A novel chemically directed route for the generation of definitive endoderm from human embryonic stem cells based on inhibition of GSK-3

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Summary

The use of small molecules to ‘chemically direct’ differentiation represents a powerful approach to promote specification of embryonic stem cells (ESCs) towards particular functional cell types for use in regenerative medicine and pharmaceutical applications. Here, we demonstrate a novel route for chemically directed differentiation of human ESCs (hESCs) into definitive endoderm (DE) exploiting a selective small-molecule inhibitor of glycogen synthase kinase 3 (GSK-3). This GSK-3 inhibitor, termed 1m, when used as the only supplement to a chemically defined feeder-free culture system, effectively promoted differentiation of ESC lines towards primitive streak (PS), mesoderm and DE. This contrasts with the role of GSK-3 in murine ESCs, where GSK-3 inhibition promotes pluripotency. Interestingly, 1m-mediated induction of differentiation involved transient NODAL expression and Nodal signalling. Prolonged treatment of hESCs with 1m resulted in the generation of a population of cells displaying hepatoblast characteristics, that is expressing α-fetoprotein and HNF4α. Furthermore, 1m-induced DE had the capacity to mature and generate hepatocyte-like cells capable of producing albumin. These findings describe, for the first time, the utility of GSK-3 inhibition, in a chemically directed approach, to a method of DE generation that is robust, potentially scalable and applicable to different hESC lines.

Key words: Embryonic stem cells, Directed differentiation, Definitive endoderm, Glycogen synthase kinase 3 (GSK-3) inhibition, Hepatic differentiation

Introduction

Human embryonic stem cells (hESCs) self-renew and have the potential to differentiate into all cells comprising the three embryonic germ layers, making them an attractive source of cells for use in regenerative medicine. The ability to efficiently generate definitive endoderm (DE), the precursor cell type to the liver, pancreas, lungs, thyroid and intestines, is of great clinical importance (Zaret, 2008). A particular interest is in the derivation of endoderm with hepatic potential for therapeutic and pharmaceutical applications. A chronic shortage of donors currently limits whole organ and isolated hepatocyte transplantation (Fox et al., 1998), and although primary human hepatocytes are used in pharmaceutical screening, they de-differentiate in culture and show wide inter-individual variation in their responses (Elaut et al., 2006). Therefore, alternative sources of human hepatocyte-like cells (HLCs) for both therapeutic purposes and for drug screening are urgently required.

Normal development provides important clues to understanding the control of ESC differentiation (Zaret, 2008). The mesoderm and DE are specified in the anterior region of the primitive streak (PS) during early embryogenesis and arise from a common mesendoderm progenitor population (Tada et al., 2005). Wnt and TGFβ signalling play crucial roles during the formation of the PS, mesoderm and DE (Tamura and Loebel, 2007). Mice lacking components of the Wnt signalling pathways fail to develop a PS and lack mesoderm (Huelsken et al., 2000; Kelly et al., 2004; Liu et al., 1999; Yoshikawa et al., 1997), whereas upregulation of Wnt target genes leads to premature epithelial–mesenchymal transition (Kemler et al., 2004). The subsequent specification of the anterior region of the PS relies on the TGFβ family member Nodal, with higher levels specifying the DE and lower levels specifying mesoderm (Lowe et al., 2001; Vincent et al., 2003).

In ESCs, manipulations of Nodal and Wnt signalling pathways have been exploited to direct differentiation towards the DE. Activin A, which activates the Nodal pathway, directs DE formation from mesendoderm precursors in mouse (Gadue et al., 2006; Kubo et al., 2004; Morrison et al., 2008; Tada et al., 2005; Takenaga et al., 2007; Yasunaga et al., 2005) and human (D’Amour et al., 2005; McLean et al., 2007) ESCs, whereas synergistic activation of Nodal and Wnt-β-catenin signalling promotes more efficient generation of DE from hESCs (D’Amour et al., 2006; Hay et al., 2008; Sumi et al., 2008). Activation of Wnt signalling can be mimicked by inhibition of glycogen synthase kinase 3 (GSK-3), and in mouse and human ESCs Wnt signalling and GSK-3 inhibition have been implicated in both self-renewal (Hao et al., 2006; Ogawa et al., 2006; Sato et al.,...
2004; Singla et al., 2006) and differentiation (Dravid et al., 2005; Gadue et al., 2006; Lindsley et al., 2006; Nakanishi et al., 2009).

The use of small molecule inhibitors represents a powerful and scalable approach for efficiently and reproducibly directing the differentiation of stem cells towards a desired cell type (Borowiak et al., 2009; Chen et al., 2009; Xu et al., 2008). We have previously synthesised and characterised a panel of compounds that inhibit GSK-3 in mouse ESCs, resulting in enhanced self-renewal (Bone et al., 2009). Given the conflicting roles reported for GSK-3 in hESCs (Dravid et al., 2005; Sato et al., 2004), here we investigated its role in hESCs in greater detail. We discovered that rather than promoting self-renewal, treatment of hESCs with GSK-3 inhibitors led to differentiation towards the PS, mesoderm and DE. Importantly, 1m-derived DE was capable of maturing into cells with a hepatic phenotype. Our studies reveal the utility of a single small molecule, with a known mechanism of action, in chemically directing differentiation of hESCs into DE with the potential to generate cells of the hepatic lineage.

Results

Treatment with the GSK-3 inhibitor 1m does not support pluripotency of hESCs but induces differentiation

We previously synthesised and characterised a panel of compounds that robustly inhibit GSK-3 in mouse ESCs and that enhance ESC self-renewal (Bone et al., 2009). Given the conflicting evidence regarding the role of GSK-3 in hESCs, we examined the effects of our most potent and specific GSK-3 inhibitor on hESC fate. Treatment of hESCs with a dose of 2 μM 1m (structure shown in supplementary material Fig. S1) did not maintain self-renewal but instead appeared to induce differentiation of the Shef-3 hESC line cultured either on mouse embryonic fibroblasts (MEFs) or in a feeder-free chemically defined system on Matrigel in mTeSR1 medium (Fig. 1A). Expression of surface markers of pluripotency (Tra-1-60 and SSEA4) decreased following 1m treatment (Fig. 1B and supplementary material Fig. S2A), whereas vehicle alone (DMSO) had no effect (Fig. 1A,B). The decrease in SSEA4 expression observed with 1m-treated Shef-3 cells co-cultured on MEFs was less than that observed with mTeSR1 (supplementary material Fig. S2A), but was nonetheless consistent (Fig. 1B). It is possible that factors produced by MEFs slow the loss of SSEA4, accounting for this observation. The reduction in Tra-1-60 and SSEA4 expression was accompanied by loss of OCT4 and NANOG gene expression (Fig. 1C) and OCT4 protein expression (Fig. 1D), and similar results were observed with Shef-1 hESCs cultured on feeders (supplementary material Fig. S3). Importantly, under chemically defined feeder-free conditions, treatment with 2 μM 1m led to a modest (~twofold) enhancement in hESC viability and proliferation (supplementary material Fig. S2B). For comparison, we also investigated the influence of the structurally unrelated GSK-3 inhibitor BIO and interestingly discovered its effects were dependent on culture conditions (Fig. 1). hESCs retained pluripotency when cultured on MEFs in the presence of BIO. However, when cultured in mTeSR1, 2 μM BIO, as for 1m, also induced differentiation.

In view of these results, it was important to confirm the ability of 1m to inhibit GSK-3 in hESCs. In vitro assays had generated an
IC_{50} value for GSK-3β inhibition by 1m of 3 nM, and in mouse ESCs 1m treatment led to activation of the canonical Wnt pathway, exemplified by decreased β-catenin phosphorylation and activation of TCF/LEF transcriptional activity (Bone et al., 2009). As shown in Fig. 2A, both BIO and 1m induced a dose-dependent increase in the levels of β-catenin phosphorylation at GSK-3-dependent sites, 1m consistently leading to a greater reduction. Similar to our observations in mouse ESCs (Bone et al., 2009), 1m did not alter ERK phosphorylation, whereas, under chemically defined conditions, treatment with high concentrations of BIO led to a reduction in ERK phosphorylation (Fig. 2B). Treatment with 2 or 5 μM 1m (doses that decrease β-catenin phosphorylation by >80%) led to increases in β-catenin-mediated TCF/LEF transcriptional activity (Fig. 2C) whereas BIO, even at doses up to 5 μM, did not lead to consistent increases in transcriptional activity. One possible explanation is that levels of unphosphorylated β-catenin have to increase above a threshold level in order to permit efficient nuclear translocation and activation of LEF/TCF transcription. This might occur when β-catenin phosphorylation is reduced by >80% (as with 1m), whereas the 60–70% reduction effected by BIO might lead to insufficient accumulation and, hence, the result being more variable. These results indicate that 1m robustly activates the Wnt–β-catenin pathway and leads to loss of pluripotency of both Shef-1 and Shef-3 hESCs under two different growth conditions. We observed a similar induction of Wnt signalling, and loss of self-renewal, following treatment with another of our GSK-3 inhibitors, compound 1o (supplementary material Fig. S4).

Treatment of hESCs with 1m induces differentiation towards the primitive streak, mesoderm and definitive endoderm

On the basis of the knowledge that Wnt–β-catenin signalling plays a pivotal role in formation of the PS, mesoderm and endoderm in ESCs (Hay et al., 2008; Nakanishi et al., 2009; Sumi et al., 2008), we investigated whether treatment of hESCs with 1m could induce differentiation towards these lineages. The following experiments were all performed on hESCs cultured feeder-free on Matrigel in chemically defined mTeSR1 medium. This system has the advantage of simplicity and reliability, leading to consistent and reproducible cell culture and differentiation. Initially, the effects of 1m treatment on gene expression in hESCs cultured over a 7-day period were examined (Fig. 3A). Induction of differentiation was indicated by loss of OCT4 and NANOGEN expression. The emergence of the PS and mesendoderm is characterised by expression of MIXL1 (Davis et al., 2008; Pearce and Evans, 1999) and GSC, the gene encoding the homeobox protein goosecoid (Tada et al., 2005), which were both rapidly upregulated following 1m treatment. In addition, T, the gene encoding the brachyury protein, which is expressed in the PS and mesendoderm, and also the early mesoderm (Kubo et al., 2004; Wilkinson et al., 1990), displayed a similar expression profile. Expression of brachyury protein emerged on day 3 and was maintained to day 7 (Fig. 3B). We also observed expression of other early mesodermal markers including KDR (also known as FIK1 and VEGFR2), MSX1 and PDGFRB (Fig. 3A). In addition, we observed some expression of PDGFRβ protein following 7 days of differentiation (Fig. 3B), indicating mesendodermal differentiation. Expression of FOXA2 [encoding hepatocyte nuclear factor (HNF) 3β] and CXCR4, expressed in the PS and maintained in definitive endoderm progenitors, were also rapidly induced, followed by the DE marker SOX17. CXCR4 is expressed in the developing mesoderm and definitive endoderm but not the visceral endoderm (VE) (McGrath et al., 1999) and has been used to distinguish DE from VE in both mouse and human ESCs (D’Amour et al., 2005; Yasunaga et al., 2005). Expression of CXCR4 along with SOX17, GSC and FOXA2, is consistent with differentiation towards the DE. Importantly, hESCs expressing CXCR4 are capable of differentiating into endodermal cells with either a pancreatic (D’Amour et al., 2006) or hepatic (Gouon-Evans et al., 2006) phenotype. Following 7 days of treatment with 1m, we observed expression of HNF4A and AFP (encoding α-fetoprotein), the latter marking the earliest stage of specification to the hepatic lineage.
HNF4A is a key transcription factor that regulates hepatic morphogenesis and differentiation (Parviz, 2003). Together, expression of AFP and HNF4A suggests transition from the DE through to an early hepatic lineage (hepatoblast). Protein expression of the endodermal markers FOXA2, SOX17 and HNF4α was confirmed by immunostaining in Shef-3 and Shef-1 hESCs and demonstrated that there was time-dependent differentiation towards the DE (Fig. 3B; supplementary material Fig. S5A). Treatment of hESCs with BIO under the same culture conditions also induced differentiation towards the DE (supplementary material Fig. S5B).

Our results indicate that inhibition of GSK-3 with 1m promotes differentiation of hESCs, first to a state resembling primitive streak and then towards both mesodermal and endodermal lineages.

**Involvement of activin and Nodal signalling in 1m-induced DE formation**

We were particularly interested in the ability of 1m to induce the generation of DE, as this could represent a novel route to generation of hepatic lineages. Genetic studies in mice have revealed a role for Nodal during initiation of the PS and formation of DE (Conlon et al., 1994; Brennan et al., 2001), with the intensity and duration of Nodal signalling influencing specification of the anterior PS to either mesoderm or, at higher levels, endoderm (Lowe et al., 2001; Vincent et al., 2003). Therefore, we were initially interested in testing whether Nodal signalling was involved in 1m-mediated effects. We found that 1m induced a rapid and transient increase in NODAL gene expression (despite replenishment every 2 days; Fig. 4A), which was partially inhibited by the inhibition of activin receptor-like kinases (ALK4, ALK5 and ALK7) with SB43125 (Fig. 4B). Treatment with SB34125 dramatically reduced 1m-induced GSC and FOXA2 expression, downstream targets of Nodal signalling, and caused a delay in expression of brachyury (T) (Fig. 4B). These data suggest that 1m-induced expression of NODAL and Nodal signalling could be required either for induction of differentiation into PS or later during specification of DE. Owing to the fact that expression of brachyury (T) is delayed, rather than blocked, we favour the latter possibility. Secondly, we compared the effects of 1m or activin A alone and in combination on the generation of DE. Activin A, another member of the TGFβ superfamily, binds to the same receptors as Nodal and is used to mimic Nodal activity in vitro. Generation of DE from hESCs has relied on activation of the activin–Nodal pathway either alone (D’Amour et al., 2005) or in combination with Wnt signalling (D’Amour et al., 2006; Hay et al., 2008; Sumi et al., 2008), which enhances efficiency. Gene expression patterns were similar for all conditions after 3 days (Fig. 4C) and after 7 days SOX17, FOXA2 and HNF4A gene expression were also similar. However, although little or no GSC expression was apparent following treatment with 1m alone for 7 days, indicative of transition through the PS, GSC expression was maintained in the presence of activin A alone or in combination with 1m. Intriguingly, AFP expression was only detected in samples treated with 1m alone for 7 days (Fig. 4C) and
consistently correlated with a decrease in NODAL expression, whereas activin A, both alone and in combination with 1m, maintained the increased NODAL levels. Nodal signalling persists during gastrulation until formation of the DE, after which expression falls (Collignon et al., 1996), and in mouse ESCs induction of Nodal expression results in DE specification, with subsequent downregulation promoting maturation of the DE (Takanaga et al., 2007). Therefore, we propose 1m promotes transient Nodal signalling, with the initial increased levels involved in promoting specification to DE and a subsequent decrease allowing maturation to an early hepatic phenotype.

To examine the efficiency of DE formation, we analysed FOXA2 and HNF4α expression by immunostaining (Fig. 4D). Following 1m treatment, FOXA2 expression was detected at a high frequency (73%±13%) in clusters of cells (Fig. 4D), whereas the overall proportion of FOXA2-positive cells was 42.5%±8.9%. Activin A treatment generated a lower proportion of cells expressing FOXA2 (20.3%±7.7%), which were dispersed throughout the population. Addition of activin A further enhanced 1m-induced FOXA2 expression (65.5%±15.2%) and the proportion of HNF4α-expressing cells (68.4%±1.7%) compared with 1m (32.1%±13.7%) or activin A (27.6%±12.4%) alone (Fig. 4D). The combination of 1m and activin A also enhanced the proportion of cells expressing CXCR4 (61.0%) compared with activin A (33.1%) or 1m (20.9%) alone (Fig. 4E). Our results suggest that, in chemically defined cultures, 1m-induced DE formation is further enhanced by activin A. However, the lack of AFP expression at later time-points suggests that the continued presence of activin A inhibits subsequent differentiation towards an early hepatic phenotype.

1m-induced DE has hepatocyte-like potential in vitro

One of our main interests is in generation of HLCs, so we next tested whether 1m-induced DE was capable of differentiating into HLCs. The ability to generate HLCs provides an indication of the developmental potential of the chemically derived DE, where the ultimate aim is the generation of hepatocytes displaying functional characteristics. To evaluate this potential, we employed a differentiation protocol based on that of Agarwal (Agarwal et al., 2008) (Fig. 5A). Untreated hESCs did not survive the hepatic induction and maturation regimes. However, following hepatic induction of 1m-induced DE, stage II cells began to adopt morphological features characteristic of cells of the hepatic lineage (Fig. 5B) and expressed the genes encoding α-fetoprotein (AFP), albumin (ALB), transferrin (TFN; officially known as TF), transthyretin (TTR) and α1-antitrypsin (AAT; officially known as SERPINA1), correlating with initial hepatic differentiation (Fig. 5C). Following hepatic maturation, stage III cells exhibited characteristics of hepatocytes including a polygonal morphology, distinct round nuclei with 1 or 2 prominent nucleoli, and the presence of binucleate cells (Fig. 5B,D). Increased ALB and cytochrome P450 3A family (CYP3A) gene expression (Fig. 5C) and increased HNF4α, AFP and TTR protein expression (Fig. 5D) were observed in stage III compared with that in stage II cells. Equivalent amounts of RNA were used for the reverse transcription reaction that were then subjected to PCR; however, human liver expresses proportionally less β-actin-encoding mRNA than hESCs, which results in the apparent absence of β-actin in the human liver samples. To assess liver-specific function, secretion of the key serum proteins, AFP and albumin, was determined. Both AFP and albumin (Fig. 5E,F) were detected in the culture medium of stage III cells derived from 1m-induced DE. These results indicate that 1m-derived definitive endoderm has the potential to differentiate into cells with hepatocyte characteristics. Similar results were also generated using BIO-derived DE cells (supplementary material Fig. S6).

**Discussion**

Human embryonic stem cells have much to offer as a source of differentiated cells for a wide variety of applications both in regenerative medicine and pharmaceutical research. One of the crucial requirements for realising this potential is the development of scalable, robust and reproducible systems that facilitate the differentiation of hESCs into specialised cell types. There is much interest in generating functional hepatocytes to meet the needs of cell replacement therapies and for use in toxicity screening (Zaret, 2008). Here, we report a novel chemically directed approach for the generation of DE with hepatic potential utilising a single small-molecule inhibitor of GSK-3 [referred to as 1m (Bone et al.,
which, when used as the only supplement to a chemically defined culture system, is capable of robustly promoting differentiation of hESCs to both DE and mesoderm. Our novel scheme of chemically directed differentiation offers a number of advantages over other published protocols; it is simple and robust, requiring a single chemical entity to direct differentiation to DE and on towards hepatoblasts, it relies on monolayer-based procedures, utilises chemically defined media, is applicable to distinct hESC lines and is readily scalable.

Chemically directed differentiation is an attractive approach for the generation of specific cell types from hESCs (Xu et al., 2008). The data presented here demonstrate that inhibition of GSK-3 by 1m seems to mimic the actions of Wnt signalling by promoting differentiation of hESCs, first to a state resembling the primitive streak and then, via mesendodermal intermediates, into both mesoderm and definitive endoderm. Thus, treatment of hESCs with 1m could find utility in generation not only of endodermal derivatives, our primary interest, but also of mesodermal cells. 1m shares some properties with the two small molecules, IDE1 and IDE2, that were identified as a result of their ability to induce differentiation of mouse and human ESCs to the endodermal lineage (Borowiak et al., 2009). Another small molecule, indolactam V, has been shown to induce the generation of pancreatic progenitors from hESCs (Chen et al., 2009). IDE1 and IDE2 both appear to activate Nodal-Smad signalling, although the direct biochemical targets of these molecules have not been reported (Borowiak et al., 2009). Here, we have shown that 1m induces a transient increase in NODAL gene expression, which is detectable within 24 hour and declines by day 4. By contrast, IDE1 and IDE2 cause sustained expression of NODAL mRNA (Borowiak et al., 2009). Importantly, we find that addition of an inhibitor to ALK 4, ALK5 and ALK7 prevents 1m-induced expression of FOXA2, suggesting that activation of Nodal signalling plays an important role in the action of 1m. Our demonstration that SB43125 delays, rather than blocks expression of brachyury (T), suggests that 1m-induced NODAL is not essential for generation of a PS-like state in hESC derivatives, as it is in vivo (Shen, 2007), but rather plays a role in specification of DE. The Nodal and Wnt pathways have well-documented interactions during early mammalian development (Shen, 2007; Tam and Loebel, 2007). In the mouse epiblast, BMP4 induces WNT3 expression, leading to activation of Wnt signalling which increases expression of Nodal and its co-receptor Cripto (Shen, 2007). Inhibition of GSK-3 could mimic this event in vitro and such crosstalk has been suggested previously in relation to maintenance of hESC pluripotency (Besser, 2004). Given that cells respond to Nodal signalling in a dose-dependent manner, in the chemically defined system we have used, it might be that after transit to mesendodermal progenitors 1m-induced NODAL expression reaches levels sufficient to promote endodermal specification in a subpopulation of cells. In contrast to IDE1 and IDE2, we have identified the biochemical target of 1m as GSK-3 and have shown that 1m activates Wnt signalling in hESCs. Furthermore, a compound in the same series as 1m, 1o (Bone et al., 2009), has a similar capacity to induce hESC differentiation.
(supplemental material Fig. S4), whereas the structurally unrelated GSK-3 inhibitor BIO, when applied to hESCs grown on Matrigel in defined medium, also directs differentiation of hESCs to DE with hepatic potential, illustrating the utility of GSK-3 inhibition for directed differentiation.

Activin A (a Nodal surrogate) either alone (Agarwal et al., 2008; D’Amour et al., 2005; McLean et al., 2007) or in combination with Wnt signalling (D’Amour et al., 2006; Hay et al., 2008; Sumi et al., 2008) leads to effective differentiation of hESCs into DE. Interestingly, 1m treatment alone led to the formation of clusters of cells within the population, a high proportion of which (73±13%) expressed FOXA2, whereas activin A alone generated FOXA2-positive cells dispersed throughout the population. These observations raise the possibility that 1m is not only directing differentiation but also promoting proliferation of progenitor cells, and our data support the ability of 1m to enhance cell viability and proliferation (supplementary material Fig. S2B). Overall, the proportion of FOXA2-positive cells we observe following 5 days treatment of either Shef-1 or Shef-3 hESCs with 1m is 35–40%. Given our findings that 1m also induces generation of mesodermal cells, it is probable that these cells account for some of the remaining population. Indeed, we cannot exclude the possibility that the mesodermal cells present play a role in promoting endodermal differentiation and hepatic specification, in a manner analogous to the role of the cardiac mesenchyme in vivo (Jung et al., 1999; Rossi et al., 2001). In comparison with 1m, following 4 days of treatment with IDE1 or IDE2, hESCs express 62% and 57% SOX17-positive cells, respectively, >90% of which are FOXA2-positive (Borowiak et al., 2009); others have reported 70% SOX17-positive cells after exposure to activin A for 4 days (Brolén et al., 2010). Activin A and WNT3A in combination enhance the efficiency of DE generation from hESCs (D’Amour et al., 2006; Hay et al., 2008; Sumi et al., 2008) and under our chemically defined conditions we generated >60% CXCR4-positive cells when 1m was used in combination with activin A, which compares favourably with recent findings (Touboul et al., 2010). To generate hepatocyte-like cells (HLCs), hESCs were initially differentiated with 2 μM 1m for 7 days; medium was refreshed every other day. These cultures were then passaged with collagenase and plated at a ratio of 1:10 on Matrigel in KO DMEM supplemented with glutamine, 50 units/ml penicillin and streptomycin, 1 mM NEAA, 2% (v/v) KOSR, 10 ng/ml hepatocyte growth factor (HGF; Peprotech) and 10 ng/ml fibroblast growth factor-4 (FGF4; Peprotech) and cultured for a further 7 days with the medium being replaced every other day. To allow for maturation of the HLCs, 10 ng/ml oncostatin M (Peprotech) and 10−7 M dexamethasone (Sigma) were added to the above medium and the cells were cultured for an additional 7 days with fresh medium every other day.

**RT-PCR**

Total RNA was isolated and purified using Trizol Reagent (Invitrogen), following the manufacturer’s instructions. All RNA samples were treated with DNase I (Ambion) before cDNA synthesis. RNA (2 μg) was reverse-transcribed using oligo(dT)17 (Promega) and SuperScript II (Invitrogen). Gene-specific PCR was performed using primers and annealing temperatures outlined in supplementary material Table S1.

**Immunoblotting**

Cell lysates (20 μg) were prepared, separated by SDS-PAGE and transferred onto nitrocellulose, as described previously (Welham et al., 1994). Immunoblotting was performed using antibodies against the following proteins: OCT4 (1:5000; sc-9081, Santa Cruz Biotechnology), GAPDH (1:2000; sc-20357, Santa Cruz Biotechnology), phosphorylated β-catenin (Ser33, Ser37 and Thr41) (1:10,000; CST 9561, Cell Signalling Technology), β-catenin (1:1000; CST 9562, Cell Signalling Technology), phosphorylated ERK1/2 (Thr202 and Tyr204) (1:1000; CST 9101, Cell Signalling Technology), ERK1 (1:1000; sc-93, Santa Cruz Biotechnology) and human α1-fetoprotein (1:10,000; A0088, DAKO). Anti-rabbit-IgG antibodies conjugated to horseradish peroxidase (HRP; DAKO) were used at 1:10,000 and blots were developed using ECL Advance (GE Healthcare) or Alka-Blue (Immotech) according to manufacturer’s directions. Blots were stripped and re-probed as described previously (Welham et al., 1994). Blots were quantified by capturing images using an ImageQuant RT ECL imaging system (GE Healthcare) and analysed using ImageQuant TL software. Levels of phosphorylated β-catenin were then normalised to β-catenin and expressed relative to the untreated sample.

**Flow cytometry**

hESCs were trypsinised, resuspended in PBS containing 2% (v/v) FBS and stained on ice for 45 minutes with antibodies against Tra-1-60 (10 μg/ml; clone MC813 Abcam), or phycoerythrin (PE)-conjugated anti-CXCR4 antibody (1:10; clone 12G5, R&D Systems). Cells were washed and stained with secondary FITC-conjugated antibodies (Sigma) where required. Flow cytometry was performed using a FACSCanto cytometer (Becton Dickenson). Data were analysed with FACSdiva software and dead cells were excluded based on forward- and side-scatter parameters.

**Luciferase reporter assay to measure β-catenin-mediated transcriptional activity**

The TOPFlash and FOPFlash luciferase reporters and Renilla luciferase control plasmid (pRL-TK) have been described previously (Bone et al., 2009). hESCs cultured on Matrigel-coated 24-well plates were transfected with 0.6 μg TOPFlash (or FOPFlash) and 0.144 μg pRL-TK (for normalisation) mixed with 3 μl Lipofectamine 2000 (Invitrogen). The transfection mixture (0.1 ml) was added to the cells in 0.5 ml fresh mTeSR1. After 24 hours, medium was replaced, compounds added and incubation continued for a further 24 hours. Cell extracts were prepared and Renilla and Firefly luciferase activities were determined using the dual-luciferase reporter assay system according to manufacturer’s instructions (Promega). TOPFlash and FOPFlash firefly luciferase activities were normalised to those of co-transfected pRL-TK Renilla luciferase activity and expressed relative to the untreated sample.
Immunofluorescence

To obtain digitally-captured images, hESCs were cultured and differentiated on Matrigel-coated LuMax (Sarstedt) trays, which have a 50-μm thick fluorocarbon gas-permeable film base. Following the culture period, hESCs were fixed with 4% (w/v) paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were permeabilised in PBST (PBS containing 0.1% Triton X-100) and blocked in 2% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were examined using a Zeiss 10 Meta confocal microscope using a 40x objective. For quantification and statistical analysis, the percentage of FOXA2- and HNF4α-expressing cells was determined from five to eight randomised fields of view for each experimental condition, across three or over four independent experiments. Results are means ±SEM.

Albumin ELISA

Anti-human-albumin antibody (Universal Biologicals, Cambridge, UK) was coated onto 96-well plates at a 1:100 dilution in 0.05 M sodium-carbonate-bicarbonate buffer pH 9.6, for 1 hour, and blocked with 1% BSA in 50 mM Tris-HCl pH 8, and 0.14% NaCl. Media and standards of human serum were diluted in 50 mM Tris-HCl pH 8, containing 0.1% NaCl, 1% BSA and 0.05% Tween-20. Plates were washed with 50 mM Tris-HCl pH 8, containing 0.14% NaCl, and 0.05% Tween-between incubations. Dilutions of the medium were incubated in wells for 1 hour, and the plates were then incubated with HRP-conjugated anti-human-albumin antibody at 1:75,000 in sample diluent, as described above, for 1 hour. Plates were visualised with a 1:1 mixture of HRP substrates and the reaction was stopped using 2 M sulphuric acid. Plates were read at 450 nm, using software that generates a four-parameter sigmoidal curve, for the quantification of samples.

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Chemically directed hESC differentiation 1999

Of the dozens of published studies using hESCs, this is the first to demonstrate chemical control of differentiation. The protocol described here can be readily adapted to other hESC lines and cell lines, and offers a powerful tool for the study of human cell development and disease. The ability to chemically control hESC differentiation will enable the development of novel therapeutic strategies for a range of disorders, including diabetes, heart disease, and neurological disorders. The approach described here provides a robust and reproducible method for the chemical control of hESC differentiation, which will be of great interest to the stem cell community.
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