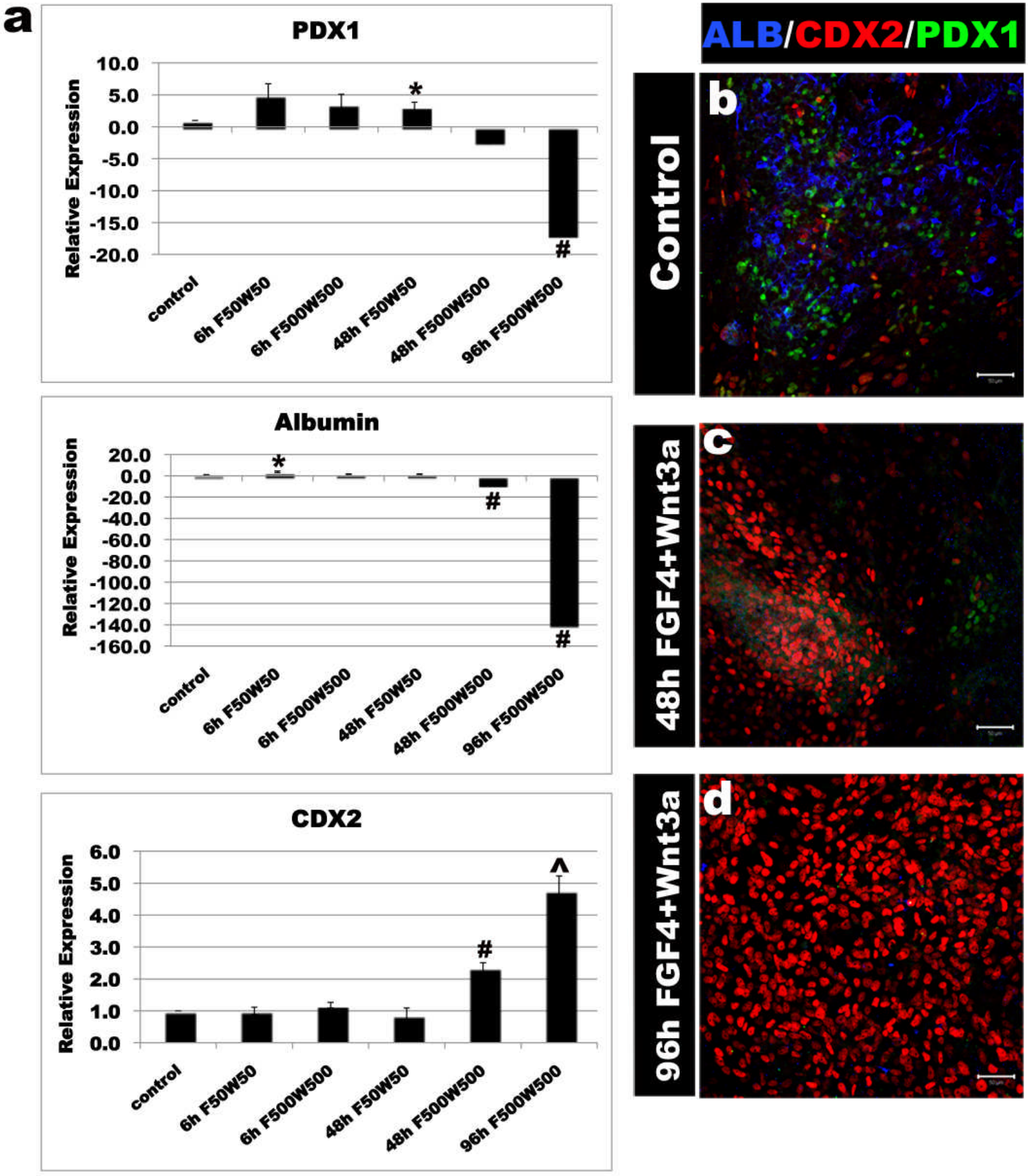
a
PDX1
Relative Expression
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
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control
6h F50W50
6h F500W500
48h F50W50
48h F500W500
96h F500W500
Albumin
Relative Expression
20.0
0.0
-20.0
-40.0
-60.0
-80.0
-100.0
-120.0
-140.0
-160.0
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6h F50W50
6h F500W500
48h F50W50
48h F500W500
96h F500W500
CDX2
Relative Expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
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control
6h F50W50
6h F500W500
48h F50W50
48h F500W500
96h F500W500
ALB/CDX2/PDX1
Control
b
48h FGF4+Wnt3a
c
96h FGF4+Wnt3a
d