Published in final edited form as:

Hepatology. 2010 January ; 51(1): 297–305. doi:10.1002/hep.23354.

Highly Efficient Generation of Human Hepatocyte–like Cells from Induced Pluripotent Stem Cells

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Abstract

There exists a worldwide shortage of donor livers available for orthotopic liver transplant (OLT) and hepatocyte transplantation therapies. In addition to their therapeutic potential, primary human hepatocytes facilitate the study of molecular and genetic aspects of human hepatic disease and development and provide a platform for drug toxicity screens and identification of novel pharmaceuticals with potential to treat a wide array of metabolic diseases. The demand for human hepatocytes, therefore, heavily outweighs their availability. As an alternative to using donor livers as a source of primary hepatocytes, we explored the possibility of generating patient specific human hepatocytes from induced pluripotent stem (iPS) cells.

Conclusion—We demonstrate that mouse iPS cells retain full potential for fetal liver development and describe a procedure that facilitates the efficient generation of highly differentiated human hepatocyte–like cells from iPS cells that display key liver functions and can integrate into the hepatic parenchyma *in vivo*.

Keywords

Liver development; reprogramming; hepatocytes; orthotopic liver transplant

INTRODUCTION

The ability to generate induced pluripotent stem (iPS) cells from somatic cells by forced expression of the reprogramming factors Oct3/4 and Sox2 along with either Klf4 1–4 or Nanog and Lin28 5 raises the possibility of generating patient specific cell types of all lineages. Differentiated cell types produced from patient's iPS cells 6 have many potential therapeutic applications including their use in tissue replacement and gene therapy. While the use of iPS–based cell therapies is a realistic long term goal, if protocols that facilitated efficient differentiation into specific cell lineages could be developed, iPS–derived cells could be used immediately for the analysis of disease mechanisms and the identification and study of pharmaceuticals.

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The generation of hepatocytes from iPS cells is a particularly appealing goal because this parenchymal cell of the liver is associated with several congenital diseases 7, is the site of xenobiotic control, and is the target of many pathogens that cause severe liver dysfunction including hepatitis B and C viruses. Moreover, unlike most other organs, the introduction of exogenous hepatocytes into the liver parenchyma is a relatively simple undertaking suggesting that the liver is highly amenable to tissue therapy using iPS cell-derived hepatocytes 8–10. We, therefore, sought to determine whether iPS cells are fully competent to adopt a hepatic cell fate in embryos and to establish a protocol using defined culture conditions for the generation of human hepatocyte-like cells from iPS cells.

EXPERIMENTAL PROCEDURES

Human ES and iPS cell culture

Human H9 (WA09) ES cells and iPS cells were cultured using standard conditions 5 that are described in supplemental information online.

Histological and Functional Assays

In most cases assays relied on well established procedures, and details are provided as supplemental material online. Antibodies used are provided in supplemental table S1.

Oligonucleotide array analyses

Each array analysis was performed on three samples that were generated through independent differentiation experiments. Specific experimental details are provided as supplemental material online. All original gene array files are available through the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) accession number GSE14897.

RESULTS

Fetal Mouse Livers Generated from iPS Cells are Indistinguishable from Wild Type Livers

We first determined whether iPS cells were competent to follow a hepatic developmental program that produced all liver cell lineages by examining embryos derived solely from mouse iPS cells by tetraploid complementation. Mouse iPS cells were generated from C57BL/6J-Tg(pPGKneobpA)3Ems/J fibroblasts as described in supplemental Figure S1. Embryos were then produced from these iPS cells by tetraploid complementation using transgenic mice (Tg(CAG-EGFP)B5Nagy/J) that ubiquitously express EGFP as donors of tetraploid embryos. Figure 1A shows that control CAG-EGFP embryos ubiquitously express EGFP, while EGFP was not detected in wild type CD1 embryos. When embryos were generated from mouse iPS cells, from which EGFP is absent, all embryos (n=5), including their livers (Figure 1B), were devoid of EGFP expression except in extra embryonic tissues that were derived from the donor tetraploid embryos 11.

Gross examination of E14.5 iPS cell-derived embryos and their livers (n=3) revealed that they appeared to be identical to controls (Figure 1C). We, therefore determined whether these livers contained the expected repertoire of hepatic cells by identifying the expression of proteins that are characteristic of specific cell types. Figure 1D shows that like control CD1 fetal livers, iPS cell-derived livers contained hepatocytes (HNF4a positive), endothelial cells (GATA4 positive), sinusoidal cells (LYVE1 positive), and Kupffer cells/macrophage (F4/80 positive). We also measured the extent of hepatocyte differentiation using RT-PCR to detect mRNAs that are key markers of the hepatocyte cell lineage. Figure 1E shows that every hepatocyte marker mRNA examined – *Alpha fetoprotein*, *Albumin*, *Aldolase b*, *Apolipoproteins A1, A2, and C2*, *Liver fatty acid binding protein (Fabp1)*,

Retinol binding protein (Rbp4), and *Transthyretin* – was expressed at a level comparable to control fetal livers. Moreover, expression of several mRNAs encoding liver transcription factors – *Gata4*, *Hnf1a*, *Hnf1b*, *FoxA1*, *FoxA2*, *Pxr (Nr1i2)*, and *Hnf4a* – was commensurate with control livers. From these cumulative results, we conclude that mouse iPS cells are fully competent to generate fetal livers *in vivo*.

Establishing a Protocol for the Efficient Production of Hepatocyte-like cells from Human Pluripotent Cells

The generation of clinically and scientifically useful hepatocytes from iPS cells requires the availability of completely defined culture conditions that support efficient and reproducible differentiation of iPS cells into the hepatocyte lineage. Existing published procedures that have been applied to the differentiation of both human and mouse ES cells generally include steps in which poorly defined components are introduced into the culture conditions. This is potentially problematic especially if such cells are to be used therapeutically. We, therefore, sought to optimize the differentiation procedure and eliminate the use of serum, fibroblast feeder cells, embryoid bodies, and undefined culture medium components initially using huES cells. We based our protocol on an understanding of the mechanisms underlying mouse embryogenesis, the availability of protocols published by others 12–14, and the use of empirically determined procedures that resulted in an increase in the number of cells expressing a combination of markers of definitive endoderm (FOXA2, SOX17 and GATA4), specified hepatic cells (FOXA2 and HNF4a), hepatoblasts (FOXA2, HNF4a, and AFP) and differentiated hepatocytes (FOXA2, HNF4a, and ALB).

Figure 2A illustrates the procedure that we have used. Undifferentiated stem cells were maintained in monolayer culture on Matrigel in ES cell culture media conditioned by mitotically inactivated primary mouse embryonic fibroblasts (MEFs) in 4% O₂/5%CO₂. Under these conditions, >95% of cells expressed pluripotency markers including Oct4 (Figure 2B) and SSEA4 (not shown). To initiate differentiation, monolayers of huES cells were cultured in RPMI media containing B27 supplements and 100ng/ml Activin A, which has been shown to efficiently induce differentiation of definitive endoderm 15–16. After five days of culture in 5%CO₂ with ambient oxygen, >90% of cells had lost expression of the pluripotency markers OCT3/4 (Figure 2B) and SSEA4 (not shown). Immunocytochemistry using antibodies to detect proteins expressed in the definitive endoderm revealed that >80% of cells expressed FOXA2, GATA4, and SOX17. Importantly, these cells did not express HNF4a, which is highly expressed in extra embryonic endodermal cells, thereby excluding the possibility that the endoderm generated by Activin A treatment was visceral (yolk sac) endoderm. Culture dishes containing induced definitive endoderm were next moved to 4% O₂/5%CO₂ in RPMI/B27 media supplemented with 20ng/ml BMP4 and 10ng/ml FGF2 for 5 days. Both BMP4 and FGF2 have been shown to have crucial roles during hepatic specification in mouse embryos 17–18. Figure 2B shows that culture in BMP4/FGF2 supplemented media resulted in reduced expression of both GATA4 and SOX17; while, FOXA2 expression was maintained and HNF4a expression was initiated. This pattern of expression closely resembles that found during development of the mouse liver. In particular, GATA4 expression is specifically down regulated in cells that are destined to follow a hepatic fate but remains expressed in the gut endoderm 19–20, whereas HNF4a expression is restricted to the nascent hepatic cells formed during hepatic specification stages of development (10 somites) 20–21. The specification of hepatic cells following addition of BMP4/FGF2 was robust with >80% of cells expressing HNF4a. Based on findings by others 12–13, we cultured the specified hepatic cells in RPMI-B27 supplemented with 20ng/ml hepatocyte growth factor (HGF), under 5%CO₂/4%O₂. HGF inclusion in the culture conditions resulted in high levels of expression of Alpha-fetoprotein, which indicates that the specified cells have committed to a hepatoblast fate (Figure 2B). Co-staining with

FoxA2 (not shown) revealed that >98% of FoxA2 expressing cells co-expressed alphafetoprotein implying that the differentiation of endoderm into the hepatic lineage was extremely efficient.

For the final stage of differentiation, cultures were transferred to 5%CO₂/ambient O₂ and the media was replaced with Hepatocyte Culture Medium supplemented with Oncostatin M (20ng/ml) 22 for an additional five days. Under these conditions, the cells were found to express high levels of Albumin that could be identified by immunocytochemistry (Figure 2B) and quantified in the media by ELISA assay (Figure 2C). On average, 80% of cells were Albumin positive based on flow cytometry analyses (Figure 2D). At the completion of the differentiation protocol, the cells were also found to display several known hepatic functions. Periodic acid–Schiff staining revealed that glycogen synthesis by the differentiated cells, oil red O staining identified the presence of lipid droplets, and incubation of the cells with fluoresceinated Low Density Lipoprotein (Dil-LDL) demonstrated the ability of the cells to accumulate LDL (Figure 2E). The differentiated cells were also capable of uptake of indocyanine green, which was metabolized overnight (Figure 2E) and analyses of the culture media revealed the ability of cells to undertake urea metabolism (Supplemental Figure S2). The morphology of the differentiated cells also shared many characteristics with primary hepatocytes including a large cytoplasmic to nuclear ratio, numerous vacuoles and vesicles, and prominent nucleoli. Several cells were found to be binucleated (Figure 2E panel c, and Supplemental Figure 3); moreover, the differentiated cells formed sheets reminiscent of an epithelial layer and were capable of actively localizing dichlorofluorescein diacetate to their plasma membranes (Figure 2E panel f, arrow). We further examined the extent of differentiation using gene array analyses, which were performed on undifferentiated H9 ES cells and cells subjected to the complete 20 day differentiation protocol in three independent experiments. Genome wide expression profiling studies by others 23 have identified a cluster of 175 genes whose expression is restricted to normal human liver compared with 35 other tissues examined. A subset of 40 of these genes have successfully been used to identify hepatic character in other studies 23 and so we believe that expression of these 40 genes provides an accurate transcriptional fingerprint of a differentiated hepatic phenotype. As expected, this cluster of genes is not expressed in undifferentiated huES cells (Figure 2F and Supplemental Table S2); however, expression of nearly the entire gene set is robustly increased following completion of the differentiation protocol. Based on our analyses shown in Figure 2, we conclude that we have in hand a protocol that can efficiently and reproducibly generate hepatocyte-like cells from huES cells under well-defined culture conditions.

Production of Hepatocyte-like Cells from Human Induced Pluripotent Stem Cells

If hepatocytes could be generated from hiPS cells with efficiencies that resembled those achieved using huES cells, the procedure would provide a reliable tool for the study and treatment of human hepatic disease as well as potentially provide human hepatocytes for toxicological studies and pharmaceutical screens. However, the effect of somatic cell nuclear reprogramming on hepatocyte differentiation from iPS cells is unknown. We, therefore, generated human iPS cells (hiPS) from foreskin fibroblasts by transduction with lentiviruses that independently expressed OCT3/4, SOX2, NANOG, and LIN28 as described by Yu et al 5. A detailed characterization of these iPS cells is shown in Supplemental Figure S4.

We next determined the ability of iPS.C2a cells to form hepatocyte-like cells. Human iPS cells were subjected to the same protocol used to induce formation of hepatocytes from huES cells, and the same analyses were performed. As was the case for huES cells, iPS cells responded to the inductive procedures by expressing all markers of definitive endoderm in response to Activin A, hepatic specification in response to BMP4/FGF2, hepatoblast

formation in response to HGF, and hepatocyte-like differentiation in response to OSM (Figure 3A). Quantification of Albumin-positive cells revealed that the kinetics and efficiency of hepatic differentiation was similar to that found for differentiation of huES cells (Figure 2A). Flow cytometry revealed that at the completion of the differentiation protocol, >80% of cells expressed Albumin (Figure 3B) and the levels of human Albumin in the media approached 1.5 µg/ml following 3-days of culture (Figure 3C). As was the case with human ES cell-derived hepatocyte-like cells, iPS cell-derived hepatocyte-like cells displayed several hepatic functions including accumulation of glycogen, metabolism of indocyanine green, accumulation of lipid, active uptake of low density lipoprotein (Figure 3D), and synthesis of urea (Supplemental Figure S2). Following differentiation, cells generated from hiPS cells shared many of the morphological characteristics associated with hepatocytes (Figure 3D and Supplemental Figure S3). In addition, oligonucleotide array analyses revealed that iPS cell-derived hepatocyte-like cells expressed the same hepatocyte mRNA fingerprint that was found for human ES cell-derived hepatocyte-like cells (Figure 3E and supplemental table S2). We also compared the expression of a series of genes encoding phase I and phase II enzymes, whose expression is characteristic of a fully differentiated hepatocyte, between cadaveric liver samples and hepatocyte-like cells derived from either huES cells or hiPS cells. In both cases, the levels of such mRNAs showed similar trends in expression. Of note, however, the levels of expression of these enzymes were lower in most cases when compared with adult liver samples (Figure 3F) suggesting that although hepatocyte-like cells derived from both huES or hiPS cells have differentiated to a state that supports many hepatic activities including expression of a subset of genes encoding phase I and phase 2 enzymes, they do not entirely recapitulate mature liver function.

Finally we sought to determine whether the differentiated hepatic-like cells generated from huES cells and hiPS cells had the capacity to contribute to the liver parenchyma *in vivo* (Figure 4). To test this, cells were collected at the completion of the 20-day differentiation protocol and approximately 3×10^5 cells were injected into the right lateral liver lobe of newborn mice. Livers were harvested seven days following injection, and human cells were identified using an antibody that specifically recognizes human but not mouse Albumin (Figure 4A). In contrast to control mice, in which no human Albumin-positive cells could be identified, mice injected with either huES cell- or hiPS cell-derived hepatocyte-like cells contained foci of cells throughout the injected lobe that strongly expressed human Albumin (Figure 4A). Uninjected lobes had no human Albumin positive cells. At high resolution, the human Albumin-positive cells in injected lobes could be seen to be integrated into the existing mouse parenchyma. Because Albumin is a secreted protein it could potentially be taken up by surrounding mouse cells giving a false positive result. We, therefore, confirmed that the cells detected as Albumin positive were indeed of human origin using PCR of genomic DNA isolated from human Albumin-positive cells collected by laser capture microdissection (Figure 4B). From these results, we conclude that hiPS cells derived from human foreskin fibroblasts can be efficiently induced to form hepatocyte-like cells in culture and that they have the inherent capacity to integrate into the hepatic parenchyma *in vivo*.

DISCUSSION

Orthotopic liver transplant (OLT) remains the primary mechanism for the treatment of both chronic and acute liver failure. However, the need for OLT far outweighs the availability of donor livers 10. For a subset of liver diseases, particularly those resulting from enzymatic disorders, hepatocyte transplantation could be a viable alternative 9. Several human trials along with the study of animal models have supported the safety and, in some cases, efficacy of using hepatocyte transplantation therapeutically 8. Although primary human hepatocytes

can be purified from donor livers, approximately $1\text{--}5 \times 10^9$ cells are required per transplant, which makes necessary access to large numbers of donor livers or the need to expand primary hepatocytes in culture. However, the ability to use primary hepatocytes either for therapeutic purposes or for basic research has been frustrated by their tendency to rapidly dedifferentiate and lose most hepatic functions after growth in a tissue culture environment 24.

The need to expand primary hepatocytes purified from donor livers could be avoided by using stem cells to produce hepatocytes. Unlike many other stem cells, ES cells and iPS cells can proliferate indefinitely without loss of potency. The appeal of using iPS cells is that they could provide a source of autologous hepatocytes. Several studies have described the differentiation of human embryonic stem cells into cells that display hepatic characteristics $7\cdot12\text{--}14\cdot25\text{--}31$; however, this is the first report demonstrating that iPS cells can also be used to efficiently generate hepatocyte-like cells. Using the described procedure, the generation of hepatocyte-like cells from hiPS cells appears to be as efficient as observed from huES cells, although it was noted that subtle differences in the timing of onset and level of expression of different hepatic genes were found (Figure 3). It is not clear at this point whether such differences in gene expression simply reflect heterogeneity between different iPS lines, as is seen for huES cells, or whether they are characteristic of all hiPS cells in general. Work is under way to address this. In addition, it is important to note that one hiPS cell line we had generated (iPS C3a), although possessing most of the hallmarks of pluripotency, immediately differentiated into a fibroblast-like morphology when plated on Matrigel and was, therefore, not competent to differentiate toward the hepatic lineages. Similarly, it has been noted by others that some hiPS cell lines appear to be incompletely reprogrammed and still others maintain expression of exogenous transgenes, which appear to interfere with differentiation protocols 32. With this in mind, we believe it is crucial that standards for the generation and characterization of hiPS cells are adopted throughout the community to ensure reproducibility of formation of differentiated cells from hiPS cells from different patients and tissue sources.

Although several groups have been able to produce hepatocyte-like cells from huES cells, we believe that the current protocol used to produce hepatocytes from either huES or hiPS cells offers a number of advances. Differentiation is extremely efficient and reproducible with between 80–85% of cells expressing hepatic markers including Albumin. In most other procedures the differentiation of cells relies on embryoid body formation, includes interactions with primary feeder cells, or requires the inclusion of serum during the differentiation procedure. While using such approaches to produce hepatocytes can be successful, the inherent variability associated with use of undefined factors reduces reproducibility. The approach we have described relies on well defined culture conditions. We believe that using such conditions will facilitate accurate analyses of molecular pathways that control human hepatocyte differentiation, comparative studies between iPS cells derived from patients suffering from various congenital liver diseases, and development of screens for novel pharmaceutical approaches to correct liver disease.

While the efficiency of generating cells that exhibit most hepatocyte characteristics is high, we noted that the repertoire of mRNAs encoding phase I and phase II enzymes, which have important roles in controlling drug metabolism and xenobiotic responses, is incomplete when compared with cadaveric livers. Loss of CYP450 enzyme expression is common when hepatocytes are grown under normal culture conditions, and this reflects the complex control of CYP450 expression and activity by several environmental and physiological parameters that are lacking in the tissue culture environment 33·34. We believe our data support the conclusion that both huES and hiPS cells are competent to differentiate toward the hepatocyte lineage; however, we also believe that to use iPS cells as a source of hepatocytes

for toxicological and drug metabolic studies will require the establishment of culture conditions that more fully support expression of a full panel of phase I and II enzymes. In this regard, recent experiments using microengineering approaches have established conditions that allow extended culture of primary hepatocytes that maintain phase I and II enzymatic activities, and we have initiated studies to determine whether such an approach could be useful for culture of hiPS–derived hepatocyte-like cells 35.

In summary, we have shown that mouse iPS cells can be induced to efficiently generate intact fetal livers and that hiPS cells can be induced in culture to produce highly differentiated hepatocytes. We acknowledge that compared with the *in vivo* environment of the liver, the conditions in culture are relatively artificial, and this is likely to impact the function of iPS–derived hepatocytes compared with the native environment. Nevertheless, the data provided above demonstrate the feasibility of generating cells with hepatic characteristics from skin cells through an iPS cell intermediate and that such cells can engraft into the mammalian liver parenchyma. Such proof-of-concept opens up the possibility of producing patient–specific hepatocytes in a relatively simple and straightforward manner with high efficiency. We are confident that such cells could be immediately useful for the study of hepatocellular disease and basic developmental mechanisms and for drug screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this project was provided by National Institutes of Health (NIDDK/NHLBI) grants to S.A.D., and S.D. (NICHD/NHLBI), Advancing a Healthier Wisconsin Fund, as well as gifts from the Marcus Family, Phoebe R. and John D. Lewis Foundation, the Sophia Wolf Quadracci Memorial Fund, and the Dr. James Guhl Memorial Fund.

We thank Charles Myers for providing frozen liver samples.

List of abbreviations

OLT	orthotopic liver transplant
iPS cells	induced pluripotent stem1 cells
ES cells	embryonic stem cells
huES cells	human ES cells
EGFP	enhanced green fluorescent protein
HNF	hepatocyte nuclear factor
GATA4	GATA binding protein 4
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
Fabp1	fatty acid binding protein 1
Rbp4	Retinol binding protein 4
Fox	Forkhead box
Pxr	Pregnane X receptor
Sox17	Sex determining region Y box 17
AFP	Alpha feto-protein

ALB	Albumin
MEFs	Mouse embryonic fibroblasts
Oct4	Octamer-4
SSEA4	Stage specific embryonic antigen 4
BMP4	Bone morphogenetic protein 4
FGF2	Fibroblast growth factor 2
HGF	Hepatocyte growth factor
OSM	Oncostatin M
ELISA	Enzyme-linked immunosorbent assay
Dil-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-low density lipoprotein
DCF-DA	Dichlorofluorescein diacetate
NANOG	NANOG homeobox
LIN28	LIN28 homolog
hiPS	human iPS
CYP450	Cytochrome P450
Pol2ra	RNA polymerase II
HPRT	Hypoxanthine-guanine phosphoribosyltransferase

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Figure 1. Fetal livers derived from mouse iPS cells

A) Brightfield (top) and fluorescent (bottom) images of CAG-eGFP^{+/−} (eGFP), eGFP negative (CD1), and iPS cell-derived (eGFP:iPS 13) E12.5 embryos. B) Brightfield and fluorescent images of CAG-eGFP^{+/−} (eGFP; left), eGFP-negative (CD1; right), and iPS cell-derived E12.5 fetal livers (eGFP:iPS 13). C) Whole mount images of E14.5 embryos (top) and their livers (middle) derived from wild-type (CD-1) and iPS cells (iPS 13). Bottom panels show hematoxylin and eosin stained sections through control (CD-1) and iPS cell-derived (iPS 13) E14.5 livers. D) Immunohistochemistry revealing marker expression in hepatocytes (HNF4a), endothelial cells (GATA4), sinusoidal cells (Lyve-1), and macrophages/Kupffer cells (F4/80) in control (CD-1) and iPS cell-derived (iPS 13) E14.5 livers. Scale bars = 100μm. E) RT-PCR analyses of two control (CD-1) and iPS-derived (iPS 13) E14.5 livers showing steady levels of characteristic liver mRNAs as well as *neomycin phosphotransferase II* mRNA, which is only expressed in iPS cell-derived livers. *RNA polymerase II mRNA (Pol2ra)* was used as a loading control, and reactions without reverse transcriptase (−RT) or template (0) confirmed the absence of contaminating DNA.

Figure 2. Generation of hepatocytes from human ES cells

A) Flow diagram outlining the procedure used to control hepatocyte differentiation. B) Hepatocyte differentiation from huES cells was monitored by immunocytochemistry at day 0, 5, 10, 15, and 20 using antibodies that recognized OCT3/4, FOXA2, SOX17, GATA4, HNF4a, Alpha-fetoprotein (AFP), and Albumin. Results are representative of three independent differentiation experiments. C) Albumin secretion by huES cell-derived hepatocytes was identified after 3-days of culture in medium by ELISA. D) Representative flow cytometry profile showing the average number of Albumin positive hepatocytes in three independent differentiation experiments = 80.9% ± 0.75. E) hES cell-derived hepatocytes were shown to store glycogen by PAS staining (a); store lipids by Oil Red O staining (b); to display hepatocyte morphology including bi-nucleated cells (black arrow) (c); efficiently uptake LDL using Dil-LDL (d); metabolize indocyanine green (e); and localize dichlorofluorescein diacetate to their plasma membranes (white arrow) (f). F) Heat map of gene array analyses demonstrating that a series of 40 mRNAs that are solely expressed in livers 23 were increased (red=high, blue=low) following differentiation (Hep) compared with undifferentiated (ES) cells.

Figure 3. Differentiation of hepatocytes from human iPS cells

A) Hepatocyte differentiation from hiPS cells was followed by detecting OCT3/4, FOXA2, SOX17, GATA4, HNF4a, Alpha-fetoprotein (AFP), and Albumin (ALB) by immunocytochemistry at day 0, 5, 10, 15, and 20 days. Results are representative of three independent differentiation experiments. B) Representative flow cytometry profile showing the average number of Albumin positive hiPS-derived hepatocytes in three independent differentiation experiments = $81.0\% \pm 4.8$. C) Albumin secretion by hiPS cell-derived hepatocytes was identified in the medium after 3-days of culture using ELISA. D) Hepatocyte-like cells derived from hiPS cells were shown to store glycogen by PAS staining (a); store lipids by Oil Red O staining (b); uptake LDL using Dil-LDL (c); and metabolize indocyanine green (d); have similar morphology to hepatocytes with some cells being bi-nucleated (black arrow) (e); and direct dichlorofluorescein diacetate to plasma membranes (white arrow) (f). E) Heat map of gene array analyses showing that expression of 40 liver-specific mRNAs 23 was increased (red) following differentiation (Hep) compared with undifferentiated (hiPS) cells where the majority of these mRNAs were expressed at relatively low levels (blue). F) Bar graphs showing the levels of mRNAs, determined by real-time qRT-PCR, encoding Phase I and II enzymes in hepatocyte-like cells derived from H9 huES cells (yellow) and C2 hiPS cells (orange) expressed as fold of levels found in cadaveric human liver samples.

Figure 4. Integration of huES and hiPS cell-derived hepatocytes into the mouse hepatic parenchyma

A) Micrographs showing the identification of cells expressing human Albumin (brown) in human livers (upper right) and in mouse livers injected with huES cell- (lower left) and hiPS cell-derived (lower right) hepatocytes but not in uninjected control mouse livers (top right). B) PCR analyses using primers that specifically recognize human *Alu* or mouse *HPRT* sequences on genomic DNA isolated from control mouse and human fibroblasts as well as cells collected by laser capture from sections through mouse liver, human tonsil, and albumin positive cells from mouse livers injected with huES cell- or hiPS cell-derived hepatocytes. Amplifications performed without template ensured the absence of contaminating DNA.