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ARTICLE

Inhibition of activin/nodal signalling is necessary for pancreatic differentiation of human pluripotent stem cells

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Abstract
Aims/hypothesis Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hIPSCs) offer unique opportunities for regenerative medicine and for the study of mammalian development. However, developing methods to differentiate hESCs/hIPSCs into specific cell types following a natural pathway of development remains a major challenge.
Methods We used defined culture media to identify signalling pathways controlling the differentiation of hESCs/hIPSCs into pancreatic or hepatic progenitors. This approach avoids the use of feeders, stroma cells or serum, all of which can interfere with experimental outcomes and could preclude future clinical applications.
Results This study reveals, for the first time, that activin/TGF-β signalling blocks pancreatic specification induced by retinoic acid while promoting hepatic specification in combination with bone morphogenetic protein and fibroblast growth factor. Using this knowledge, we developed culture systems to differentiate human pluripotent stem cells into near homogenous population of pancreatic and hepatic progenitors displaying functional characteristics specific to their natural counterparts. Finally, functional experiments showed that activin/TGF-β signalling achieves this essential function by controlling the levels of transcription factors necessary for liver and pancreatic development, such as HEX and HLXB9.
Conclusion/interpretation Our methods of differentiation provide an advantageous system to model early human endoderm development in vitro, and also represent an important step towards the generation of pancreatic and hepatic cells for clinical applications.

Keywords Activin/nodal signalling · Beta cell · Hepatocyte · Human pluripotent stem cells · Liver · Pancreas · PDX1 · TGF-β

Abbreviations

BMP	Bone morphogenetic protein
CDM	Chemically defined medium
DE	Definitive endoderm
DF	Dorsal foregut
FGF	Fibroblast growth factor
hESC	Human embryonic stem cell
HEX	Homeobox protein
hIPSC	Human induced pluripotent stem cell
HLXB9	Homeobox HB9
hPSC	Human pluripotent stem cell
PE	Pancreatic endoderm

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qPCR	Quantitative PCR
RA	Retinoic acid
SB	SB431542
SCID	Severe combined immunodeficiency
shRNA	Small hairpin RNA
VF	Ventral foregut

Introduction

Production of pancreatic beta cells is a major objective of regenerative medicine. An increased supply of these cells will enable the development of cell-based therapy against diabetes, which is currently limited by the lack of donated organs and difficulties with increasing insulin-secreting cells in vitro. Human pluripotent stem cells (hPSCs) of embryonic origin (human embryonic stem cells [hESCs]) [1] or generated from reprogrammed somatic cells (human induced pluripotent stem cells [hIPSCs]) [2] offer the prospect of bypassing these restrictions. These cells are capable of proliferating indefinitely in vitro while maintaining the capacity to differentiate into a broad number of cell types, including pancreatic progenitors [3–6]; however, robust protocols allowing for the production of homogenous populations of these cells in defined culture conditions have not yet been established. Current methods involve undefined animal products such as feeders, FBS and Matrigel and only allow for the generation of heterogeneous populations of cells, thus increasing the risk of teratoma formation after transplantation [7, 8]. They also appear to work efficiently on a limited number of hPSC lines [3], which hinders their use in a large number of laboratories.

Most of the culture systems currently used to direct the differentiation of hPSCs mimic normal development, since this approach could facilitate the generation of fully functional cell types. Consequently, the knowledge coming from studies on mice or other vertebrate animal models has been used to inform strategies driving hPSCs towards specific lineages. The pancreas and liver arise at approximately embryonic day 8.5–9.5 from adjacent regions of the developing primitive foregut under the influence of inductive signals secreted by the nearby mesoderm [9]. These signals probably command the production of transcription factors necessary for pancreatic specification, such as HLXB9, which marks the dorsal foregut (DF) prior to the formation of the pancreatic bud [10, 11], and PDX1, which marks regions of the foregut from which the ventral and dorsal pancreatic buds arise [12, 13]. The newly specified pancreatic endoderm quickly produces additional markers, including PTF1A, NKX6.1 and SOX9, and these progenitors give rise to both endocrine (islets of Langerhans) and exocrine (acinar and ductal cells) cells of the pancreas. Similar mechanisms control hepatic specification, although they involve a different set of transcription factors, such as homeobox protein (HEX), GATA-binding factor 6 (GATA6), Prospero homeobox protein 1 (PROX1) and hepatic nuclear factor 4α (HNF4A) [14], and signalling pathways, such as bone morphogenetic protein (BMP) and fibroblast growth factors (FGFs) [15]. Despite this broad knowledge, the molecular mechanisms enabling extracellular signalling pathways to orchestrate the transcriptional networks characterising pancreatic or hepatic progenitors remain to be elucidated. Especially in humans, hPSCs could present a unique advantage in completing this major task.

In the current study, we screened defined culture conditions to differentiate human definitive endoderm (DE) from multiple hPSC lines into a near homogenous population of pancreatic endoderm cells. Our analyses revealed that activin/TGF-β controls DE cell fate choice between the pancreatic and hepatic lineages by controlling the levels of key transcription factors. These observations facilitated the development of defined culture systems to differentiate DE cells into near homogenous populations of pancreatic and hepatic endoderm following a natural path of development. Therefore, the method described here not only provides a unique in vitro model of development for basic studies, but also represents a first step towards the production of pancreatic and hepatic cell types for therapeutic use.

Methods

hESC and hIPSC culture conditions hESCs (H9 from WiCell, Maddison, WI, USA) and hIPSCs (BBHX8, A1ATD-1, JRO1D; University of Cambridge, Cambridge, UK) [16] were passaged weekly using collagenase IV and maintained in chemically defined medium (CDM) supplemented with activin A (10 ng/ml) and FGF2 (12 ng/ml), as described previously [17]. Differentiation was carried out as described in Fig. 1 and is explained in detail in the electronic supplementary material (ESM) Methods.

RT-qPCR, immunostaining, FACS analyses and HEX/HLXB9 knockdown The methods used for RT-quantitative (q)PCR, immunostaining and FACS analyses have been described elsewhere [18, 19] and are outlined in the ESM Methods and ESM Table 1. All data are presented as average of three independent biological triplicates and error bars indicate SDs.

C-peptide release, cytochrome P450 activity, periodic acid Schiff and LDL-cholesterol uptake assays C-peptide ELISA (Mercodia, Uppsala, Sweden), Cyp3A4 P450-Glo assays (Promega, Maddison, WI, USA), periodic acid Schiff

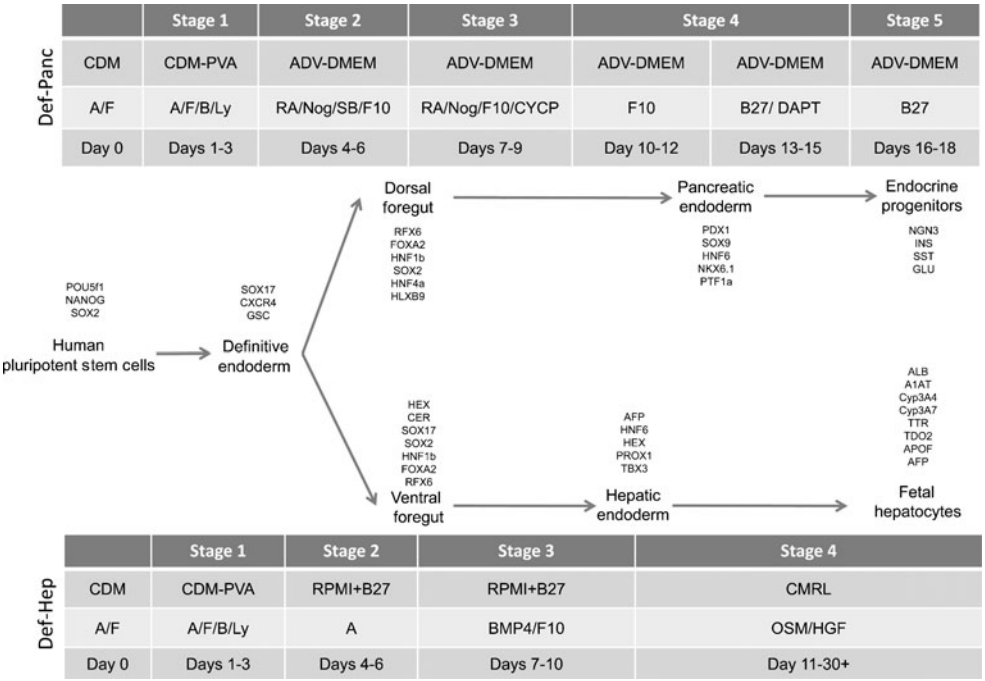

Fig. 1 Protocols to generate hepatic and pancreatic endoderm from hESCs and hIPSCs. Successive culture conditions driving differentiation of pluripotent stem cells towards pancreatic endoderm and hepatic endoderm. A, activin; ADV, advanced DMEM; B, BMP; CMRL, Connaught Medical Research Laboratories medium; CYCP, cytochrome P450; DAPT, *N*-(*N*-[3,5-diflurophenylacetyl]-L-alanyl)-*S*-phenylglycine *t*-butyl ester; F, FGF; HGF, hepatocyte growth factor; Ly, LY294002; Nog, noggin; OSM, oncostatin M

staining (Sigma, St Louis, MO, USA) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labelled acetylated LDL (Dil-LDL) uptake (Cayman Chemicals, MA, USA) were measured in triplicate according to the manufacturers' instructions.

Microarray profiling RNA was hybridised to Illumina (San Diego, CA, USA) HumanHT-12 v4.0R1 Expression BeadChips using the 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. Array processing was performed using the BeadArray package of the Bioconductor suite of software (www.bioconductor.org) for the *R* statistical programming language (www.r-project.org). See ESM for more detailed methods.

Animal studies Differentiated cells (5×10^6) were grafted under the kidney capsule of NOD/severe combined immunodeficiency (SCID) mice using a 24G catheter attached to a positive displacement pipette. Blood samples were removed from the tail at various time intervals for C-peptide analysis. Kidneys were harvested at the indicated time points, and a section containing the grafted cells was fixed in 4% paraformaldehyde, wax embedded and processed for immunohistochemistry. Antibody binding was visualised using 3,3′-diaminobenzidine.

Ethics approvals Ethics approval was obtained for hPSCs (authorisation 09/H0306/73 and 08/H0311/201) and hESCs were imported under the guidelines of the UK Stem Cell Bank Steering Committee (authorisation SCSC10-44).

All animal experiments were conducted following UK regulations under the project licence PPL60/4242. Animals were sourced from Harlan laboratories (Indianapolis, IN, USA).

Results

Inhibition of activin and BMP signalling in the presence of retinoic acid induces PDX1 expression in hESC-derived endoderm cells grown in fully defined culture conditions We recently established a defined culture system to differentiate hESCs and hIPSCs into near homogenous populations of DE cells [20]. This culture system relies on a CDM devoid of animal product including BSA, serum, complex extracellular matrix such as Matrigel (BD Biosciences, NJ, USA) or feeder cells, thereby avoiding the presence of unknown factors that might interfere with experimental outcomes. To further extend this protocol, we screened a broad number of combinations of growth factors and inhibitors of signalling pathways to identify defined culture conditions driving the differentiation of DE cells into pancreatic progenitors. These analyses revealed that a combination of retinoic acid (RA), FGF10, noggin (a BMP inhibitor) and SB431542 (SB; an activin/TGF-β receptor antagonist) was able to induce expression of the pancreatic markers *PDX1*, *HNF6*, *PTF1A*, *SOX9* and *HLXB9* (also known as *MNX1*), while inhibiting the expression of gut (*CDX2*) and liver (*AFP*) markers in hESC-derived DE cells (Fig. 2a–g). Importantly, this cocktail of factors only induced PDX1 in specific basal medium (advanced DMEM) while the presence of serum, Matrigel or feeders (ESM

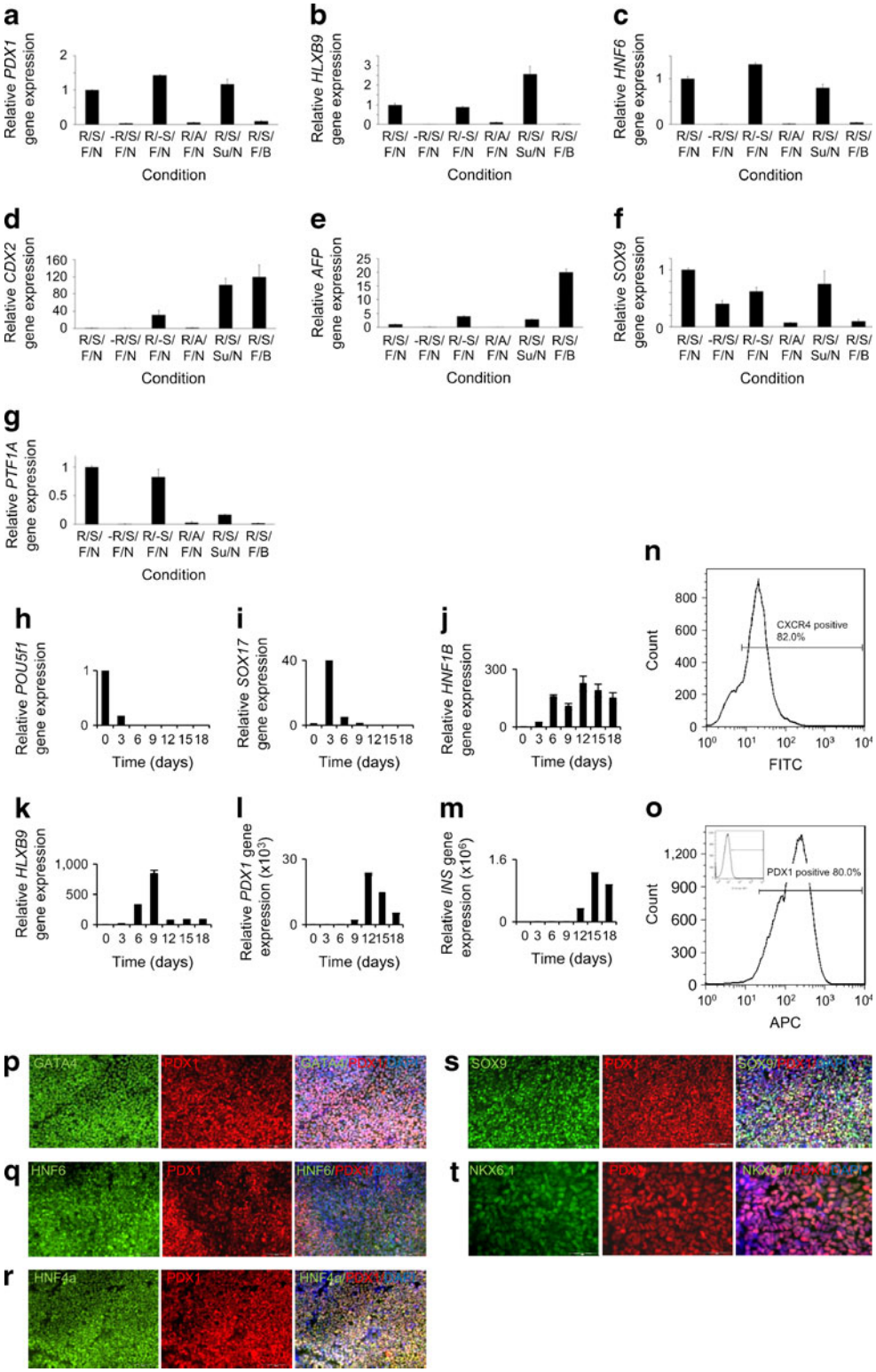

Fig. 2 Differentiation of hESC-derived DE into pancreatic endoderm in defined culture conditions. (**a**–**g**) Function of RA, BMP, FGF10 and activin/TGF-β on pancreatic differentiation of DE cells generated from hESCs. qPCR analyses showing the expression of *PDX1*, *HLXB9*, *HNF6*, *CDX2*, *AFP*, *SOX9* and *PTF1A* genes in DE cells grown for 6 days in the presence of diverse combinations of RA (R), SB (S), activin 50 ng/ml (A), FGF10 50 ng/ml (F) or SU5402 10 μmol/l (Su), and noggin 150 ng/ml (N) or BMP4 10 ng/ml (B). Gene expression was normalised to DE cells differentiated for 6 days in the presence of RA, FGF10, noggin and SB. (**h**–**m**) Successive expression of markers showing patterning from pluripotency (**h**) to DE (**i**), into the foregut (**j**) and then successive differentiation towards pancreatic endoderm (**k**, **l**) and hormone-expressing cells (**m**). All time points were normalised to undifferentiated hESC (time day 0). (**n**, **o**) FACS analyses showing expression of CXCR4 (**n**) in DE cells (day 3) and PDX1 (**o**) in pancreatic endoderm (day 12). Insert shows isotype control. Conjugated isotype control were used as negative controls to gate the positive population. (**p**–**t**) Coexpression of PDX1 and diverse pancreatic endoderm markers in DE cells grown for 12 days in culture conditions inductive for pancreatic specification. Scale bar 50 μm

Fig. 1a–c and data not shown) inhibited pancreatic endoderm differentiation, confirming that DE differentiation can be influenced by a diversity of factors.

We then sought to validate and optimise the role of each of these additives. Absence of RA in the presence of noggin, FGF10 and SB inhibited the expression of pancreatic markers (Fig. 2a–c and ESM Fig. 1d–f), confirming that RA is necessary for the induction of pancreatic specification [21]. Absence of noggin or addition of BMP4 at any time during the differentiation process (Fig. 2a–g and ESM Fig. 1g–i) resulted in a significant decrease in the expression of pancreatic endoderm markers while inducing gut (*CDX2*)

and liver (*AFP*) markers, thereby reinforcing previous studies showing that BMP signalling inhibits pancreatic specification to promote alternative cell fate [22]. Inhibition of FGF signalling using SU5402 (an FGF receptor antagonist) or increasing the dose of FGF10 did not affect the expression of pancreatic endoderm markers such as *PDX1*, *SOX9* and *HLXB9* (Fig. 2a–g, ESM Fig. 1j–o). However, the expression of the gut marker *CDX2* [23] was augmented and the expression of *PTF1A* was strongly reduced in the absence of FGF signalling, suggesting that FGF10 might block the specification of *PDX1*-expressing cells towards duodenum [23, 24] during pancreatic bud specification. Furthermore, FGF inhibition caused significant cell death, implying that FGFs are also necessary for the proliferation and survival of pancreatic endoderm in vitro. More importantly, we observed that the addition of activin abolished the expression of pancreatic markers while inhibition of activin/TGF-β signalling by SB had the opposite effect (Fig. 2a–g), demonstrating for the first time that activin/TGF-β signalling inhibits pancreatic specification in vitro. Interestingly, the presence of SB was only required for the first 3 days of differentiation, indicating that activin/TGF-β signalling acted on the earliest steps of pancreatic specification preceding *PDX1* expression. Together, these results show that RA acts as an inductive signal driving the differentiation of DE cells towards the pancreatic lineage, while TGF-β signalling pathways (i.e. activin and BMP) act as a potent inhibitor of this cell fate choice.

Inhibition of activin/TGF-β induces differentiation of endoderm into a near homogenous population of pancreatic endoderm following a native path of development Based on the results described above, we established a four-step protocol to differentiate hESCs into pancreatic endoderm using defined culture media (Def-Panc, Fig. 1). During the first step (days 1–3), hESCs were grown in CDM supplemented with activin/BMP/FGF2/LY294002 (a phosphoinositide 3-kinase inhibitor) [20]. The resulting cells were positive for the expression of DE markers including *SOX17*, *CXCR4*, *HEX* (also known as *HHEX*), *FOXA2* and *EOMES*, and simultaneously negative for the expression of the pluripotency markers *POU5f1* (also known as *OCT3/4*), *NANOG* and *SOX2* and the primitive streak markers *T* (brachyury) and *MIXL1* (Fig. 2h–m and ESM Fig. 1p–af). The second step of the Def-Panc protocol involved growing DE cells in the presence of RA/noggin/FGF10/SB431542 for 3 days (days 4–6). The resulting cells expressed *HNF1B FOXA2*, *HNF4A*, *RFX6* and *HLXB9* (Fig. 2i–m and ESM Fig. 1p–z), all of which mark the foregut during early mammalian development.

Notably, the expression of *HLXB9* and the absence of *HEX* expression suggested a dorsal identity for these foregut cells, while the absence of *CDX2* excluded the presence of midgut or hindgut cells (Fig. 2k, ESM Fig. 1x and data not shown). In the third step of the protocol, DF cells were grown for three additional days in the presence of RA/noggin/FGF10/cyclopamine (days 7–9). The resulting cells expressed a combination of foregut markers (*HNF1B*, *SOX2*, *FOXA2* and *HLXB9*) and pancreatic endoderm markers (*SOX9*, *HNF6*, *PTF1A* and *PDX1*) (Fig. 2i–l and ESM Fig. 1s–af). The expression of pancreatic endoderm markers was further reinforced in the fourth step of the protocol by the addition of FGF10 for 3 days (days 10–12). The resulting cells expressed *NKX6.1*, *SOX9*, *HNF6*, *PTF1A*, *PDX1*, *HNF1B*, *SOX2* and *FOXA2*, while the expression of *HLXB9* was strongly diminished (Fig. 2i–m and ESM Fig. 1a,b). FACS analyses performed at the end of step 1 showed that the DE-enriched cells were homogeneously positive for *CXCR4* and after the fourth step of the protocol (day 12), 80% of cells expressed *PDX1* (Fig. 2n,o). Immunostaining analyses also confirmed that *PDX1* was coexpressed with *SOX9*, *HNF6*, *HNF4*, *NKX6.1* and *GATA4* (Fig. 2p–t) in approximately 80% of the cells generated. Together, these results indicate that the Def-Panc protocol drives differentiation of hESCs towards a near homogenous population of pancreatic endoderm cells following successive events of specifications reminiscent of those that occur during pancreatic development.

PDX1 endoderm generated in defined culture conditions can differentiate into insulin secreting cells in vitro and in vivo To confirm the capacity of pancreatic endoderm cells to differentiate further towards the endocrine lineage, *PDX1*-expressing cells obtained at the end of stage 4 were grown for six additional days in culture conditions previously shown to stimulate endocrine cell differentiation [3]. qPCR analyses showed that *PDX1* expression decreased after 3 days, while expression of *NGN3* and hormonal markers (insulin, glucagon and somatostatin) progressively increased (Fig. 2i–m and ESM Fig. 1ac–af). By day 18, 11% of cells stained positive for C-peptide (Fig. 3a). Interestingly, these hESC-derived insulin-expressing cells were able to release C-peptide upon glucose stimulation, mimicking insulin release by pancreatic beta cells (Fig. 3c). Nonetheless, the expression of hormonal markers (insulin, somatostatin and glucagon, ESM Fig. 2a–k) was relatively low when compared with that of human adult islets cells, while the expression of markers specific to endocrine progenitors was maintained (*NKX6.1*, *NGN3* and *SOX9*, ESM Fig. 2a–k). Furthermore, a fraction of C-peptide-expressing cells was also positive for glucagon or somatostatin (Fig. 3b).

Polyhormonal expression could mark islet cells of embryonic origin [25], thereby confirming that our in vitro culture conditions are not sufficient to generate fully functional endocrine cells. To overcome this limitation of the in vitro system, pancreatic endoderm cells obtained after

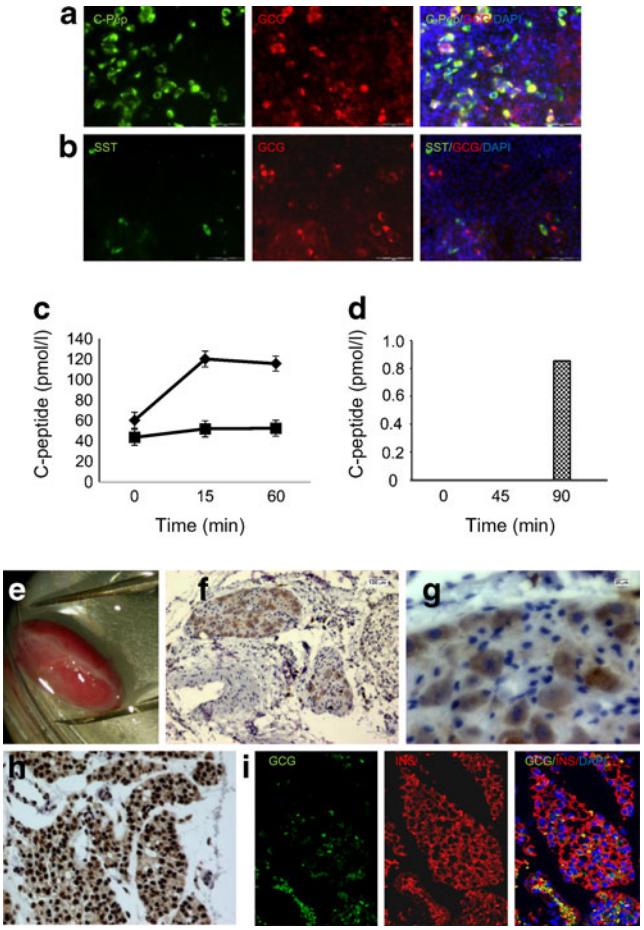

Fig. 3 Pancreatic progenitors generated from the DF can differentiate into hormone-expressing cells in vitro and in vivo. (**a**, **b**) Expression of hormonal markers C-peptide (C-Pep), glucagon (GCG) and somatostatin (SST) in pancreatic endoderm grown for 6 days (day 18) in culture conditions permissive for endocrine specification. Scale bar 50 μm. (**c**) C-peptide secretion upon high-glucose (diamond) (22 mmol/l) stimulation in culture medium of endocrine cells generated from pancreatic endoderm (day 18). Data are presented as average of three biological replicates and error bars indicate standard deviation. Cells grown in low glucose (square) (2.2 mmol/l) were used as negative control. (**d**) Mice transplanted with pancreatic progenitors (day 12) were injected intraperitoneally with glucose 20 weeks after transplantation. Blood samples were taken at indicated time for C-peptide measurement using ELISA. (**e**–**i**) Immunostaining showing the expression of pancreatic markers in the kidney capsule of a mouse engrafted with pancreatic endoderm cells (day 12) and then differentiated for 20 weeks in vivo. (**e**) A kidney capsule 20 weeks after transplantation, showing the absence of teratomas or overgrowth. (**f**) A graft containing an islet-like structure with cells expressing C-peptide. (**g**) Higher magnification of the C-peptide expressing cells shown in (**f**). (**h**) Graft-containing cells expressing glucagon. (**i**) Immunostaining analyses for glucagon (green fluorescence, GCG) and insulin (red florescence, INS) of pancreatic progenitors on day 12, differentiated in vivo for 19 weeks. DAPI staining (blue fluorescence) marks nuclei

12 days of differentiation were injected under the kidney capsule of NOD/SCID mice to provide an environment known to favour their differentiation into endocrine cells [3]. A low level of human C-peptide was detected in the blood stream of three out of eight transplanted animals as soon as 12 weeks after transplantation (negative control= 0.021 ng/ml; mouse 1=0.1 ng/ml, mouse 2=0.43 ng/ml, mouse 3=0.1635 ng/ml) (Fig. 3d). In addition, histology analyses performed after 19 weeks of differentiation in vivo revealed the presence of islet-like clusters with cells containing glucagon and C-peptide (Fig. 3e–i). Together, these results demonstrate that pancreatic endoderm cells generated with the Def-Panc protocol have the capacity to differentiate further into endocrine cells and thus represent early pancreatic progenitors. Finally, similar results were obtained with three hIPSCs lines (ESM Fig. 3), indicating that the Def-Panc protocol could be successfully used to produce pancreatic progenitors from diverse hPSCs.

Activin/TGF-β drives differentiation of endoderm cells into hepatic progenitors, which can differentiate into fetal hepatocytes During screening of the culture conditions described above, we noted that DE cells grown in the presence of activin acquired the appearance of fetal hepatocytes with large darkened cytoplasmic space and canaliculi-like structures (data not shown). Further analyses confirmed that DE cells grown in the presence of activin for 5 days expressed genes marking the ventral foregut (VF), the site of liver bud formation (*HEX*, *SOX17*, *HNF4A*, *FOXA1*, *FOXA2* and *TBX3* in Fig. 4a–h). Conversely, inhibition of activin by SB decreased the expression of *HNF4A*, *SOX17*, *HEX* and *TBX3*, while blocking known hepatic inducers such as FGF signalling also decreased the expression of liver bud genes such as *HEX*, *SOX17* and *TBX3* (Fig. 4i–l). Surprisingly, noggin only induced a moderate decrease in *HNF4A* expression, suggesting that BMP signalling might have a limited function in hepatic specification in vitro. Alternatively, unknown signalling pathways could activate the same programme of differentiation. Considered together, these results suggest that the combined effect of activin, BMP and FGF is necessary to fully promote hepatic specification of DE cells in vitro.

Based on this observation, we developed a three-step protocol to generate hepatocytes from hPSCs in defined culture conditions (Def-Hep, Fig. 1). The first step of the Def-Hep protocol consisted of differentiating hESCs into DE cells, as described above, while the second step involved promoting DE specification towards the hepatic lineage using first activin alone for 3 days and then activin combined with BMP4 and FGF10. In the third step of the Def-Hep protocol, hepatic endoderm cells were grown for 15 additional days in the presence of oncostatin M and hepatocyte growth factor, two growth factors known to control hepatoblast differentiation into hepatocytes. Accordingly, the cells generated with the Def-Hep protocol expressed hepatocyte markers such as *ALB*, *AAT*, *αAPOF*, *TAT*,

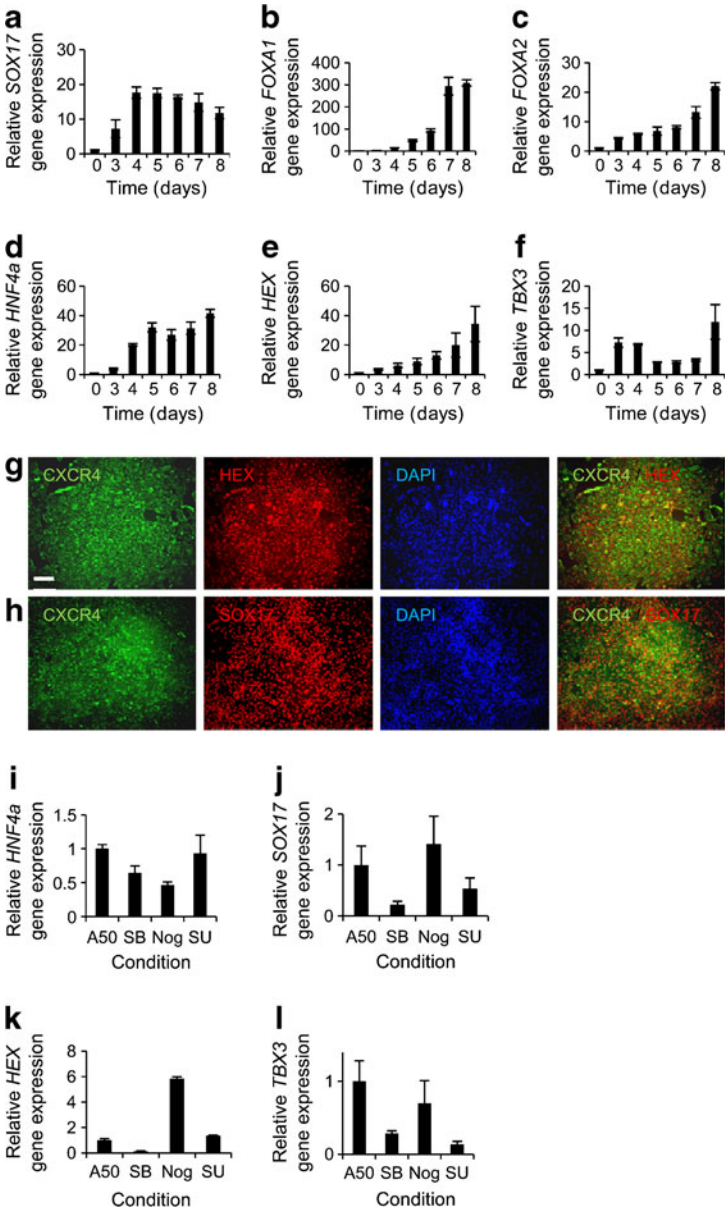

Fig. 4 Activin induces specification of DE into VF. (**a**–**f**) Expression of VF and liver bud markers in DE cells grown for 5 days in the presence of activin. All time points normalised to undifferentiated hESC (day 0). (**g**, **h**) Immunostaining analyses showing the coproduction of HEX, SOX17 and CXCR4 in hESCs differentiated into VF using activin (day 6). Scale bar 50 μm. (**i**–**l**) qPCR analyses showing that inhibition of activin signalling by SB, BMP by noggin (Nog) and FGF by SU5402 (SU) decreases the expression of hepatic markers in DE cells. All data points normalised to VF cells cultured in activin (50 ng/ml; A50)

TDO2, *TTR*, *HNF4A* and *HEX* (Fig. 5a–j). These observations were confirmed by immunostaining and FACS analyses, which showed homogenous coexpression of *ALB*, *CK18*, *AAT* and *AFP* (Fig. 5k–m). These cells also displayed functional characteristic of hepatocytes, such as: (i) albumin and α-1-antitrypsin secretion (Fig. 5n–o); (ii) CYP3A4 activity inducible by dexamethasone (Fig. 5p); (iii) cholesterol uptake; and (iv) glycogen storage (Fig. 5q–r). Together, these data demonstrate that activin drives DE specification towards VF-like cells and then hepatic endoderm, which has the capacity to differentiate into cells displaying characteristics of fetal hepatocytes.

HEX and HLXB9 knockdown during pancreatic and hepatic differentiation of hESCs blocks hepatic and pancreatic differentiation, respectively We then decided to take advantage of the Def-Panc and Def-Hep culture systems to study the mechanisms by which activin can control the cell-fate choice between the pancreatic and hepatic lineages. For that, we performed gene-expression profiling experiments to identify genes that were up- or downregulated by the presence of activin during pancreatic specification. These analyses revealed that activin could activate or block the expression of a broad number of genes, including *HEX* and *HLXB9* (Fig. 6a), that are known to be essential for foregut development. Thus, we hypothesised that activin could direct DE specification by controlling the expression of these transcription factors. To test this hypothesis, we knocked down HEX or HLXB9 levels in hESCs using stable expression of small hairpin (sh)RNA. The resulting hESC sublines (sh*HEX*-hESCs and sh*HLXB9*-hESCs) were then differentiated as described in Fig. 1. qPCR analyses

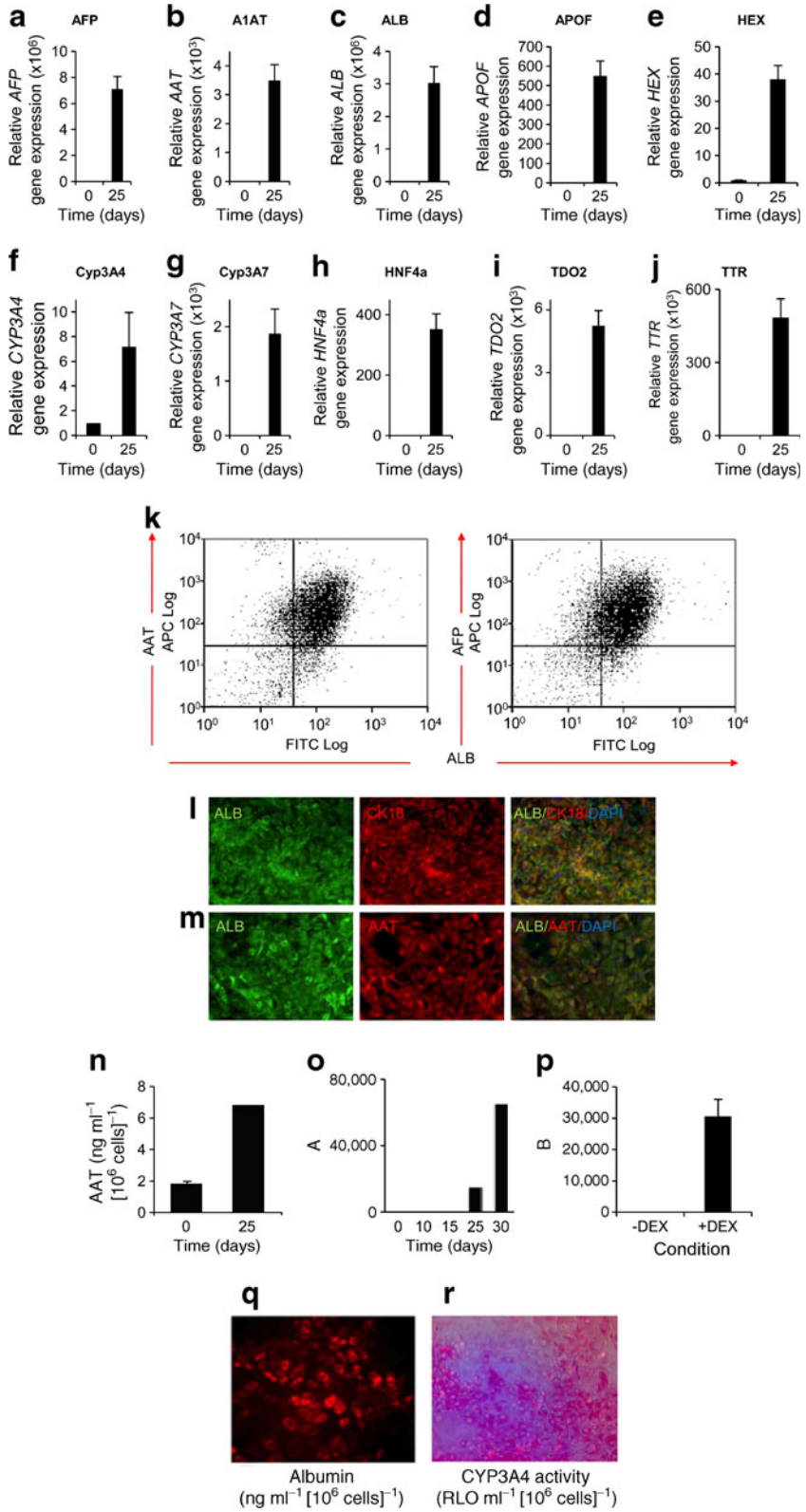

Fig. 5 Differentiation of hESC-derived DE into fetal hepatocytes in defined culture conditions. (**a**–**j**) Expression of hepatocyte markers in DE cells grown for 25 days in conditions inductive for hepatic differentiation. Data were normalised to undifferentiated hESCs (day 0). (**k**) FACS analyses showing the coproduction of albumin (ALB), α1-antitrypsin (AAT) and α1-fetoprotein (AFP) in hESC-derived fetal hepatocytes (day 25). (**l**, **m**) Immunostaining analyses showing the levels of ALB, cytokeratin 18 (CK18) and AAT in hESC-derived fetal hepatocytes (day 25). Scale bar 50 μm. (**n**, **o**) ELISA analyses showing AAT (**n**) and albumin (**o**) secretion in culture media of hESC-derived fetal hepatocytes. Fresh culture medium was used as negative control. (**p**) Inducible activity of CYP3A4 by dexamethasone (DEX) in hESC-derived fetal hepatocytes. (**q**, **r**) Diagnostic Immunology Laboratories assay showing uptake of cholesterol (**q**) and periodic acid Schiff staining showing glycogen storage (**r**) in hESC-derived fetal hepatocytes. Scale bar 50 μm

showed that knockdown of HEX levels during VF differentiation (Fig. 6b) was systematically associated with down-regulation of hepatic markers such as *AFP* and albumin (Fig. 6c–k). A similar decrease was not observed with DE cells derived from sh*HLXB9*-hESCs or DE cells derived from hESCs stably overexpressing a non-targeting shRNA (shScramble-hESCs) (Fig. 6c, f and i, and data not shown). However, we also observed that reduced *HEX* expression

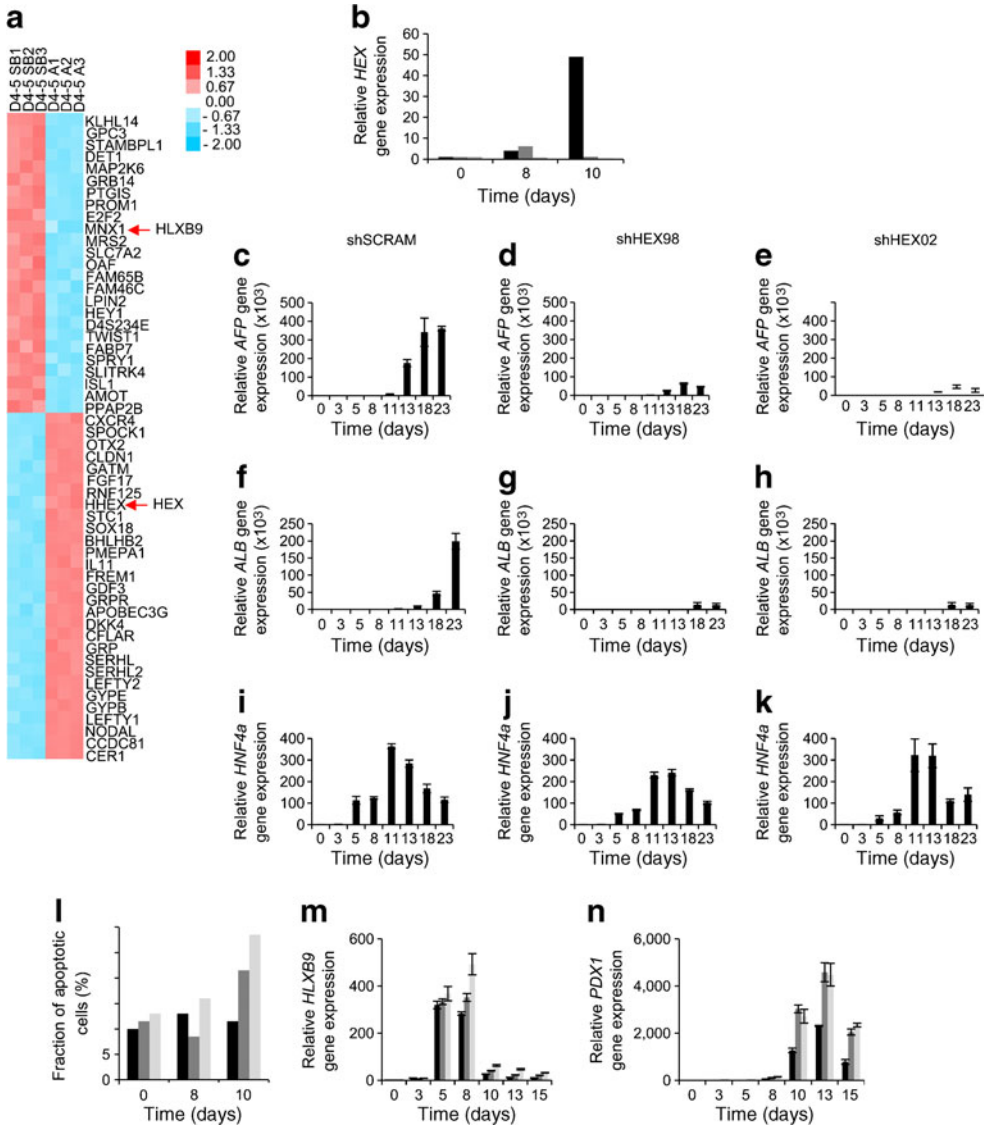

Fig. 6 HEX is necessary for the hepatic specification of VF in vitro. (**a**) Gene expression profile of DE cells grown for 36 h in the presence activin/RA/noggin/FGF (D4.5A) or SB/RA/noggin/FGF (D4.5SB). (**b**) qPCR analyses showing knockdown of HEX in sh*HEX*-hESCs (grey bar, sh*HEX*98; white bar, sh*HEX*02 [not visible as there was no expression compared with control]) differentiating into hepatic endoderm. ShScramble-hESCs were used as negative control (black bar). All time points normalised to undifferentiated shScramble. (**c**–**k**) qPCR analyses showing the effect of HEX knockdown on the hepatic specification of VF cells. All time points normalised to undifferentiated shScramble (shSCRAM). shScramble (**c**, **f**, **i**), sh*HEX*98 (**d**, **g**, **j**) and sh*HEX*02 (**e**, **h**, **k**). (**l**) Fraction of apoptotic cells in shScramble-hESCs (black bar) and in sh*HEX*-hESCs (dark grey bar, HEX98; light grey bar, HEX02) differentiating into hepatic endoderm. (**m**, **n**) Expression of pancreatic markers in sh*HEX*-hESCs differentiating into pancreatic endoderm (black bar, shScramble; dark grey bar, HEX98; light grey bar, HEX02 open box)

increased cell death during VF differentiation (Fig. 6l). Therefore, *HEX* expression appears to be necessary for the survival and differentiation of VF-like cells towards the hepatic lineage in vitro. Finally, sh*HEX*-hESCs were able to differentiate into pancreatic endoderm expressing successively *HLXB9* and *PDX1* (Fig. 6m,n).

Similar experiments performed with sh*HLXB9*-hESCs showed that knockdown of HLXB9 levels during foregut differentiation strongly decreased the expression of pancreatic endoderm markers, including *PDX1*/*SOX9* (Fig. 7a–g). Interestingly, a decrease in *HLXB9* expression did not affect the expression of foregut makers such as *HNF4A*, *FOXA2* and *HNF1B* (Fig. 7a–f), suggesting that HLXB9 is not required for DF specification while being necessary for its differentiation towards the pancreatic lineage. Importantly, *HLXB9* is not expressed during hepatic differentiation (Fig. 7h), and thus DE cells generated from sh*HLXB9*-hESCs were able to differentiate into VF-like cells and into hepatic endoderm when grown in the presence of activin (data not shown).

Collectively, these results recapitulate studies performed in the mouse embryo showing that absence of HEX disrupts hepatic bud development without affecting dorsal pancreatic specification, while HLXB9 is necessary for induction of *PDX1* expression in the pancreas [26]. Therefore, they demonstrate the general application of our culture system in modelling DE development and studying early organogenesis of the pancreas and liver in vitro.

Discussion

Robust protocols allowing for the production of homogenous populations of liver and pancreatic progenitors from hPSCs under culture conditions compatible with clinical applications have not yet been established. Available

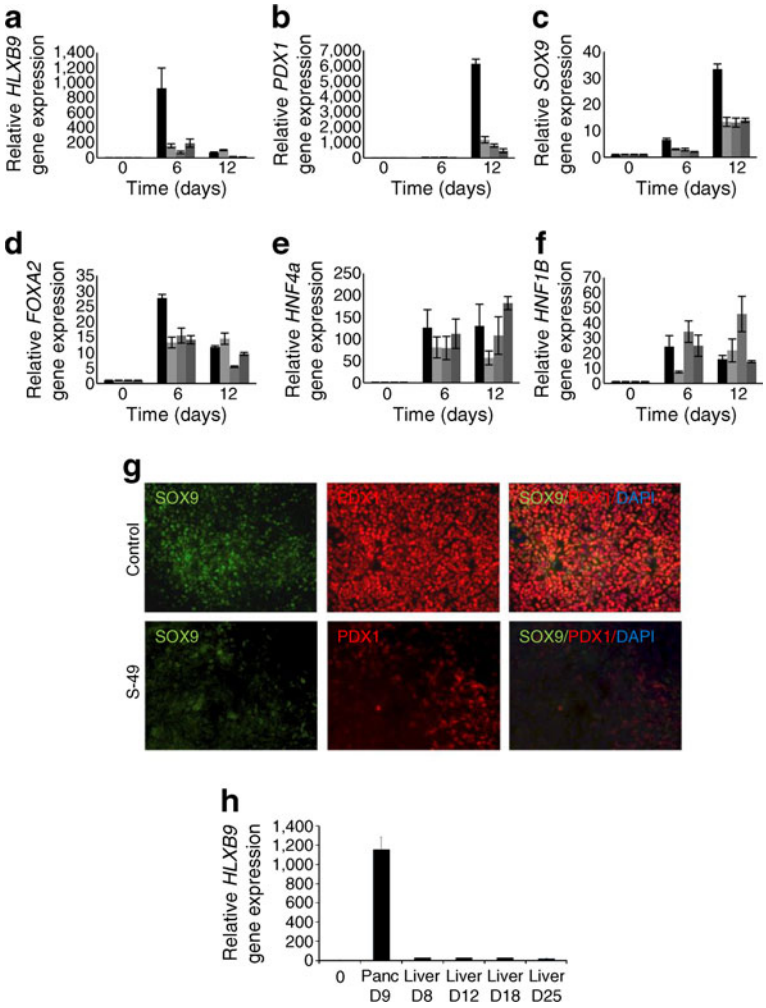

Fig. 7 HLXB9 is necessary for pancreatic specification of DF in vitro. (**a**–**f**) qPCR analyses showing the effect of hHLXB9 knockdown on pancreatic differentiation. All points normalised to undifferentiated shScramble. (**g**) Expression of pancreatic endoderm markers in sh*HLXB9*-hESCs grown for 12 days in culture conditions inductive for pancreatic differentiation. Scale bar 50 μm. (**h**) Expression of *HLXB9* during hepatic differentiation. hESCs were grown for 25 days in culture inductive for hepatic differentiation and analysed for the expression of *HLXB9* every 6 days using qPCR. hESCs were used as negative control and pancreatic progenitors differentiated for 9 days (Panc D9) were used as a positive control

methods often contain undefined animal products such as feeders or FBS. To address these challenges, we screened defined culture conditions to differentiate human DE into a near homogenous population of pancreatic and liver endoderm from multiple hPSC lines. The result of this screening shows that RA has an essential function in promoting pancreatic specification while BMP signalling blocks the expression of the pancreatic marker *PDX1*, reinforcing previous studies [21, 22]. However, our results concerning the function of FGF signalling contradict those of previous studies [27] by suggesting that FGF acts as a permissive rather than an inductive signal of pancreatic specification.

This apparent divergence might be explained by the absence in our culture conditions of feeders, serum and Matrigel, all of which contain unknown components that are likely to interfere with FGF signalling. In addition, we observed that inhibition of FGF signalling decreases cell survival of pancreatic progenitors, thus justifying the use of FGFs in our protocol. More importantly, our analyses also revealed that activin/TGF-β controls DE cell fate choice towards the pancreas lineage by inhibiting DF specification while promoting the hepatic lineage. Previous studies have shown that TGF-β signalling controls ventral pancreatic bud induction in the mouse embryo [15] and, thus, our data demonstrate for the first time that similar mechanisms could occur in the dorsal pancreas, confirming the interest of our culture system in modelling foregut development in vitro. Interestingly, a recent report has shown that inhibition of TGF-β signalling can block gut specification induced by WNT signalling [24] and also promote endocrine differentiation of *PDX1*-expressing progenitors [28, 29]. Thus, TGF-β can control different stages of pancreatic development. Further studies will be necessary to fully understand the successive functions of TGF-β pathways during primitive tube patterning and organogenesis.

Our results also show that activin/TGF-β signalling directs hepatic vs pancreatic specification by controlling transcription factors directing foregut differentiation, such as HEX and HLXB9. Interestingly, we recently identified HEX as a direct target of Smad2/3 using chromatin immunoprecipitation-sequencing analyses in differentiating DE cells [30], and thus activin/TGF-β signalling could

'directly' control hepatic specification by maintaining *HEX* expression during DE development. Our findings are in keeping with a recent demonstration that HEX plays a pivotal role in the induction of liver development from mouse embryonic stem-cell-derived endoderm [31], and the observation that transient overexpression of *HEX* in hESCs and hIPSCs enhances the formation of hepatoblasts [32]. However, our findings also suggest an essential function for HEX in the early stage of hepatic specification, which differs from the results obtained with mouse embryos showing that HEX is not necessary for liver bud induction [33]. Nevertheless, these experiments were performed either with whole embryos or with culture explant, mixing several tissues grown in culture media containing serum and unknown factors. Our culture system is based on defined culture conditions that might not contain all of the necessary factors to maintain hepatic endoderm in the absence of HEX. This hypothesis is in agreement with recent studies indicating that BMP and HEX display a synergistic effect on hepatic specification [31].

Similarly, activin/TGF-β signalling could inhibit pancreatic specification by blocking *HLXB9* expression, which is necessary for the subsequent induction of PDX1 [10, 11]. This mechanism is likely to involve other factors since Smad2/3 was not detected on the HLXB9 promoter upon DE differentiation and thus cannot play a 'direct' repressive function. The importance of HLXB9 also indicates that cells generated in vitro have a DF origin, since HLXB9 is not necessary for ventral bud development [10, 11]. Nevertheless, further studies will be necessary to confirm this hypothesis and to identify additional culture conditions for generating ventral pancreatic cells.

Finally, these results have important practical significance since protocols currently available to generate pancreatic cells from hPSCs often rely on feeders, Matrigel and serum, all of which represent a potential source of TGF-β signalling with the capacity to compromise pancreatic specification. Moreover, recent studies have shown that endogenous levels of nodal expression might determine the capacity of specific hIPSC lines to differentiate into mesodermal derivatives [34]. Such differences in the endogenous level of nodal/TGF-β growth factors could affect the capacity of diverse hPSC lines to differentiate into pancreatic endoderm, and the inhibition of this signalling pathway with SB could bypass this limitation. Accordingly, we recently differentiated ten hIPSC lines into pancreatic endoderm using our four-step protocol, and observed that only those hIPSC lines that failed to differentiate into DE (two out of ten) also lacked the ability to differentiate into pancreatic cells (M. Brimpari, University of Cambridge, Cambridge, UK and L. Vallier, personal observations).

Another advantage of inhibiting TGF-β signalling during pancreatic specification resides in the possibility of eliminating contaminating pluripotent cells. Indeed, we and others have extensively demonstrated that inhibition of activin/nodal/TGF-β signalling induces differentiation of hPSCs [35]. Thus, inhibition of activin during DE specification could decrease contamination by undifferentiated cells. Accordingly, we have failed to observe teratoma formation in mice transplanted with pancreatic progenitors. Therefore, inhibiting activin signalling during pancreatic specification could allow for the generation of 'safer' pancreatic progenitor for potential cell-based therapy.

To conclude, our study could greatly facilitate the production of homogenous populations of pancreatic and liver cells in defined culture conditions for clinical applications. However, this culture system also provides a robust and efficient in vitro model of development to study human endoderm differentiation.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement CH-HC and NR-FH contributed equally to this study. All authors participated in concept and design of the study as well as collection, assembly and interpretation of data. CH-HC, NR-FH, KD and LV prepared the manuscript and all authors revised, edited and approved the final version of the manuscript.

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

ELECTRONIC SUPPLEMENTARY MATERIALS.

Candy Cho*, Nicholas Hannan*, and colleagues.

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.

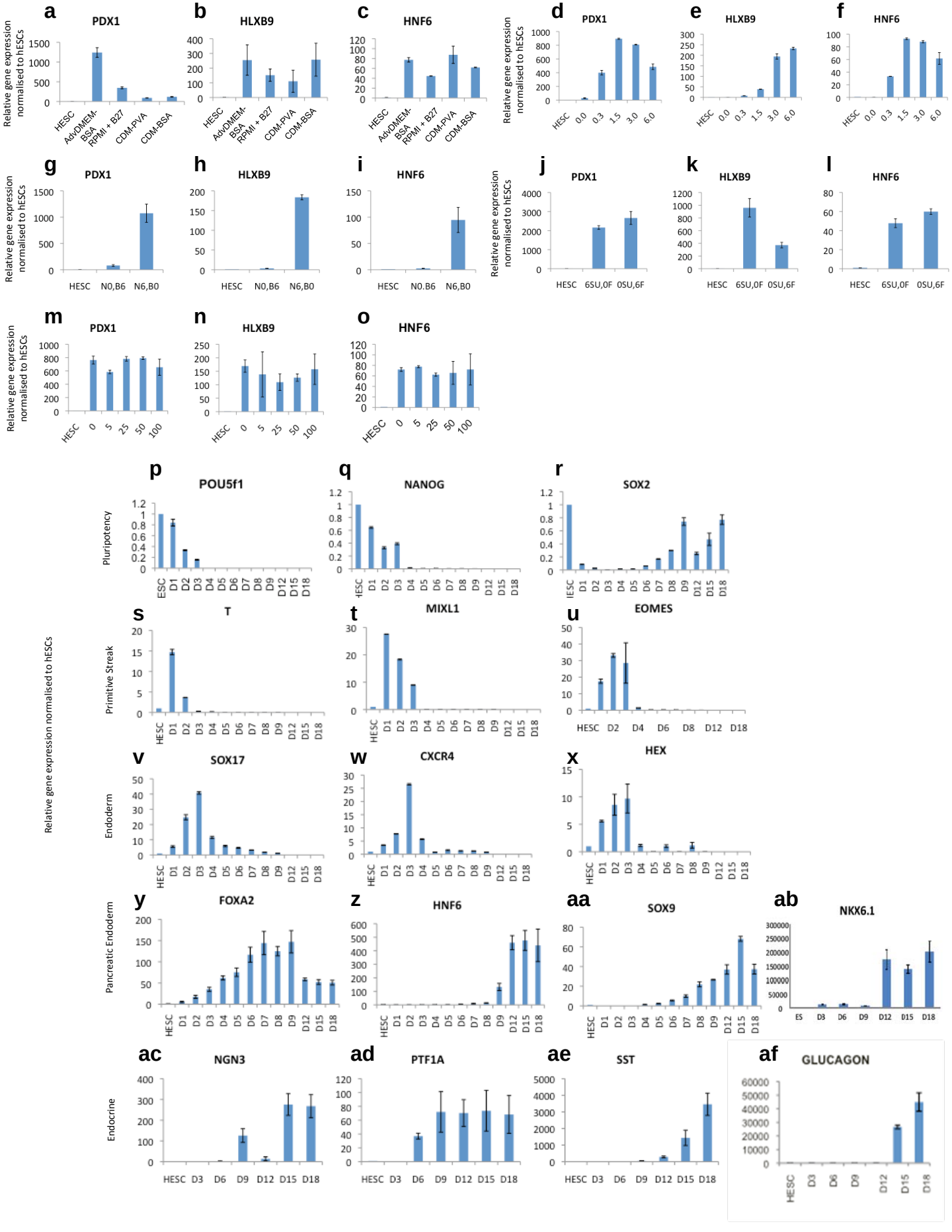
Flow Cytometry

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.

Table 1: Antibodies and corresponding dilution

Target protein	Dilution	Company
C-PEPTIDE	ICC 1:300	ACRIS ANTIBODIES
CYTOKERATIN 18	ICC 1:100	SANTA CRUZ
GATA-4 (G-4)	ICC 1:100	SANTA CRUZ
GATA6	ICC 1:100	ABCAM
GLUCAGON (N-17)	ICC 1:100	SANTA CRUZ
HNF1B (C-20)	ICC 1:100	SANTA CRUZ
HNF3B/FOXA2	ICC 1:100	R&D SYSTEMS
HNF4 (H-171)	ICC 1:100	SANTA CRUZ:
HNF-6 (H-100)	ICC 1:100	SANTA CRUZ
NGN3	ICC 1:300	R&D SYSTEMS
NKX6.1 (GS-9A8)	ICC 1:50	THE DEVELOPMENTAL STUDIES HYBRIDOMA BANK
PDX-1/IPF1	ICC/ 1:300	R&D SYSTEMS
PDX-1/IPF1 (IC2419A)	FC	R&D SYSTEMS
PRO-INSULIN	ICC 1:50	THE DEVELOPMENTAL STUDIES HYBRIDOMA BANK
SOMATOSTATIN	ICC 1:200	DAKO
SOX9 H-90	ICC 1:100	SANTACRUZ:
ALEXA FLUOR® 488 DONKEY ANTI-MOUSE IGG (H+L) *2 MG/ML*	ICC 1:2000	INVITROGEN
ALEXA FLUOR® 488 GOAT ANTI-GUINEA PIG IGG (H+L) *2 MG/ML*	ICC 1:2000	INVITROGEN
ALEXA FLUOR® 488 DONKEY ANTI-GOAT IGG (H+L) *2 MG/ML*	ICC 1:2000	INVITROGEN
ALEXA FLUOR 568 DONKEY ANTI GOAT IGG (H+L) *2 MG/ML*	ICC 1:2000	INVITROGEN
ALEXA FLUOR 568 DONKEY ANTI RABBIT IGG (H+L) *2 MG/ML*	ICC 1:2000	INVITROGEN
ALEXA FLUOR® 647 DONKEY ANTI-GOAT IGG (H+L) *2 MG/ML*	FL 1:2000	INVITROGEN
ALEXA FLUOR® 647 DONKEY ANTI-RABBIT IGG (H+L) *2 MG/ML*	FL 1:2000	INVITROGEN
APC-conjugated isotype control	FL	R&D SYSTEMS

#


a
PDX1
b
HLXB9
c
HNF6
d
PDX1
e
HLXB9
f
HNF6
Relative gene expression normalised to hESCs
HESC
Adv/DMEM-BSA
RPMI + B27
CDM-PVA
CDM-BSA
0.0
0.3
1.5
3.0
6.0
g
PDX1
h
HLXB9
i
HNF6
j
PDX1
k
HLXB9
l
HNF6
N0,B6
N6,B0
6SU,0F
0SU,6F
m
PDX1
n
HLXB9
o
HNF6
0
5
25
50
100
Relative gene expression normalised to hESCs
p
POU5f1
q
NANOG
r
SOX2
Pluripotency
ESC
D1
D2
D3
D4
D5
D6
D7
D8
D9
D12
D15
D18
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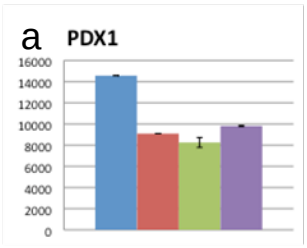
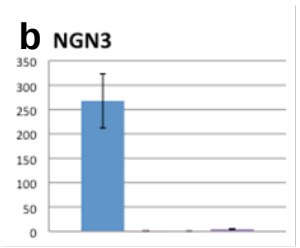
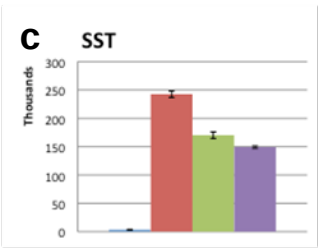
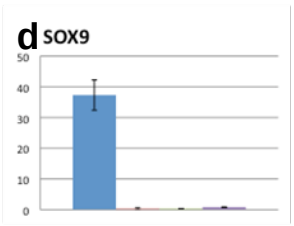
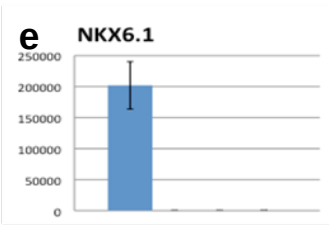
t
MIXL1
u
EOMES
Primitive Streak
HESC
D2
D4
D6
D8
v
SOX17
w
CXCR4
x
HEX
Endoderm
y
FOXA2
z
HNF6
aa
SOX9
ab
NKX6.1
Pancreatic Endoderm
ES
D3
ac
NGN3
ad
PTF1A
ae
SST
af
GLUCAGON
Endocrine

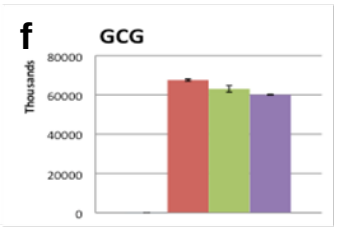

a
PDX1
16000
14000
12000
10000
8000
6000
4000
2000
0

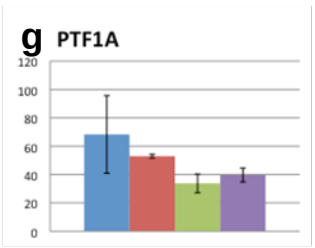

b
NGN3
350
300
250
200
150
100
50
0

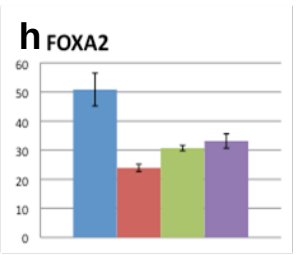

c
SST
Thousands
300
250
200
150
100
50
0

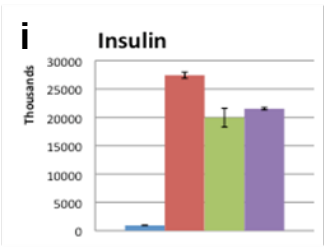

d
SOX9
50
40
30
20
10
0

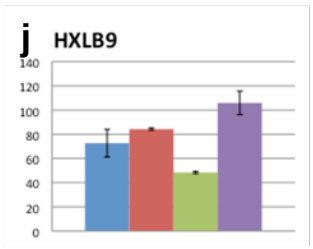

e
NKX6.1
250000
200000
150000
100000
50000
0

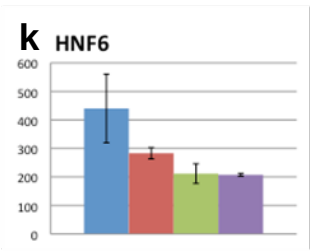

f
GCG
Thousands
80000
60000
40000
20000
0

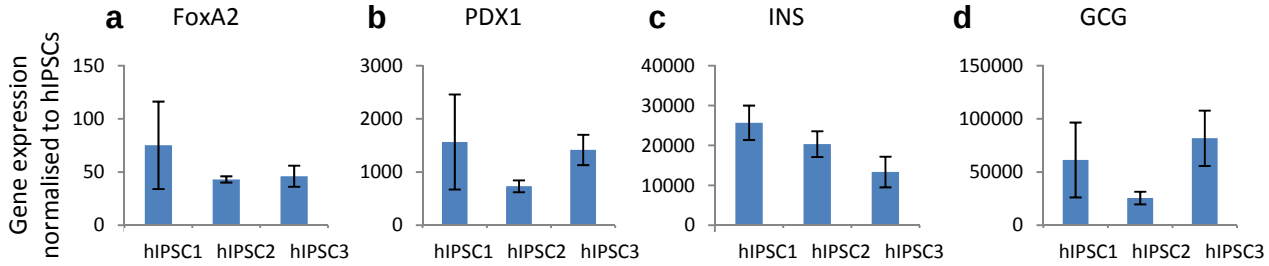

g
PTF1A
120
100
80
60
40
20
0

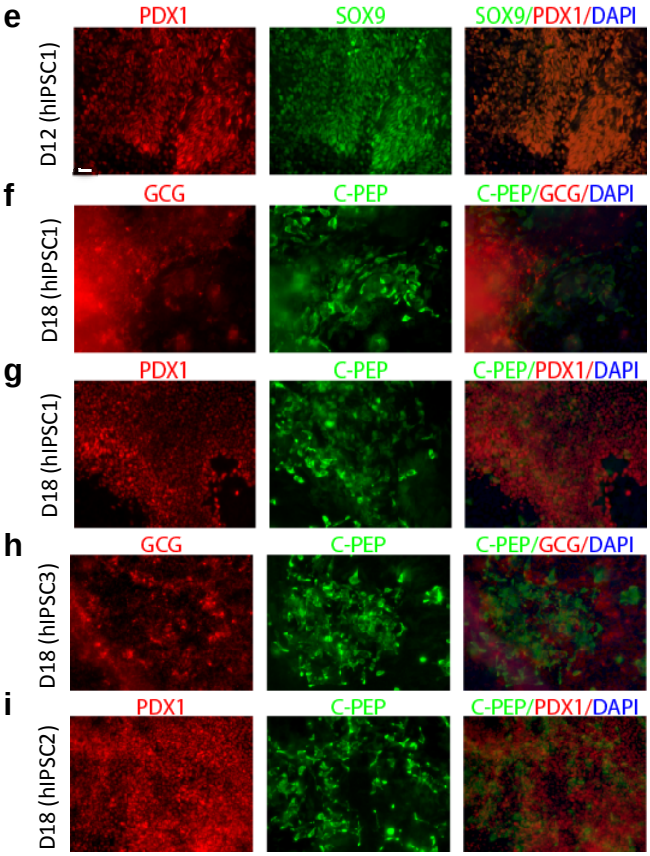

h
FOXA2
60
50
40
30
20
10
0

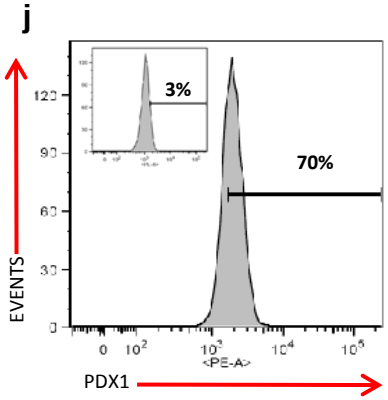

i
Insulin
Thousands
30000
25000
20000
15000
10000
5000
0

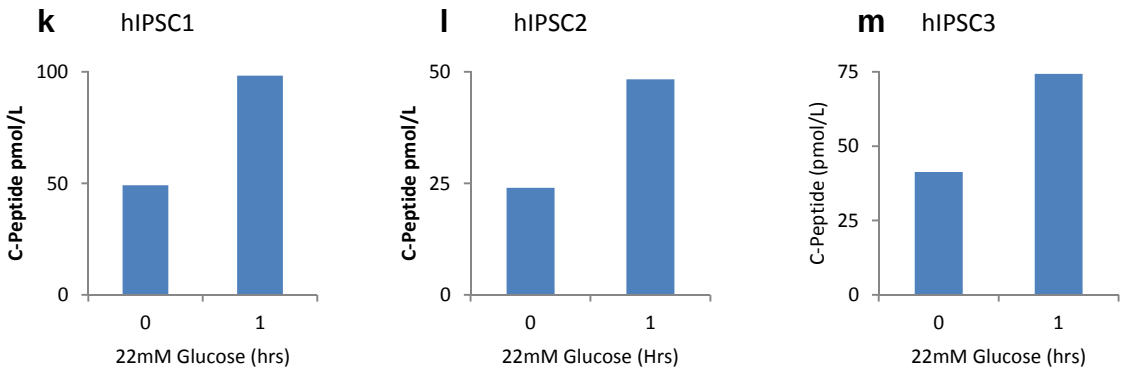

j
HXLB9
140
120
100
80
60
40
20
0


k
HNF6
600
500
400
300
200
100
0


a
FoxA2
b
PDX1
c
INS
d
GCG
Gene expression normalised to hIPSCs
150
100
50
0
3000
2000
1000
0
40000
30000
20000
10000
0
150000
100000
50000
0
hIPSC1
hIPSC2
hIPSC3


e
PDX1
SOX9
SOX9/PDX1/DAPI
D12 (hIPSC1)
f
GCG
C-PEP
C-PEP/GCG/DAPI
D18 (hIPSC1)
g
PDX1
C-PEP
C-PEP/PDX1/DAPI
D18 (hIPSC1)
h
GCG
C-PEP
C-PEP/GCG/DAPI
D18 (hIPSC3)
i
PDX1
C-PEP
C-PEP/PDX1/DAPI
D18 (hIPSC2)


j
3%
70%
EVENTS
PDX1
<PE-A>


k
hIPSC1
l
hIPSC2
m
hIPSC3
C-Peptide pmol/L
C-Peptide (pmol/L)
22mM Glucose (hrs)
22mM Glucose (Hrs)