Inhibition of Activin/Nodal signalling is necessary for pancreatic differentiation of human pluripotent stem cells.

ELECTRONIC SUPPLEMENTARY MATERIALS.

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SUPPLEMENTARY METHODS

hESCs and hIPSCs culture conditions.

hESCs and hIPSCs maintained in chemically defined medium (CDM) supplemented with Activin A (10ng/ml) and FGF2 (12ng/ml) . Cells were generally grown on tissue culture plates coated with gelatine and 10% FBS containing medium. However, human fibronectin was used occasionally as described previously. CDM contains 250 ml DMEM-F12 (GIBCO 31765-027) and 250mL IMDM (GIBCO 21980-032) mixed 1:1, 5mL concentrated lipids (GIBCO 11905), 20uL Mercapto-thio-Glycerol (Sigma M6145), 350uL Insulin (ROCHE 11376497001), 250uL Transferrin (ROCHE 10652202001), 5mL Pen/Strep (GIBCO 15140-122), and 2.5gr of bovine serum albumin, cohn Fraction V (Europa Bioproducts). BSA can be replaced by 0.5gr Polyvinylalcohol (SIGMA P8136) to obtain a fully defined medium. Prior to splitting, cells were washed once with PBS, collagenase was added and cells incubated at 37C for 20 minutes. When colonies detached from the tissue culture plate they were transferred to a 15ml tube, washed twice with 10ml of CDM and then resuspended in a volume to obtain a 1:6-10 split ratio. Differentiation was carried out as described in Figure 1. DE was induced by growing hESCs in CDM-PVA + Activin-A (100ng/mL), BMP4

(10ng/mL), bFGF (20ng/mL) and LY (10 mM) (AFBLy). The CDM-PVA AFBLy cocktail was replenished daily, and daily media changes were made during the entire differentiation protocol.

hESCs and hIPSCs maintenance and differentiation culture conditions.

hESCs (H9 from WiCell) and hIPSCs (BBHX8, A1ATD-1, JRO1D) [16] were passaged weekly using collagenase IV and maintained in chemically defined medium (CDM) supplemented with Activin A (10ng/ml) and FGF2 (12ng/ml) as described previously [17]. Differentiation was carried out as described in Figure 1. DE was induced by growing hESCs in CDM-PVA + Activin-A (100ng/mL), BMP4 (10ng/mL), bFGF (20ng/mL) and LY (10 μM) (AFBLy). The CDM-PVA AFBLy cocktail was replenished daily, and daily media changes were made during the entire differentiation protocol. After the DE stage (stage 1), cells were cultured in Advanced DMEM (Invitrogen) supplemented with SB-431542 (10 μM; Tocris), FGF10 (50 ng/ml; AutogenBioclear), all-trans retinoic acid (RA, 2 μM; Sigma) and Noggin (50 ng/ml; R&D Systems) for 3 days. For stage stage 3, the cells were cultured in Advanced DMEM supplemented with human FGF10 (50 ng/ml; AutogenBioclear), all-trans retinoic acid (RA, 2 uM; Sigma), KAAD-cyclopamine (0.25 uM; Toronto Research Chemicals) and Noggin (50 ng/ml; R&D Systems) for 3 days. For Stage 4, the cells were cultured in human FGF10 (50 ng/ml; R&D Systems) for 3 days. For maturation of pancreatic progenitors, cells were grown in Advanced DMEM + 1% vol/vol B27 and DAPT (1 mM) for 3 days and for 3 additional days in Advanced DMEM + 1% vol/vol B27.

HEX and HLXB9 Knockdown.

hESCs (H9) were stably transfected with expression vectors for ShRNA directed against HEX and HLXB9 (Open Biosystem) using Lipofectamine 2000 (Invitrogen) [19]. Stably

transfected cells were then selected using puromycin and the resulting colonies were individually picked for further analyses. 100 hESC sublines (10 hESC sublines for each ShRNA expression vector) were analysed for the knock down of HEX and HLXB9 after differentiation into hepatic or pancreatic endoderm respectively. Further analyses were systematically performed on at least 2 hESCs sublines expressing different ShRNA sequences.

Microarray profiling

Total RNA was extracted using RNeasy® Mini Kit according to manufacturer's protocol (Qiagen). RNA samples were first assessed for their RNA integrity prior to hybridisation on the microarray. Five biological replicate samples for each condition among Day 4.5 and Day 4.5 -Activin+SB differentiated hESCs were hybridised to Illumina Human HT-12 v4.0R1 Expression BeadChips using manufacturer's standard protocols. BeadChip probe-sets that did not pass the Illumina signal detection statistic at a threshold of $p < 0.01$ in all sample replicates of at least one sample group were removed from further analysis. For all samples, the remaining probe-sets were background corrected, normalized and summarized using default parameters of the RMA model [23]. Array processing was performed using the *beadarray* package of the *Bioconductor* (*http://www.bioconductor.org*) suite of software for the *R* statistical programming language (*http://www.r-project.org*). Probe-sets were annotated using transcript information made available by the manufacturer (*http://www.switchtoi.com/annotationfiles.ilmn*). The raw microarray data described has been uploaded to the ArrayExpress repository (EBI; *http://www.ebi.ac.uk/microarray-as/ae/*). Experiment name: Vallier hESC Endoderm. ArrayExpress accession: E-MEXP-2373

Analysis of Differential Regulation: The moderated t-statistic of [24], implemented in the *limma*

package of *Bioconductor*, was employed to assess the significance of differential gene (probe-set) expression between sample groups. In order to reduce errors associated with multiple hypothesis testing on such a scale, the significance *p-values* obtained were converted to corrected *q-values* using the FDR method of [25]. Probe-sets with associated $q < 0.001$ (FDR 0.1%) were deemed to exhibit significant differential expression between sample groups.

Data Visualisation: Heat maps of gene expression were created by importing relevant subsets of RMA processed microarray gene expression data into the *Java Treeview* data visualisation package (*http://sourceforge.net/projects/jtreeview/*). In the case wherein a gene is represented by more than one probe-set on the array, a single probe-set was chosen to represent gene expression in the heat map according to highest mean expression over all samples (i.e. the most reliable sample hybridization regardless of group membership).

The raw microarray data described has been uploaded to the ArrayExpress repository (EBI; *http://www.ebi.ac.uk/microarray-as/ae/*).

Enzyme linked immunosorbent assay (ELISA).

hESCs grown for 18 days in culture conditions inductive for pancreatic specification were cultured in differentiation medium without insulin for 24 h prior to Glucose stimulation. Cells were then washed three times in PBS and pre-incubated in DMEM supplemented with 2.2 mM glucose (Invitrogen) for 60 min at 37ºC. To estimate glucose-induced insulin secretion, pre-incubated cells wre grown in DMEM containing 22 mM glucose or alternatively 2.2 mM glucose for 15 or 60 minutes. Supernatants were collected for determination of C-peptide release. ELISA analyses were performed using the Mercodia Ultrasensitive C-peptide ELISA kit (Mercodia).

Concerning Abumin and ATT secretion assays, High binding surface COSTAR 96-well plates (Corning, NY, USA) were coated overnight with affinity-purified rabbit

polyclonal antibodies against α_1-antitrypsin (Abcam 31657, Cambridge, UK) and Albumin (Abcam 87564, Cambridge, UK) at 2 μg/ml in carbonate/bicarbonate buffer (Na2CO3/NAHCO3, pH 9.5). After washing (0.9% w/v NaCl, 0.05% v/v Tween 20), the plates were blocked for two hours in blocking buffer (PBS, 0.25% w/v BSA, 0.05% v/v Tween 20). Culture medium were diluted in blocking buffer and 50μl added to each well then incubated for two hours. After washing, the wells were incubated with corresponding monoclonal antibodies (1μg/ml diluted in blocking buffer), and incubated for two hours. Bound monoclonal antibodies were detected with rabbit anti-mouse IgG HRP-labelled antibody (Sigma Aldrich, Haverhill, UK, 1:20,000) for one hour. The reaction was developed with TMB liquid substrate (Sigma Aldrich, Haverhill, UK) for 10 minutes in the dark and the reaction was stopped with 1 M H_2SO4. Absorbance was read at 450nm on a Thermo-max microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Immunostaining

hESCs or their differentiated progenitors were fixed for 20 minutes at 4^oC in 4% paraformaldehyde and then washed three times in PBS. Cells were incubated for 20 minutes at room temperature in PBST (0.1% Triton X100; Sigma; in PBS) containing 10% donkey serum (Serotec Ltd.) and subsequently incubated overnight at 4^oC with primary antibody (Table 11) diluted in 1% donkey serum in PBST. Cells were then washed three times in PBS and incubated with secondary antibodies (Table 11) in 1% donkey serum in PBST for 2 hours at room temperature. Unbound secondary antibody was removed by three 5 minutes washes in PBS. Hoechst 33258 was added to the first wash (Sigma-Aldrich; 1:10,000). For lipid visualization a lipid specific stain BODIPY (boron-dipyrromethene; BODIPY® 493/503 Invitrogen.D-3922) was used.

Flow Cytometry

Adherent cells at the specific stage of the pancreatic differentiation protocol were washed twice in PBS and then incubated for 20 minutes at 37^{o}C in cell dissociation buffer (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Cells were dissociated by gentle pipetting and resuspended at approximately 0.1-1 x 10^{5} cells per milliliter in PBST + 3% normal donkey serum (NDS) containing 0.1% azide (Serotec Ltd.,Oxford, U.K., http://www.serotec.com). Cells were then fixed for 20 minutes at 4^{o}C in 4% paraformaldehyde and then washed three times in PBS. Cells were pelleted and resuspended in 2mL of SAP buffer (0.1% (w/v) saponin In Hanks' Balanced Salt Solution). Cells were incubated for 2 hours at room temperature with primary antibody (Table 11) in SAP buffer. Cells were then washed three times in PBS +3% NDS and then incubated with secondary antibodies (Table 1) in SAP buffer for 2 hours at room temperature. Unbound secondary antibody was removed by three washes in PBS. Cells were then analyzed using a FACS Calibur machine (BD Biosciences, San Jose, California, USA, http://www.bdbiosciences.com). Number of positive cells was recorded as the average from three separate experiments.