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HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells

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SUMMARY

The availability of pluripotent stem cells offers the possibility of using such cells to model hepatic disease and development. With this in mind, we previously established a protocol that facilitates the differentiation of both human embryonic stem cells and induced pluripotent stem cells into cells that share many characteristics with hepatocytes. The use of highly defined culture conditions and the avoidance of feeder cells or embryoid bodies allowed synchronous and reproducible differentiation to occur. The differentiation towards a hepatocyte-like fate appeared to recapitulate many of the developmental stages normally associated with the formation of hepatocytes in vivo. In the current study, we addressed the feasibility of using human pluripotent stem cells to probe the molecular mechanisms underlying human hepatocyte differentiation. We demonstrate (1) that human embryonic stem cells express a number of mRNAs that characterize each stage in the differentiation process, (2) that gene expression can be efficiently depleted throughout the differentiation time course using shRNAs expressed from lentiviruses and (3) that the nuclear hormone receptor HNF4A is essential for specification of human hepatic progenitor cells by establishing the expression of the network of transcription factors that controls the onset of hepatocyte cell fate.

KEY WORDS: Hepatocyte differentiation, Human pluripotent stem cells, HNF4A

INTRODUCTION

Substantial headway has been made in our understanding of the molecular events that control development of the liver from studies using chick, *Xenopus*, zebrafish and the mouse as model organisms (Zaret and Grompe, 2008; Lemaigre, 2009; Si-Tayeb et al., 2010a). Advances using these models have been considerable because many of the key pathways that control hepatogenesis are evolutionarily conserved. Although such models offer numerous advantages, each has its own restrictions. For example, the ability to conduct genetic experiments in birds and frogs is limited, the extent to which biochemical experiments can be performed in zebrafish is minimal, and although biochemical, molecular and genetic analyses have been successful in mice, it remains a time consuming, expensive and often tedious undertaking.

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells can be cultured indefinitely and generally maintain a normal karyotype. In contrast to cultured primary cells, these pluripotent stem cells retain the capacity to differentiate into all cell types and this has been definitively established by using mouse ES and iPS cells to generate viable animals through tetraploid embryo complementation (Nagy et al., 1993; Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009). The fact that pluripotent stem cells can be induced to differentiate in culture into a plethora of somatic cell types has raised the possibility of using pluripotent stem cells as an alternative to embryos to investigate the fundamental molecular processes that govern cell differentiation. Early experiments using mouse embryonic stem cells relied on the use of embryoid bodies to produce differentiated cells; however, this system is somewhat chaotic, resulting in the generation of heterogeneous cell types that commonly require cell sorting to obtain useful cell populations. Even with such caveats, the study of mouse embryoid bodies has successfully provided insight into liver cell differentiation, and recent analyses using *Hex*$^{-/-}$ mouse ES cells successfully recapitulated the phenotype associated with *Hex*$^{-/-}$ mouse embryos (Keng et al., 2000; Martinez Barbera et al., 2000; Bort et al., 2004; Bort et al., 2006; Kubo et al., 2010).

As mouse ES cells are capable of reproducing the differentiation of mouse hepatocytes, it raises the issue of whether human ES (huES) cells could be used to model human hepatocyte formation. Several laboratories have recently described protocols using huES cells that allow the production of cells that display functional and gene expression characteristics that are normally associated with hepatocytes (Cai et al., 2007; Agarwal et al., 2008; Chiao et al., 2008; Shiraki et al., 2008; Basma et al., 2009). Based on such studies, we developed a protocol that facilitates differentiation of hepatocyte-like cells from both huES cells and iPS cells with efficiencies >85% (Si-Tayeb et al., 2010b). This approach avoids the use of embryoid bodies, feeder cells, fetal calf serum and other undefined components within the culture medium, which results in the differentiation being highly reproducible and synchronous. Cells generated using this approach can synthesize glycogen, secrete albumin, synthesize urea, metabolize indocyanine green, form cell-cell junctions with apical characteristics, store lipid and uptake low density lipoprotein. Importantly, the formation of hepatocyte-like cells from huES or hiPS cells closely resembles the process through which hepatocyte differentiation occurs *in vivo* (Agarwal et al., 2008; Si-Tayeb et al., 2010b). In response to specific inductive cues that are added to the medium, the human pluripotent stem cell-derived cells sequentially acquire characteristics of ventral endoderm (FOXA2, GATA4, SOX17), specified hepatic progenitor cells (HNF4A), hepatoblasts (AFP) and hepatocytes (Albumin). Because the differentiation takes place *ex vivo*, this system potentially offers a means to manipulate and address the molecular events controlling

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human hepatocyte differentiation. In the current study, we therefore sought to (1) define a characteristic mRNA fingerprint that could be used to follow the differentiation process, (2) determine whether gene function could be manipulated in this system to facilitate mechanistic studies and (3) ascertain whether the nuclear hormone receptor HNF4A is required for the onset of human hepatocyte differentiation from pluripotent stem cells.

MATERIALS AND METHODS

Cell culture

HuES cell (WA09, H9) and hiPS cell (C2A) culture was approved by the MCW SCRO committee. Cells were cultured as described elsewhere (Si-Tayeb et al., 2010b) except Matrigel was substituted for a recombinant E-cadherin-IgG Fc fusion protein matrix (Nagaoka et al., 2010) (StemAdhere STEMCELL Technologies, Vancouver, BC), which ensured homogeneity of pluripotency within the starting stem cell population. The general protocol used for differentiation has been described in detail elsewhere (Si-Tayeb et al., 2010b). However, we noted that the efficiency of definitive endoderm formation using the original protocol had declined after obtaining new batches of commercially available B-27 supplement. The possibility that B-27 quality can be variable was supported by data showing that the efficacy of culturing primary neurons is dependent on specific batches of B-27 supplement (Chen et al., 2008). It has been demonstrated that inhibition of PI-3 kinase is essential for differentiation of definitive endoderm from human pluripotent stem cells (McLean et al., 2007). Insulin, which can activate the PI-3-kinase pathway, appears to be present in B-27 at relatively high concentrations (Price and Brewer, 2001). We, therefore, included the PI-3-kinase inhibitor LY294002 during endoderm formation and found that, as reported elsewhere (McLean et al., 2007), the efficiency of definitive endoderm formation was restored to over 80%. Moreover, B-27 supplement that is free of insulin has recently become available and this also supports efficient differentiation of human pluripotent stem cells towards a definitive endoderm fate.

Plasmid construction and generation of lentiviruses

Oligonucleotides encoding shRNAs (HNF4i2: TGCAGATGTGTGTGAG-TCCATTCAAGAGATGGACTCACACACATCTGCTTTTTGGAAAC, TCGAGTTTCCAAAAAGCAGATGTGTGTGAGTCCATCTCTTGAAT-GGACTCACACACATCTGCA; HNF4i3: TGAAGATTGCCAGCAT-CGCATTCAAGAGATGCGATGCTGGCAATCTTCTTTTTGGAAAC, TCGAGTTTCCAAAAAGAAGATTGCCAGCATCGCATCTCTTGAAT-GCGATGCTGGCAATCTTCA) were annealed and cloned into pLL3.7 puro (Rubinson et al., 2003). Lentiviruses were produced by Fugene 6 (Roche Indianapolis, IN) -mediated transfection of HEK293T cells with plasmids encoding helper functions, (VSVG, RSV/REV and RRE) as described previously (Konopka et al., 2007). Transduced stem cells were selected using puromycin (2-8 μg/ml) added 2 days post-infection and 'lines' were maintained as polyclonal cultures with continuous selection.

Oligonucleotide array analysis

Total RNA was isolated from three independent experiments at each stage of hepatocyte differentiation using the RNeasy mini kit (QIAgen, Valencia, CA). Biotinylated cRNA was generated and hybridized to GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA). Images were acquired using a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA) and normalized data analyzed using DNA-Chip analyzer software (Li and Wong, 2001). CEL files (GSE25417) are available through Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

Immunostaining

Cells fixed with 4% paraformaldehyde for 30 minutes were permeabilized with 0.5% Triton X-100, blocked with 3% BSA/PBS and incubated at 4°C overnight with the following antibodies: HNF4A, 1:500 (sc-6556; Santa Cruz Biotechnology, Santa Cruz, CA); SOX17, 1:250 (AF1924; R&D Systems, Minneapolis, MN); FOXA2, 1:250 (H00003170-M12, Novus Biologicals, Littleton, CO); GATA4, 1:250 (sc-1237; Santa Cruz Biotechnology, Santa Cruz, CA); or OCT4, 1:500 (sc-9081; Santa Cruz Biotechnology, Santa Cruz, CA).

RT-PCR

RNA was isolated from stem cell-derived hepatocytes and adult cadaveric livers using the RNeasy mini kit (QIAgen, Valencia, CA), and human fetal RNA was purchased from BioChain (BioChain, Hayward, CA). Real-time quantitative polymerase chain reaction (RT-PCR) was performed on an Applied Biosystems StepOne Plus real time PCR machine using Taqman (Applied Biosystems, Foster City, CA) or PrimeTime (Integrated DNA Technologies, Coralville, IA) assays following the manufacturers' protocols. Data analysis was performed using RT2 Profiler PCR Array Data Analysis software (SABiosciences, Frederick, MD). Semi-quantitative reverse-transcriptase PCR was performed as described previously (Duncan et al., 1997). Oligonucleotide sequences are provided in Table S1 in the supplementary material.

Proliferation and apoptosis

Proliferation assays were performed using the Click-iT EdU imaging kit (Invitrogen, Carlsbad, CA) and proliferating cell numbers calculated by FACS using a Guava EasyCyte Mini System (Millipore, Billerica, MA). Apoptotic cells were measured using Guava ViaCount Reagent (Millipore, Billerica, MA). Three independent experiments were performed for each assay at each stage of differentiation.

Immunoblotting

Total cellular protein extracts were separated using Nu-PAGE Bis-Tris 4-12% gradient gels (Invitrogen, Carlsbad, CA), transferred to PVDF membrane (Bio-Rad, Hercules, CA) using NuPAGE transfer buffer (Invitrogen, Carlsbad, CA) with 10% methanol/0.01% SDS. Antibodies were used to detect HNF4A (sc6556; Santa Cruz Biotechnology, Santa Cruz, CA; 1/100) and β-actin (A5441, Sigma Aldrich, St Louis, MO; 1:10,000).

RESULTS

Expression profiles reflecting differentiation of huES cells into hepatocyte-like cells

We have previously described a protocol (Fig. 1A) in which the differentiation of human pluripotent stem cells occurs in a synchronous and stepwise fashion that recapitulates many of the steps known to occur during hepatogenesis (Si-Tayeb et al., 2010b). For convenience, we named each differentiation stage based on the expression of proteins that are characteristic of defined developmental time points. As shown in Fig. 1A, the process initiates with 'pluripotent stem cells', is followed by the formation of 'definitive endoderm', then 'hepatic specification', production of 'immature hepatocytes' and finally the generation of 'mature hepatocytes'. We use the term 'mature hepatocyte' to describe the relative maturation of the cells and recognize that hepatocyte-like cells derived from human pluripotent stem cells are not fully mature and more closely resemble a neonatal state (Si-Tayeb et al., 2010b). The generation of hepatocyte-like cells from huES cells is controlled by the sequential addition of activin A, bone morphogenetic protein (BMP4)/fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF) and oncostatin M (OSM) at 5-day intervals (Fig. 1A).

We believed that a description of the complete gene expression profile that accompanies each stage of differentiation in this model would allow us to assemble a characteristic mRNA fingerprint that would facilitate the phenotypic characterization of human hepatocyte differentiation. We therefore isolated RNA from each stage of the differentiation process and used it to probe Affymetrix U133 plus 2.0 arrays in three independent experiments; data were analyzed using DNA-Chip array analysis (D-Chip) software (Li and Wong, 2001). We initiated our study by determining whether each stage of differentiation could be marked by expression of a unique gene set using cluster analyses. As illustrated by the heat map shown in Fig. 1B, we identified several genes with expression

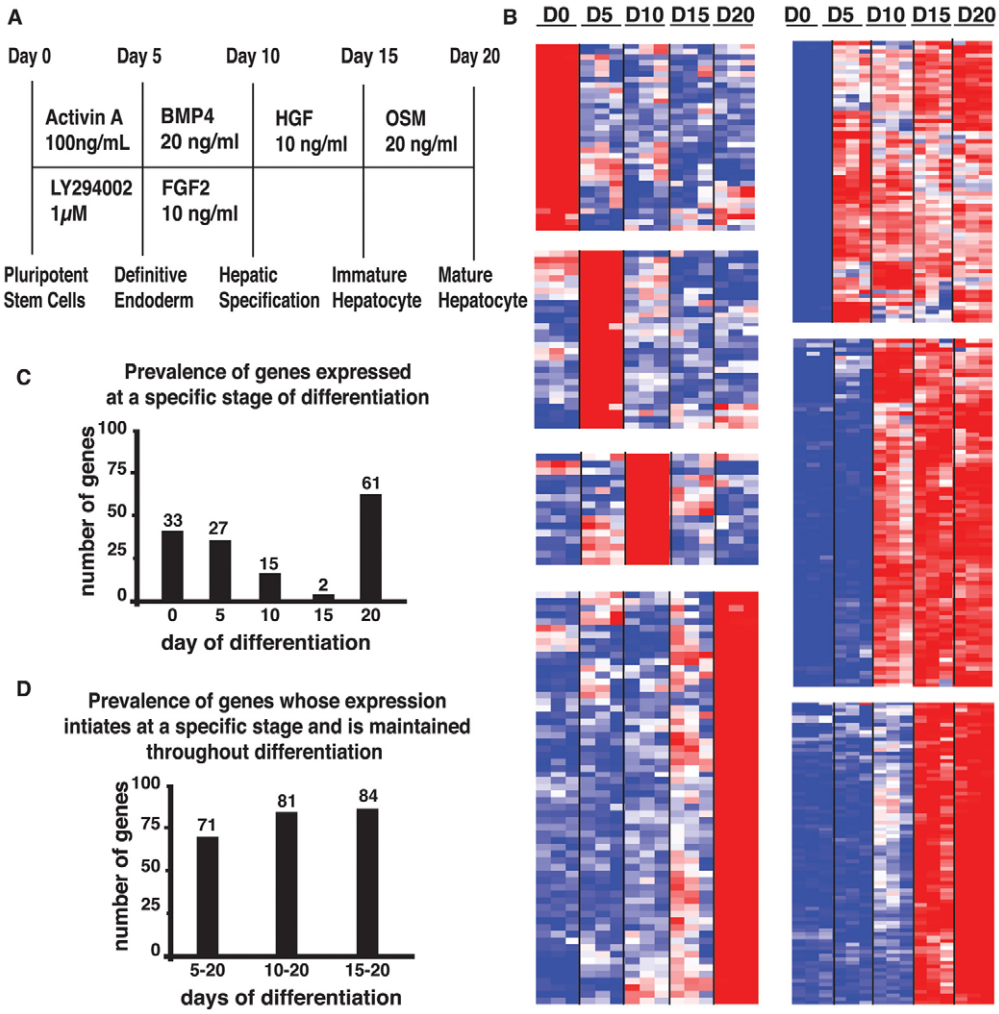

Fig. 1. Identification of mRNA profiles that are characteristic of the differentiation of hepatocyte-like cells from human ES cells. (**A**) The differentiation procedure. (**B**) Heat map summarizing relative changes in mRNA levels at each stage of differentiation (red, high; blue, low). (**C**,**D**) The total number of genes whose expression is predicted to (C) increase at least fourfold ($P \leq 0.05$, Affymetrix signal of at least 200) on a specific day of differentiation or (D) whose expression initiates on a specific day (fourfold, $P \leq 0.05$, Affymetrix signal of at least 200, compared with the previous stage) and is maintained throughout differentiation.

profiles that characteristically identified each specific stage of differentiation. In addition, clusters of genes were revealed with expression profiles that initiated at a specific differentiation stage and remained expressed as differentiation towards a hepatic fate was completed (Fig. 1B). The complete gene list showing the names and expression profiles of genes shown in the heat map are provided in Table S2 in the supplementary material. Gene ontology analyses of the array data sets revealed that, as differentiation progresses, there is a corresponding increase in the number of expressed genes that encode proteins with roles in biological processes that were typically associated with hepatocytes, such as lipid and carbohydrate metabolism, which is consistent with the cells adopting a hepatic character. Among the genes whose expression dynamically changes are several that have established roles in controlling hepatocyte formation and gene expression, including *FOXA2*, *TBX3*, *HHEX*, *HNF4A*, *GATA4* and *GATA6*, which gave confidence that the approach could not only identify markers, but could potentially reveal authentic regulators of hepatocyte differentiation.

We next applied two criteria with the goal of identifying genes whose expression would reliably define each differentiation stage. First, we considered only genes with expression levels that were predicted to reproducibly increase by at least fourfold compared with successive stages of differentiation ($P \leq 0.05$). Second, we reasoned that a fourfold difference in expression is not necessarily physiologically relevant if the mRNA level encoded by the gene is extremely low; moreover, we expected that genes with robust expression would most probably represent easily detectable markers of a given differentiation stage. We, therefore, used Affymetrix signal values as an indicator of expression levels. As a comparative standard, we chose to relate signal levels to those measured for *TBX3* because TBX3 has been shown to be required for mouse liver development (Suzuki et al., 2008; Ludtke et al., 2009). The raw signal values for *TBX3* obtained from the oligonucleotide array data appear to mimic *Tbx3* mRNA levels described during mouse hepatogenesis (Ludtke et al., 2009), with an average signal value of 943.76±145 at day 10, decreasing to 291.42±29 at day 20. We, therefore, discarded any genes whose signal value was 200 or less at stages of differentiation in which the gene was considered to be expressed. When these criteria were applied, a limited number of genes were identified whose expression initiated at each stage of differentiation (Fig. 1C,D; see Table S2 in the supplementary material).

Generation of an mRNA signature that defines hepatocyte differentiation from huES cells

Although oligonucleotide array analyses are useful for capturing large amounts of information, we felt that we could simplify phenotypic analyses of the formation of hepatocyte-like cells from pluripotent stem cells using a subset of representative markers

whose induction could be measured by qRT-PCR. We first considered genes that displayed expression that was specific to a given differentiation stage (fourfold, $P \leq 0.05$, Affymetrix signal of at least 200). A subset of those genes was selected on the basis of robust induction followed by strong suppression during the differentiation process. Additionally, we favored genes with known roles in cell differentiation or an established expression profile that is consistent with a role in hepatogenesis. Quantitative real-time RT-PCR (qRT-PCR) was then performed on RNA isolated from each stage of differentiation in two independent experiments. Fig. 2 shows that a specific mRNA signature could be measured for day 0 (pluripotent cells), day 5 (definitive endoderm), day 10 (hepatic specification) and day 20 (mature hepatocyte). In contrast to these stages, we were unable to detect a specific expression profile for day 15 (immature hepatic) because genes with induced expression at this stage generally remained expressed at day 20 (Fig. 1B). We addressed this problem by developing a second series of qRT-PCR assays that detected mRNAs whose expression initiated at a specific stage of differentiation and remained elevated as the cells completed their adoption of a hepatocyte cell fate. As shown in Fig. 3, qRT-PCR analyses revealed the presence of mRNA signatures that were characteristic of days 5-20, days 10-20 and days 15-20. Finally, to ensure that the developed signatures were not merely specific to hepatocyte differentiation from H9 ES cells, we confirmed that each signature was reliably expressed at the appropriate stages during hepatic differentiation from the human iPS cell line C2a that we had generated previously (Si-Tayeb et al., 2010b) (see Figs S1 and S2 in the supplementary material).

We predicted that if the mRNAs we had selected as being characteristic of hepatocyte differentiation were bona fide hepatic markers, then we should be able to identify their expression in vivo. We therefore first examined the marker sets that identified huES cell-derived hepatocyte-like cells at days 10 to 20, 15 to 20 and day 20 by performing real time qRT-PCR on two human fetal liver samples derived from a 20-week-old and a 38-week-old conceptus, respectively, as well as one male and one female adult liver sample (Si-Tayeb et al., 2010b). As expected, with the exception of AFP, the expression of which was dramatically higher in the fetal liver samples, all markers were robustly expressed in all samples tested (see Fig. S3 in the supplementary material). In support of these qRT-PCR results, we were also able to confirm the presence of the mRNA marker sets in both fetal and adult human livers by examining published array data (Guo et al., 2009). To test for the presence of the d10 marker mRNAs in humans is challenging because access to an appropriate tissue source is limited. As an alternative, we addressed whether the mRNAs that we defined as characteristic of the nascent hepatic progenitors were expressed in E10.5 mouse livers. RT-PCR analyses indeed revealed the presence of the corresponding mouse mRNAs in isolated liver buds (see Fig. S3 in the supplementary material). Cumulatively these data demonstrate the existence of a marker set that can be used in conjunction with immunostaining to phenotype the differentiated status of hepatic cells derived from human pluripotent stem cells. It is important to note, however, that many of the genes identified in the marker set are not specific to hepatocytes and so to be useful each set should be considered as a whole rather than as individual genes. Although it is difficult to compare differentiation protocols, we note that a subset of the markers we have identified have encouragingly been described as being expressed in the endoderm and its derivatives during hepatocyte differentiation from ES cells by others (McLean et al., 2007; Chiao et al., 2008).

Depletion of HNF4A prevents the formation of hepatic progenitors from huES cell-derived endoderm

We next sought to test the suitability of using human pluripotent stem cells to analyze the molecular mechanisms underlying human hepatocyte formation. We chose to focus our studies on the transcription factor HNF4A because of our extensive understanding of the role of HNF4A during development of the mouse liver (Li et al., 2000; Parviz et al., 2003; Battle et al., 2006). HNF4A is a member of the nuclear hormone receptor transcription factor family, and loss of HNF4A results in an extensive disruption to expression of genes encoding all aspects of mouse hepatocyte function (Battle et al., 2006; Bolotin et al., 2010). Although HNF4A directly controls expression of many hepatic genes (Battle et al., 2006; Bolotin et al., 2010), it is also crucial in maintaining the network of transcription factors that is essential for normal hepatocyte function (Kyrmizi et al., 2006).

Previous studies using mouse embryos have shown that, although HNF4A is expressed at the onset of extra-embryonic endoderm formation and continues in the extra-embryonic visceral endoderm, it is not expressed in the definitive endoderm before

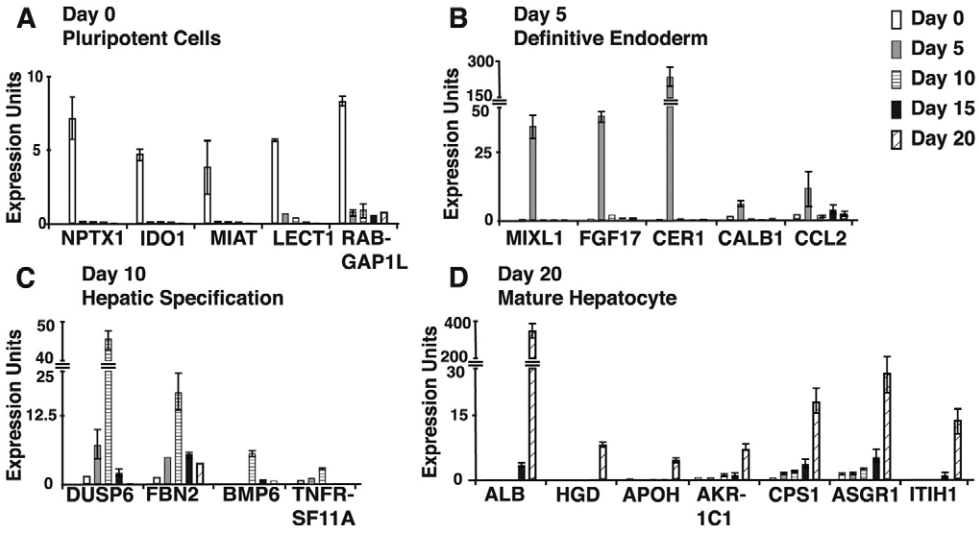

Fig. 2. Quantitative RT-PCR analyses of differentiation stage-specific mRNAs. (**A-D**) Changes in mRNA levels with characteristic expression profiles at (A) day 0, (B) day 5, (C) day 10 and (D) day 20 of differentiation. Graphs represent the relative mean expression value and s.d. normalized to GAPDH from two independent differentiations.

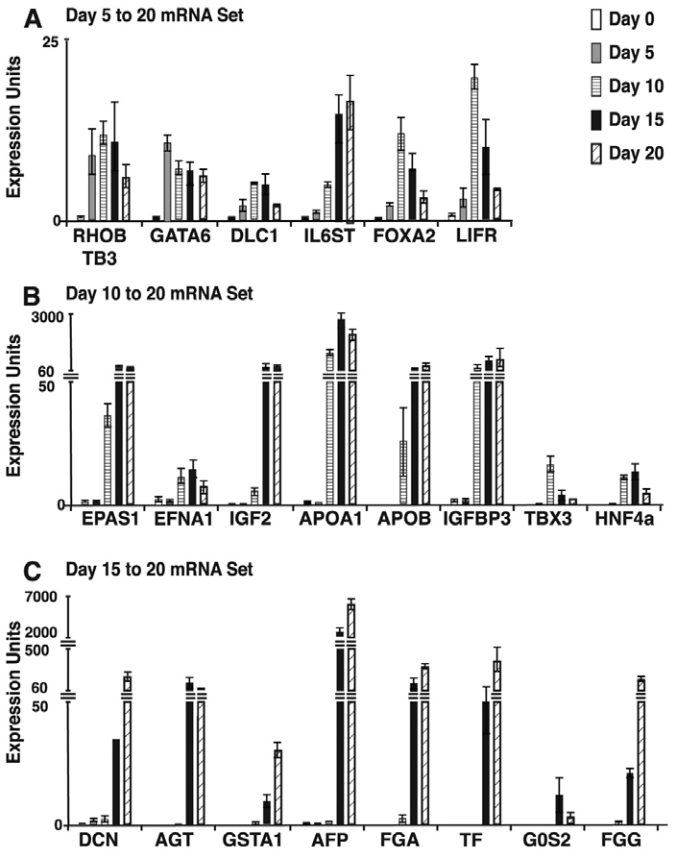

Fig. 3. Quantitative RT-PCR analyses of mRNAs with maintained expression. (**A-C**) Changes in mRNA levels that are induced at a specific stage of differentiation and are maintained throughout the differentiation process: (A) day 5 to 20, (B) day 10 to 20 and (C) day 15 to 20. Graphs represent the mean expression value and s.d. normalized to GAPDH levels from two independent differentiations.

hepatic specification (Duncan et al., 1994; Taraviras et al., 1994; Watt et al., 2007). Consistent with the expression profile in the mouse, we had previously shown by immunocytochemistry that HNF4A protein is absent from definitive endoderm generated by our differentiation of human pluripotent stem cells at day 5, but is detected after specification of hepatic progenitors by day 10 (Si-Tayeb et al., 2010b). To confirm that expression of *HNF4A* within this system did not initiate until after specification of the hepatic progenitors, we measured *HNF4A* mRNA levels by real-time qRT-PCR and protein levels by immunoblot analyses in day 0 pluripotent cells, day 5 definitive endoderm cells and day 10 specified hepatic progenitors. Fig. 4 shows that both *HNF4A* mRNA and protein were undetectable in undifferentiated huES cells and after formation of definitive endoderm (D5). However, after addition of BMP4/FGF2 and removal of activin A, *HNF4A* mRNA and protein were readily detected at day 10 of the differentiation procedure (Fig. 4A,B). The onset of *HNF4A* expression is therefore strictly associated with the specification of the hepatic lineage from human pluripotent stem cells.

To determine any role for HNF4A in regulating the formation of hepatocytes from human pluripotent stem cells, we generated stable polyclonal huES cell lines expressing shRNAs designed to target *HNF4A* by lentiviral transduction. We chose to work with polyclonal cells to avoid the concern that a clonal line may have an inherent deficiency. To ensure continued expression of the shRNA during the entire differentiation process, the *puromycin N-acetyl-transferase* (*pac*) gene, which confers resistance to puromycin, was included within the provirus, and cells were maintained in puromycin throughout the differentiation time course. Because *HNF4A* is not expressed in pluripotent stem cells, the presence of shRNAs against *HNF4A* did not affect the culture of undifferentiated huES cells. We next compared HNF4A expression by immunocytochemistry in differentiated hepatic cells derived from control huES cells infected with lentiviruses that lack any shRNA (vector), that express a control *HNF4A* shRNA that fails to deplete *HNF4A* mRNA in HepG2 cells (HNF4i2), or that express an shRNA that efficiently depletes *HNF4A* in HepG2 cells (HNF4i3). Fig. 4C shows that, in contrast to control cells, in which HNF4A can be detected in at least 85% of nuclei upon differentiation (day 20), HNF4A was undetectable in cells derived from HNF4i3 ES cells. We confirmed that *HNF4A* mRNA was absent during the entire time-course of HNF4i3 differentiation using real-time qRT-PCR (Fig. 4B). Based on the observation that HNF4A was undetectable in the ES cell-derived definitive endoderm (Fig. 4A,B), we predicted that HNF4i3 cells should be capable of effectively generating definitive endoderm in response to 5 days of treatment with activin A. As expected, HNF4i3 cells efficiently differentiated to cells that expressed SOX17, FOXA2 and GATA4 protein, which were detected by immunocytochemistry (see Fig. S4A in the supplementary material). In addition, real-time qRT-PCR analyses revealed that mRNAs encoding FOXA2, FOXA3, SOX17, HHEX and GATA4 (see Fig. S4B in the supplementary material) as well as our panel of endoderm markers *MIXL1*, *CALB1*, *FGF17*, *CER1* and *CCL2* (Fig. 5A) were expressed at levels that were comparable with control cells. These data show that HNF4A is dispensable for differentiation of definitive endoderm from human pluripotent stem cells.

We next addressed whether the requirement for HNF4A during differentiation of mouse hepatocytes was conserved in human cells. We examined the expression of genes with familiar hepatic functions by semi-quantitative RT-PCR following day 20 of differentiation of H9 ES cells, control HNF4i2 cells and HNF4A-depleted HNF4i3 cells. Expression of hepatic mRNAs were detected at similar levels in both H9 cells and HNF4i2 cells, demonstrating that the introduction of shRNA *per se* has little impact on the differentiation of the ES cells toward a hepatic fate (Fig. 4D). However, in contrast to control differentiations, hepatic mRNA levels were severely diminished following differentiation of HNF4i3 cells, which lacked detectable *HNF4A* mRNA. We also performed oligonucleotide array analyses on HNF4i3 cells after completing the 20-day differentiation protocol (n=3 independent experiments). Hepatic character was then defined in an unbiased manner by using a set of 40 genes whose mRNAs were previously shown to be expressed only in human livers and were induced following the formation of hepatocyte-like cells from huES and iPS cells (Ge et al., 2005; Si-Tayeb et al., 2010b). H9 huES-derived cells reproducibly expressed the majority of these characteristic hepatic mRNAs by completion of the differentiation protocol. However, when HNF4A was depleted, the ability of HNF4i3 cells to express these mRNAs by day 20 of differentiation was completely blocked (Fig. 4E). The loss of hepatic character associated with HNF4i3 cells was confirmed by gene ontology analysis using Ingenuity Pathway Analyses software (see Fig. S5A in the supplementary material). Following differentiation of control cells, the expression of many genes associated with hepatic functions, including lipid metabolism, small molecule biochemistry, carbohydrate metabolism

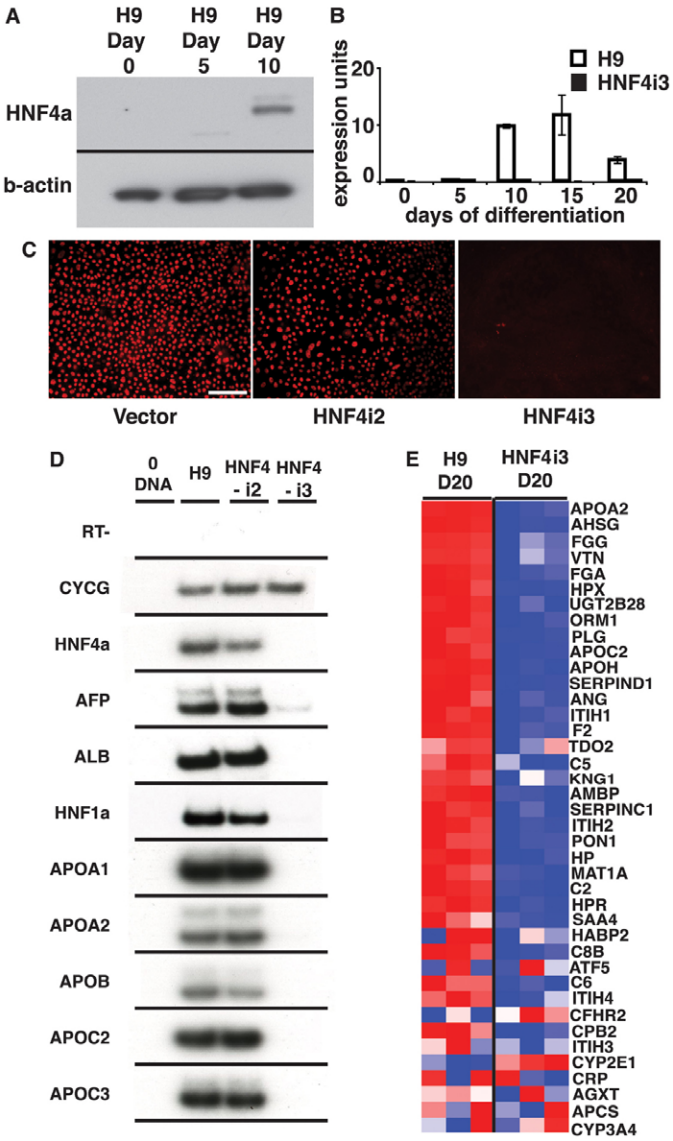

Fig. 4. HNF4A is essential for differentiation of hepatocyte-like cells from human ES cells. (**A**) Immunoblot analyses comparing HNF4A protein levels during differentiation of H9 ES cells (day 0) to definitive endoderm (day 5) and hepatic progenitor cells (day 10). (**B**) Real-time qRT-PCR comparing HNF4A mRNA levels during differentiation of HNF4i3 cells (black bars) and control H9 ES cells (white bars). Data are mean±s.d. (**C**) Immunocytochemistry identified the presence of HNF4A (red) in control cells (Vector, HNF4i2), but not in HNF4i3 cells, following differentiation. Scale bar: 100 μm. (**D**) Semi-quantitative RT-PCR revealed that, in contrast to hepatocyte-like cells derived from control cells (H9, HNF4i2), expression of characteristic hepatocyte mRNAs was severely disrupted in HNF4i3 cells. CYCG was used as a loading control. (**E**) Heat map summarizing oligonucleotide array analyses (red, high expression; blue, low expression) that confirmed the loss of expression of hepatocyte-specific mRNAs in differentiated HNF4i3 cells in contrast to control cells (H9).

and molecular transport, was robust. In HNF4i3-derived cells, however, expression of genes important in such functions was lost. Finally, we attempted to determine whether the HNF4A-depleted cells had adopted an alternate fate that represented a specific lineage. Studies by others have categorized sets of genes that are uniquely expressed in specific organs (Ge et al., 2005). As expected, these analyses confirmed loss of expression of liver genes from differentiated HNF4i3 cells; however, there was no clear evidence to suggest that the HNF4i3-derived cells switch to an alternative lineage-specific fate, at least based on the expression profile (see Fig. S5B and Table S3 in the supplementary material). These data imply that whereas HNF4A is crucial for formation of hepatocytes, in its absence the cells adopt a stable state that cannot be easily categorized.

Finally, HNF4A has been shown to bind elements within an extensive list of target genes in both the mouse and human genomes (Odom et al., 2004; Odom et al., 2007; Bolotin et al., 2010). We, therefore, sought to identify whether any of the genes whose expression was downregulated in the HNF4i3-derived hepatic cells had previously been shown to contain elements that were occupied by HNF4A. By cross referencing our list of genes whose expression is impacted by loss of HNF4A with previously identified HNF4A targets, we found that 108 of the 562 genes whose expression is reduced have been shown to house elements that are occupied by HNF4A in HepG2 cells and/or human hepatocytes (see Table S4 in the supplementary material).

HNF4A is essential for BMP4/FGF2-induced specification of hepatic progenitors from huES cell-derived definitive endoderm

We have previously demonstrated that HNF4A is essential for hepatocyte differentiation and liver morphogenesis in the mouse (Parviz et al., 2003; Battle et al., 2006); however, it has been difficult to determine the exact developmental stage at which HNF4A first acts during liver development because HNF4A is essential for gastrulation (Chen et al., 1994; Duncan et al., 1997). Using tetraploid complementation, we were able to rescue these gastrulation defects and produce *Hnf4a*$^{-/-}$ embryos that survived until around E10.5 (Li et al., 2000). Analysis of the liver buds of these embryos revealed a loss in expression of the majority of hepatic mRNAs examined, although, with the exception of PXR and HNF1A, the expression of genes encoding liver-enriched transcription factors seemed to be relatively unaffected. Nevertheless, because HNF4A is expressed at the onset of hepatogenesis in both the mouse (Duncan et al., 1994; Taraviras et al., 1994) and during huES cell differentiation, we considered the possibility that HNF4A could control the earliest stages of hepatic progenitor cell formation during the differentiation of human pluripotent stem cells. We therefore examined the impact of HNF4A depletion at each stage of the differentiation of hepatocytes from huES cells by comparing the mRNA signature (Figs 2, 3) that was characteristic of each stage between control and HNF4i3 cells (Fig. 5).

As expected, and consistent with the array data, loss of HNF4A resulted in a complete disruption to expression of all genes encoding mRNAs that are characteristic of the day 20 (mature hepatocyte) stage of differentiation (Fig. 5B). Expression of genes that initiated at day 15 was also dramatically reduced by the absence of HNF4A, although expression of one gene, *DCN*, was not changed (Fig. 5C). *HNF4A* mRNA is first detected at day 10 of the differentiation process, which coincides with the formation of hepatic progenitors in response to BMP4/FGF2 signaling (Si-Tayeb et al., 2010b). When expression of the four genes that characterize the hepatic progenitor stage (day 10) was examined, in contrast to control cells, depletion of HNF4A prevented induction of every one of these mRNAs (Fig. 5D). We also determined the abundance of mRNAs whose expression initiated at day10 and remained expressed throughout the differentiation time course and found that,

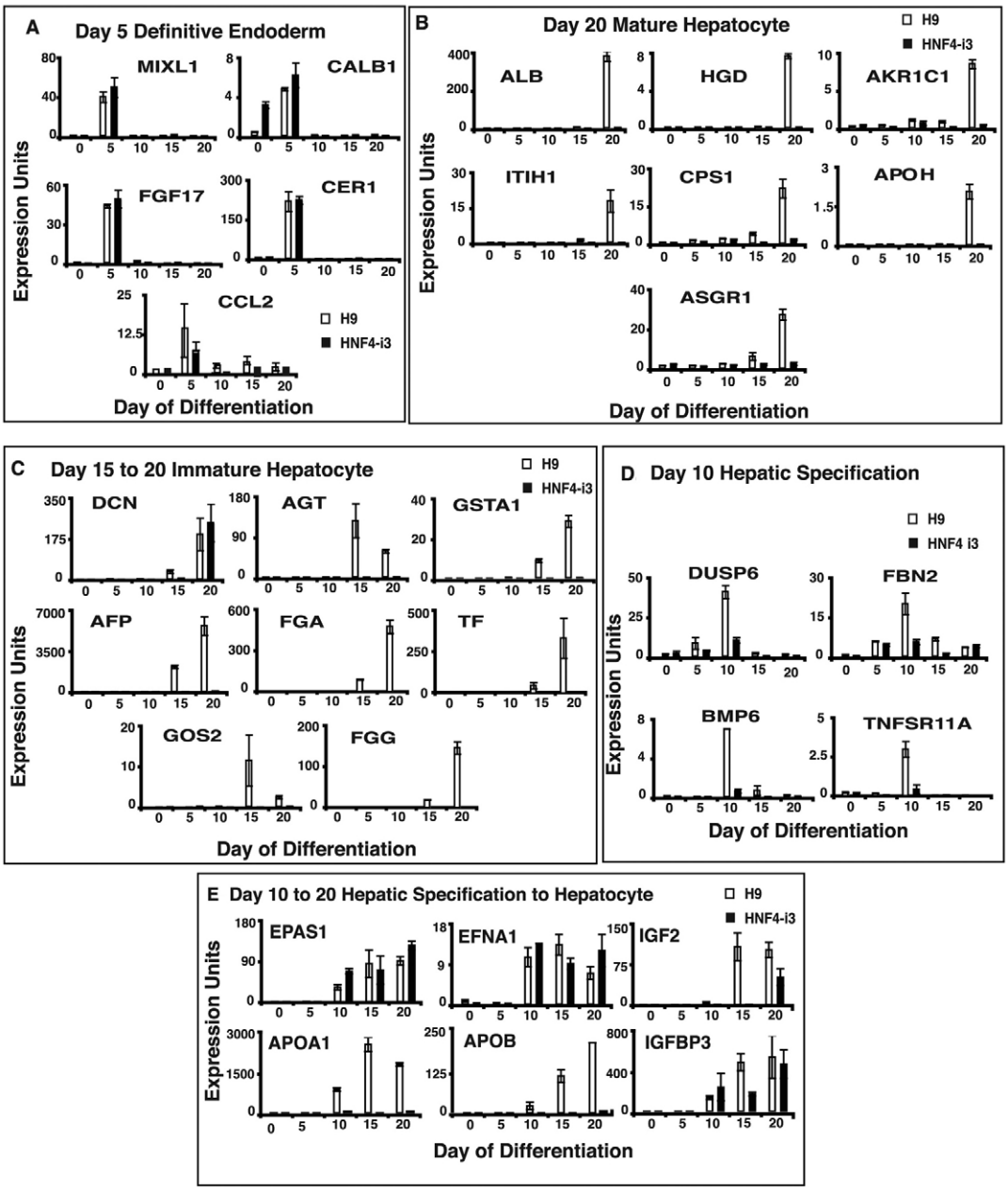

Fig. 5. HNF4A is required for hepatic specification of human ES cells. Real-time qRT-PCR identified the levels of mRNAs defined as being characteristic of (A) mature hepatocyte, (B) immature hepatocyte, (C) hepatic specification and (D) definitive endoderm throughout the differentiation of control (H9) or HNF4i3 cells. Results plotted are the mean. Error bars represent s.d. generated from two independent differentiations.

in contrast to control cells, most of these mRNAs were generally reduced in HNF4i3 cells following differentiation. Interestingly, we identified some exceptions to this general finding, in that *EPAS1*, *EFNA1*, *IGF2* and *IGFBP3* mRNA levels remained expressed after depletion of HNF4A; however, it should be noted that expression of these genes is not restricted to hepatocytes. Additionally, the gene expression profile of the differentiated HNF4A-depleted cells was considerably altered as early as day 10 of differentiation; however, the rates of proliferation and apoptotic cell death were unaffected (see Fig. S6 in the supplementary material), making it unlikely that the observed change in expression profile reflects a selective loss of hepatocytes within the cultures.

During midgestation stages of development, HNF4A has been shown to play a central role in controlling the stability of a network of transcription factors that regulate hepatocyte gene expression (Kyrmizi et al., 2006). However, given that we had observed what amounted to a loss of hepatic character as early as day 10 of differentiation, we determined whether HNF4A was essential for the onset of hepatocyte transcription factor expression by examining the expression of transcriptional regulators with known roles during liver development (Si-Tayeb et al., 2010a) in control and HNF4A-depleted ES cell-derived hepatic progenitors throughout the differentiation protocol. As shown in Fig. 6, qRT-PCR revealed that the levels of mRNAs encoding *FOXA1*, *FOXA2*, *FOXA3*, *HNF1B*,

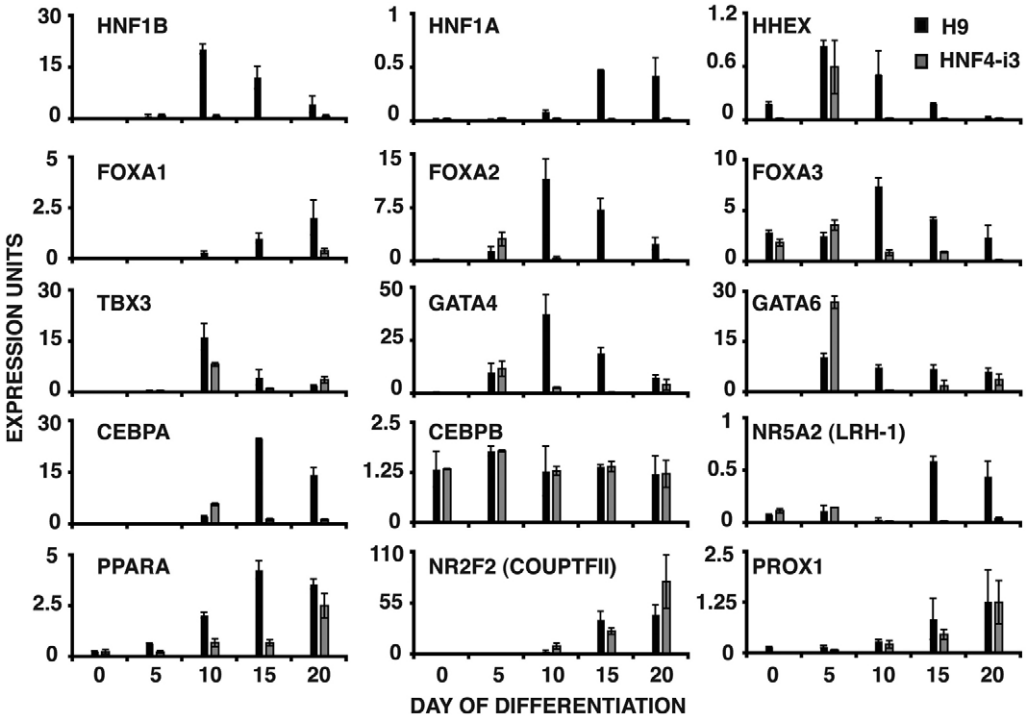

Fig. 6. HNF4A is essential for expression of transcription factors with roles in controlling the formation of hepatic progenitor cells. The level of mRNAs encoding transcription factors that have been associated with differentiation of mouse hepatocytes was measured by real-time qRT-PCR in either control H9 (black bars) or HNF4i3 (gray bars) cells throughout differentiation. Data are mean±s.d.

HNF1A, *GATA4*, *GATA6* and *HHEX*, all of which have crucial roles during early stages of hepatic development, were reduced to close to undetectable levels. *TBX3* and *PROX1* were reproducibly, yet more modestly, depleted and their expression recovered as differentiation progressed. From these data, we conclude that HNF4A is essential for specifying the fate of the earliest hepatic progenitor cells from huES and is necessary for both establishing and maintaining the network of transcription factors that controls the onset of human hepatocyte cell fate (Fig. 7).

DISCUSSION

Understanding organogenesis clearly requires analyses to be conducted in an intact developing organism because one must consider the role of cell-cell interactions, cell movements and the establishment of tissue architecture before a reasonable understanding of organ or tissue formation can be achieved. However, the specific study of cell differentiation historically has been advanced by molecular analyses using a variety of cell culture systems. For example, in the case of hepatocyte differentiation, rat hepatoma-human fibroblast hybrid cell lines were used to reveal the ability of HNF4A and HNF1A to control hepatic gene expression (Griffo et al., 1993). Indeed, it took close to 10 additional years before this result could be recapitulated using conditional knockout mouse models (Hayhurst et al., 2001; Parviz et al., 2003). Using cell culture rather than animals not only reduces the time through which valuable data can be generated, but also provides relatively homogeneous cell populations and large amounts of experimental material, which in turn increases the accuracy of high resolution molecular and biochemical analyses. Unfortunately, most cells in culture are derived from tumors or are immortalized, and although such cells have been valuable, tumor cells commonly have chromosomal abnormalities that promote proliferation and cell survival, which often confounds interpretation. In addition, differentiation is a dynamic process, a consequence of sequential changes in gene expression profiles and the competency of cells to respond to environmental cues. In most cases, however, tumor cells are poorly differentiated, or even de-differentiated, and this limits their usefulness. In the current study, we demonstrate that human pluripotent stem cells can be used to efficiently analyze the molecular basis of human hepatocyte differentiation. As part of the study, we determined the global gene expression profiles for each stage of the differentiation process. Based on these data, we defined a subset of mRNAs whose detection can be used to determine phenotypic changes in differentiation. In addition to being useful for phenotypic analyses, defining the gene expression profile for each differentiation stage will probably facilitate the identification of molecular pathways with undefined roles during human hepatocyte differentiation; such studies are currently under way.

We have shown previously that the differentiation of human pluripotent stem cells into hepatocyte-like cells results in expression of a large repertoire of genes associated with hepatocyte function; these data were confirmed in the current study. Indeed, 92% of mRNAs detected in adult and 93% detected in fetal human livers by oligonucleotide array analyses were also present in the day 20 huES cell-derived hepatocytes (see Fig. S7 in the supplementary material). Although the expression of such an extensive repertoire of hepatic mRNAs is encouraging, we also noted that the stem cell-derived hepatocyte-like cells expressed several mRNAs that were not normally associated with adult or fetal livers (see Fig. S7 in the supplementary material). Although it is difficult to realize the impact of these results without further analyses, the observation that such ectopic expression of mRNAs exists is an important caveat that must be considered if pluripotent human stem cells are to be considered a source of cells for transplant. Nevertheless, the ability to deplete candidate developmental factors efficiently by shRNA in human pluripotent stem cells and effectively examine the impact on hepatic cell differentiation is likely to accelerate the discovery of novel molecular events that control human hepatocyte cell fate.

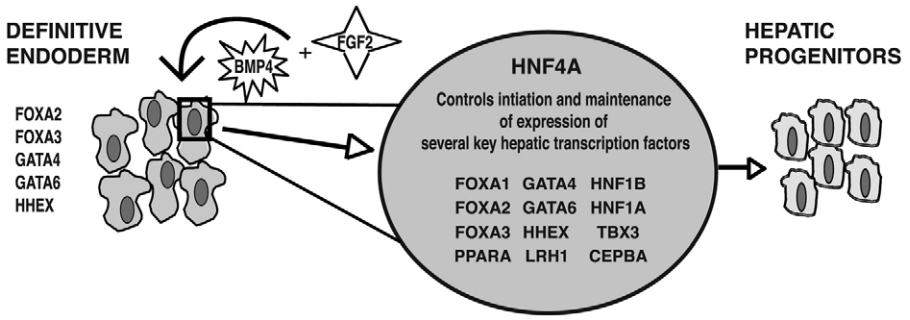

Fig. 7. Schematic of the role of HNF4A during the differentiation of human ES cells into hepatocyte-like cells. Definitive endoderm generated from human ES cells expresses key hepatic transcription factors, including FOXA2, FOXA3, GATA4, GATA6 and HHEX that, based on studies in the mouse, probably regulate the ability of the endoderm to adopt a hepatic fate. In response to inductive cues, including FGFs and BMPs, the receptive endoderm expresses HNF4A within the nascent hepatic progenitor cells where it is responsible for the initiation and maintenance of expression of several hepatic transcription factors, including HNF1B, that control formation of hepatoblasts and their differentiation towards functional hepatocytes.

In addition to providing proof-of-concept that human pluripotent stem cells can be used as an efficient tool to probe human cell fate, we exploited the model to reveal a requirement for HNF4A in controlling the onset of human hepatocyte differentiation. Our previous studies in the mouse revealed that HNF4A is required for the hepatoblasts to differentiate to a mature state (Parviz et al., 2003) and that *Hnf4a*$^{-/-}$ E10.5 liver buds failed to express many genes that are characteristic of hepatocyte function (Li et al., 2000). Although this requirement for HNF4A during differentiation is conserved during the formation of human hepatocytes from pluripotent stem cells, the phenotype associated with HNF4A-depleted huES-derived hepatocytes seems to have an earlier onset and is more severe compared with that observed in the mouse. In the mouse, the E10.5 liver bud, which, based on marker expression, appears approximately equivalent to day 15 of the huES differentiation protocol, was observed to express the majority of liver-enriched transcription factors; in the huES cells differentiation model, however, expression of most liver transcription factors is severely disrupted coincident with specification of the hepatic progenitor cells. Although we are unable to answer definitively why there are differences between the mouse and human models, we can consider a number of possible explanations. For example, it is possible that there exist inherent differences between human and mouse and that the mouse has in place mechanisms that are capable of at least partially compensating for loss of HNF4A during hepatic specification. Such an explanation may be supported by the observation that a haploinsufficiency of HNF4A in humans results in diabetes (Yamagata et al., 1996), whereas *Hnf4a*$^{+/-}$ mice are euglycemic (Chen et al., 1994). It is also possible that in the tetraploid experiments that we previously performed to generate *Hnf4a*$^{-/-}$ mouse embryos, there existed a selective pressure for cells that initiated expression of transcription factors in the absence of HNF4A. It is also worth considering that the differentiation of huES cells towards the hepatic fate occurs under relatively simple conditions compared with the complex *in vivo* environment of the mouse embryo. Because of the defined nature of the culture conditions, it is possible that the loss of HNF4A results in a more severe phenotype in the huES cell model because signals that rely on, for example, matrix interactions and three-dimensional structure are likely to be lacking in the culture system. We are currently exploring this possibility.

HNF4A has been shown to directly regulate the expression of a large number of target genes in hepatocytes (Battle et al., 2006; Bolotin et al., 2010). It therefore seems likely that the global loss of expression of hepatocyte mRNAs partly reflects a direct requirement for HNF4A in transcriptional regulation through target promoters. However, it is also important to note that the expression of several transcription factors that have been implicated in defining hepatic cell fate in mouse embryos is severely reduced in the HNF4A-depleted cells. Somewhat surprisingly, the depletion of HNF4A also significantly reduced expression of several factors that help establish a state of hepatic competence within the ventral endoderm, including the FOXA proteins (Lee et al., 2005) and GATA factors 4 and 6. All of these factors are expressed in the ventral endoderm before the onset of *HNF4A* expression in the liver progenitor cells and their expression is not affected in human endoderm derived from HNF4A-depleted huES cells (Fig. 5; see Fig. S4 in the supplementary material). This implies that the transcription factor network that governs the transition from an endodermal cell to that of hepatic progenitor cell is in a relatively plastic state and must be actively maintained by HNF4A as cell differentiation progresses.

The loss of HNF1B in HNF4A-depleted cells may be particularly important because Lokmane *et al.* have recently shown that liver specification is blocked in *Hnf1b*-null mouse embryos (Lokmane et al., 2008). Interestingly, *Hnf1b*$^{-/-}$ embryos failed to express *HNF4A*, suggesting that the closely intertwined regulation between these factors is a primary mechanism through which the hepatic transcription factor network is initially established during specification of the hepatic progenitors. Analyses of promoter regions of *HNF1B* and *HNF4A* have identified reciprocal binding sites within the regulatory regions of each factors, implying that the regulatory relationship between the two factors is direct. The issue of why the expression of so many genes is affected by the loss of HNF4A is an important one. One explanation is that HNF4A directly controls the expression of these genes through interacting with their transcriptional regulatory elements. However, given that the expression of many of these genes, such as *GATA4*, *GATA6*, *HHEX* and *FOXA2*, precedes that of *HNF4A* in the definitive endoderm we feel that this explanation may at best be only partly true. Instead, we favor an alternative explanation, in which depletion of HNF4A results in the complete loss of hepatic character by preventing the specification of hepatic progenitors. As

a consequence of their inability to follow a hepatic developmental program, the endodermal cells, in response to inductive signals in the medium, adopt an alternate stable state. Although the mRNA levels of some genes whose expression is associated with hepatocyte function, including *IGF2*, *IGFBP3*, *EPAS1* and *EFNA1* is maintained, such genes are commonly expressed in other additional cell types, which may explain why their mRNAs can still be detected in HNF4A-depleted cells.

In summary, our data demonstrate that HNF4A is essential for establishing hepatic progenitor cells from human pluripotent stem cell-derived definitive endoderm and that human pluripotent stem cells offer a valid model with which to study the molecular mechanisms underlying human hepatocyte differentiation. Although we noted that a subset of genes appears to be ectopically expressed and that some mRNAs, for example those encoding phase 1 and phase 2 enzymes, are expressed at low levels compared with adult livers, we find that the majority of hepatic mRNAs are expressed in the expected fashion, and their onset appears to closely recapitulate the normal developmental profile, as has been discussed previously (Agarwal et al., 2008). This system is also one of the few that offers direct access to the differentiation of human hepatocytes. As techniques continue to improve for the generation of human iPS cells (Anokye-Danso et al., 2011), we believe that similar procedures will facilitate the study of the mechanisms and possible treatments of inborn errors in hepatic metabolism.

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Competing interests statement
The authors declare no competing financial interests.

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