BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Differentiation and Transplantation of Human Embryonic Stem Cell–Derived Hepatocytes

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Background & Aims: **The ability to obtain unlimited numbers of human hepatocytes would improve the development of cell-based therapies for liver diseases, facilitate the study of liver biology, and improve the early stages of drug discovery. Embryonic stem cells are pluripotent, potentially can differentiate into any cell type, and therefore could be developed as a source of human hepatocytes.** ***Methods:*** **To generate human hepatocytes, human embryonic stem cells were differentiated by sequential culture in fibroblast growth factor 2 and human activin-A, hepatocyte growth factor, and dexamethasone. Functional hepatocytes were isolated by sorting for surface asialoglycoprotein-receptor expression. Characterization was performed by real-time polymerase chain reaction, immunohistochemistry, immunoblot, functional assays, and transplantation.** ***Results:*** **Embryonic stem cell–derived hepatocytes expressed liver-specific genes, but not genes representing other lineages, secreted functional human liver–specific proteins similar to those of primary human hepatocytes, and showed human hepatocyte cytochrome P450 metabolic activity. Serum from rodents given injections of embryonic stem cell–derived hepatocytes contained significant amounts of human albumin and α1-antitrypsin. Colonies of cytokeratin-18 and human albumin–expressing cells were present in the livers of recipient animals.** ***Conclusions:*** **Human embryonic stem cells can be differentiated into cells with many characteristics of primary human hepatocytes. Hepatocyte-like cells can be enriched and recovered based on asialoglycoprotein-receptor expression and potentially could be used in drug discovery research and developed as therapeutics.**

Transplantation of isolated hepatocytes has been shown in the laboratory and in patients to be a minimally invasive intervention that can augment the function of the liver.[1–3] This is an especially exciting alternative to organ transplantation for patients with acute decompensation of the liver or life-threatening liver-based genetic disorders. A shortage of human livers, however, presently limits the use of hepatocytes for transplantation. Thus, the availability of an unlimited supply of human hepatocytes would facilitate the development and clinical application of hepatocyte transplantation. An abundant source of human hepatocytes also could be used to repopulate the livers of immune-deficient mice,[4] facilitating the study of human liver disease, hepatitis virus biology,[5] and human drug metabolism.[6,7]

Human hepatocyte-like cells have been generated from a variety of putative stem cell sources.[8–14] However, although investigators have generated cells with some characteristics approaching that of human hepatocytes, to date, no approach to generating hepatocytes from stem cells has succeeded in generating a homogeneous population of cells that could provide levels of function comparable with primary liver cells. Here we report a

Abbreviations used in this paper: AAT, α1-antitrypsin; AFP, α-fetoprotein; ASGPR, asialoglycoprotein receptor; CF VII, coagulation factor VII; CYP, cytochrome P450; DMEM/F12, Dulbecco's modified Eagle medium F-12; EB, embryoid bodies; ES, embryonic stem; hES, human embryonic stem; KSR, Knockout Serum Replacement; RT-PCR, reverse-transcription polymerase chain reaction.

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differentiation strategy that produces a relatively homogeneous population of cells from human embryonic stem (hES) cells that show the morphologic and phenotypic properties of human hepatocytes.

Materials and Methods

Differentiation Program

hES cells (H1) were obtained from WiCell Research Institute (Madison, WI) and maintained on mitotically inactive mouse embryo fibroblast feeder layers in Dulbecco's modified Eagle medium/Ham's F-12 medium (DMEM/F12), high (20%) Knockout Serum Replacement (KSR), 4 ng/mL human recombinant fibroblast growth factor-2, 1 mmol/L nonessential amino acids, L-glutamine, and 0.1 mmol/L 2-mercaptoethanol (Invitrogen, Carlsbad, CA). Embryonic bodies (EBs) were generated by plating collagenase-passaged cells at a density of 1–5 $\times$ 10^4 cells per cm^2 on low-attachment Petri dishes for 48 hours in DMEM/F12 supplemented with 15% KSR, 1 mmol/L nonessential amino acids, and L-glutamine. For differentiation, EBs were plated on 5% Matrigel-Growth Factor Reduced (R&D Systems Inc, Minneapolis, MN), and maintained for 3 days in DMEM/F12 media supplemented with 100 ng/mL Recombinant activin-A (R&D Systems Inc) and 100 ng/mL fibroblast growth factor-2 (Invitrogen). The concentration of serum (fetal bovine serum or KSR) was 0% for the first 24 hours, 0.2% for the second 24 hours, and 2.0% for the last 24 hours. Cells then were grown for 8 days in DMEM/F12 containing 10% fetal bovine serum or KSR, 1 mmol/L nonessential amino acids, L-glutamine, 1% dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO), and 100 ng/mL hepatocyte growth factor (R&D Systems Inc), followed by culture for 3 additional days in DMEM/F12 containing 10% fetal bovine serum or KSR, 1 mmol/L nonessential amino acids, L-glutamine, and 10^{-7} mol/L dexamethasone (Sigma–Aldrich).

Animals

Male nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, albumin-urokinase-type plasminogen activator (Alb-uPA SCID) mice,[15] and Nagase analbuminemic rats[16] were bred in the animal breeding facility at the University of Nebraska Medical Center and were used for transplantation procedures. All procedures performed on animals were approved by the University of Nebraska Medical Center Animal Care and Use Committee, and thus were within the guidelines for humane care of laboratory animals.

Transplantation

One million hES cells, recovered after 18 days of differentiation, were transplanted into the spleens of NOD/SCID mice using a 25-gauge needle. Recipient NOD/SCID mice were injected on 2 occasions before transplantation with 70 mg/kg retrorsine, separated by 2 weeks, and underwent 50% partial hepatectomy 4 weeks later, at the time of transplantation.[17] Nagase rat recipients similarly were treated twice with 30 mg/kg of retrorsine separated over 2 weeks, and underwent a 70% partial hepatectomy just before transplantation.[18] Administration of FK506 at 2 mg/kg/day in these animals controls rejection of xenogeneic hepatocytes for at least 2 months (unpublished data). After transplantation of 1 million asialoglycoprotein receptor (ASGPR)-sorted differentiated hES cells into the spleen of a Nagase analbuminemic rat, human albumin levels were measured weekly by enzyme-linked immunosorbent assay. Three-week-old Alb-uPA SCID mice underwent transplantation without treatment with retrorsine or partial hepatectomy by intrasplenic injection. Graft survival was assessed by serum measurements of human albumin and α1-antitrypsin (AAT) levels, which were measured by enzyme-linked immunosorbent assay.

Polymerase Chain Reaction

RNA was prepared using a Qiagen RNeasy Mini Kit (Hilden, Germany). Chromosomal DNA was removed using RNase-free DNase (Qiagen), and complementary DNA was prepared using the Superscript First-Strand synthesis system (Invitrogen), using 1 μg of total RNA. Specific primers used for reverse-transcription polymerase chain reaction (RT-PCR) are described in supplementary Table 1 (see supplementary material online at www.gastrojournal.org). PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. Human glyceraldehyde-3-phosphate dehydrogenase and cyclophilin served as internal controls. Real-time RT-PCR was performed as previously described.[19]

Flow Analysis and Sorting of ASGPR-Expressing ES-Derived Hepatocytes

ES-derived cells were trypsinized and resuspended in DMEM/F12 supplemented with 10% KSR. Cells were allowed to recover for 30 minutes and then centrifuged at 1200 rpm. Nonspecific binding was blocked using 100 μL human Fc for 30 minutes, and then cells were treated for 30 minutes with a 100-μL solution containing a goat-anti-human ASGPR1 antibody diluted 1:50 (Santa Cruz Biotechnology Inc, Santa Cruz, CA). A fluorescein isothiocyanate–labeled mouse anti-goat antibody was added to the cells for 30 minutes at a concentration of 0.5 μg/mL. Cells were washed 3 times with 1 mL fluorescence-activated cell sorter buffer (1% sodium azide and 2% fetal bovine serum in phosphate-buffered saline), resuspended in 500 μL fluorescence-activated cell sorter buffer, and sorted by flow cytometry. Cell sorting was performed on a Becton–Dickinson FACSVantage DiVa flow cytometer and data acquisition and analysis was performed using Becton–Dickinson DiVa software (Becton–Dickinson, San Jose, CA).

Electron Microscopy

Cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in Millonig's phosphate buffer, pH 7.38,

postfixed in buffered 1% osmium tetroxide, washed in triple-distilled water, dehydrated in a graded ethyl alcohol series, infiltrated and embedded with Epon resin (Electron Microscopy Sciences, Fort Washington, PA), and polymerized at 60°C for 48 hours. Multiple 1-μm sections were stained with 0.5% Tolidine blue and 0.5% borax, and evaluated by light microscopy. Representative areas were selected. Silver sections, 70 nm, mounted on 300 mesh Athene copper grids (Ted Pella, Inc, Redding, CA), were stained with uranyl acetate followed by Reynold's lead citrate (Sigma–Aldrich) and examined with a JEOL 1230 transmission electron microscope (JEOL, USA, Peabody, MA) at 60 kV. Representative digital images were taken using a SIS Keenview camera and software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

Measurement of Coagulation Factor VII Activity

Coagulation factor VII (CF VII) activity was measured by monitoring the release of p-nitroanilide from Spectrozyme (American Diagnostics, Inc, Stamford, CT) FXa by factor Xa, generated from factor X by tissue factor-bound CF VII.[20] A Tmax plate reader (Molecular Devices, Sunnyvale, CA) was used to monitor the assays conducted in 96-well plates (Falcon/BD Biosciences, San Jose, CA). Stocks of purified CF VIIa (Novo Nordisk, Princeton, NJ) and factor X (purified from human plasma[21]) and homogenized human brain powder[22] were diluted before use in Tris-buffered saline (0.05 mol/L Tris, 0.1 mol/L NaCl, pH 7.6) containing 1 mg/mL bovine serum albumin (Sigma). For measurement of CF VII, 20 uL of spent medium was combined with 10 uL of the Tris buffer with bovine serum albumin, 50 uL of 1/100 dilution of brain homogenate, 20 uL factor X (concentration in the final 160-uL assay volume was approximately 100 nmol/L), 50 uL of 25 mmol/L calcium chloride, and 10 uL of Spectrozyme FXa (final concentration 310 umol/L). A CF VIIa calibration curve was generated concurrently using serial dilutions of the pure CF VIIa in place of the spent medium. The plot of factor Xa [pmol/L]/min vs CF VIIa [pmol/L] was described by $y = (1141[\times^{0.73}])/([\times^{0.73}] + 45.8) - 0.44$ in the second assay, for

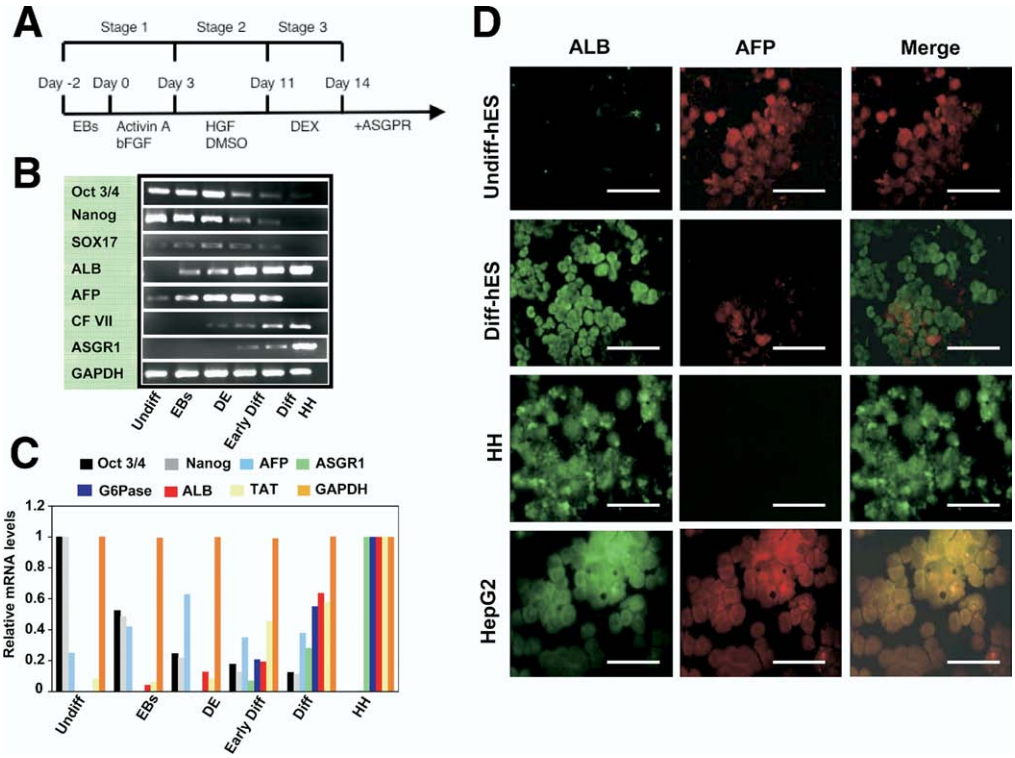

Figure 1. Differentiation of hES cells towards a hepatocyte phenotype and characterization of hepatocyte-directed cells. (*A*) Strategy for differentiation of hES cells to hepatocytes. (*B*) RT-PCR and (*C*) real-time analysis for expression of lineage-specific hepatic markers (albumin [ALB], ASGPR 1, glucose-6-phosphatase [G6Pase], tyrosine aminotransferase [TAT], and CF VII), endoderm markers (SOX 17 and AFP), and markers for undifferentiated cells (Pou5f1 and Nanog). *Lanes*: (1) undifferentiated hES cells (Undiff), (2) after EB formation (EBs), (3) after treatment with fibroblast growth factor-2 (FGF-2) and activin-A or at the definitive endoderm (DE) stage, (4) after culture in HGF and dimethyl sulfoxide (Early Diff), and (5) after culture in dexamethasone (Diff). Primary human hepatocytes served as the positive control. Forward and reverse primers used for these studies are listed in supplementary Table 1 (see supplementary material online at www.gastrojournal.org). (*D*) Immunohistochemistry of hES cells at day 18 of the differentiation protocol, after final exposure to dexamethasone (Diff-hES), for albumin, AFP, and merged albumin and AFP expression. Undifferentiated hES (Undiff hES), primary human hepatocytes (HH), and HepG2 cells were used as controls. Scale bar, 50 μm. Approximately 55% of cells expressed albumin whereas only 12% co-expressed AFP.

which the CF VIIa dilutions covered the range of activities measured with the spent medium.

Metabolic Activity

ES-derived or normal human hepatocytes were plated in hepatocyte maintenance medium (HMM, Lonza, Walkersville, MD) media at a density of 1.5 million cells per well in collagen-coated, 6-well plates. Cells were cultured for 72 hours in the presence of 1 mmol/L phenobarbital or 25 μmol/L β-naphthoflavone as previously described.[23,24] After the induction period, cells were washed 3 times to remove inducers, and media was replaced with fresh media (without inducers). For the measurement of cytochrome P450 (CYP) 1A activity, cells were exposed to media containing 20 μmol/L 7-ethoxyresorufin and 1.5 mmol/L salicylamide for 1 hour. The conversion of 7-ethoxy to 7-hydroxyresorufin in the media was quantified by the fluorescence of the 7-hydroxy metabolite measured at 535 nm (fluorescence excitation) and 581 nm (fluorescence emission).[24] Salicylamide is used to prevent the conjugation of resorufin formed by de-ethylation. The analysis of CYP3A activity was measured by exposing cells to 350 μmol/L testosterone for 1 hour. The conversion of testosterone to 6β-hydroxytestosterone was measured by a high-pressure liquid chromatographic method as previously described.[23]

Results

Differentiation of hES Toward a Hepatocyte Phenotype

To generate ES cell–derived hepatocytes, human (H1) ES cells were plated on low-attachment Petri dishes to form embryoid bodies. EBs then were plated on 5% Matrigel for 3 days in recombinant activin-A and fibroblast growth factor-2. Cells then were grown for 8 days in 10% serum (or KSR) containing HGF, followed by culture for 3 additional days in 10% serum or KSR, and 10^{-7} mol/L dexamethasone (Figure 1*A* and supplementary Figure 1; see supplementary material online at www.gastrojournal.org).

Real-time and RT-PCR analysis was performed on cell preparations at various times during the culture process to determine the time course and degree to which the ES cells differentiated toward a hepatocyte phenotype. Analysis included markers for endoderm-specific gene expression (sox 17 and α-fetoprotein [AFP]), hepatocyte-specific gene expression [albumin, ASGPR 1, and/or CF VII], and markers for undifferentiated hES cells (Pou5f1 and Nanog). As shown in Figure 1*B* and *C*, endoderm-specific gene expression increased early whereas Pou5f1 and Nanog expression gradually decreased and hepatocyte-specific gene expression progressively increased over the course of the differentiation program.

To determine the percentage of cells differentiating toward a hepatic phenotype, immunohistochemistry was performed for albumin and AFP. As shown in Figure 1*D*, undifferentiated H1 hES cells had large nuclei, as shown by 4′,6-diamidino-2-phenylindole staining, and expressed some AFP, probably representing low-grade spontaneous differentiation in culture, but no albumin. After culture in growth factors, approximately 55% of cells expressed albumin, only 12% of which co-expressed AFP. There was also a dramatic change in morphology during the course of the differentiation process. As shown in Figure 2, polygonal-shaped cells containing multiple nuclei formed and transmission electron microscopy revealed cells containing glycogen granules (Figure 2*B*), mature round nuclei with evenly distributed chromatin, Golgi complexes (Figure 2*C*), and well-developed bile canaliculi with apical microvilli and tight junctions (Figure 2*D* and *E*).

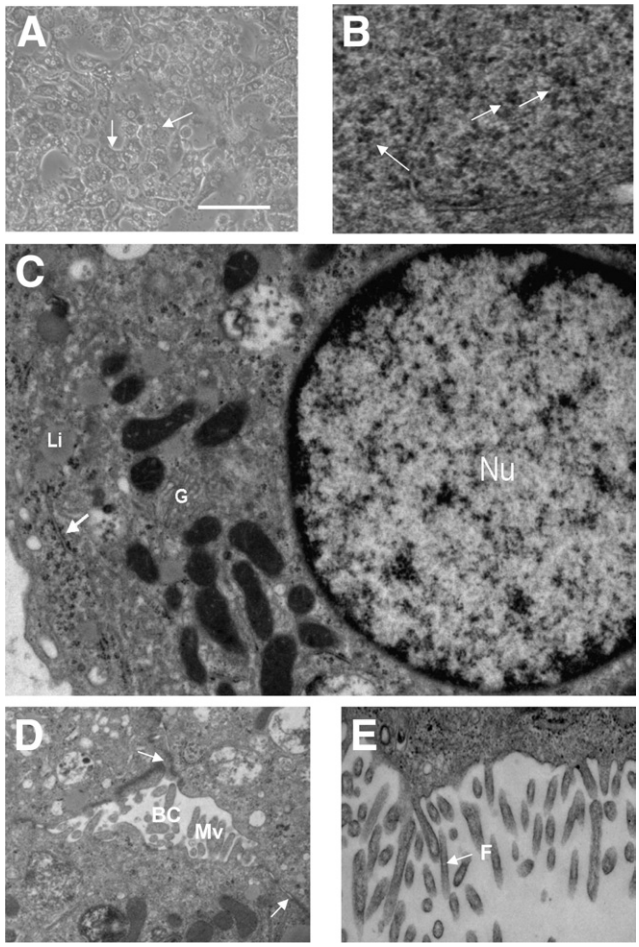

Figure 2. Morphologic analysis of differentiated hES cells. Morphologic analysis of differentiated hES cells in culture indicate that the differentiation program generated cells morphologically similar to hepatocytes, (*A*) being polygonal in shape with multiple nuclei (*arrows*). Transmission electron microscopy of differentiated hES showed (*B*) accumulation of glycogen rosettes (*arrows*), (*C*) round nuclei (NU) with evenly distributed chromatin, Golgi complexes (G), and liposomes (LI), with glycogen rosettes identified again by an *arrow*, and (*D* and *E*) well-developed bile canaliculi (BC) with apical microvilli (MV) containing filaments (F), and tight junctions (*arrows*). *Scale bar*, 50 μm. Original magnification, 30,000×.

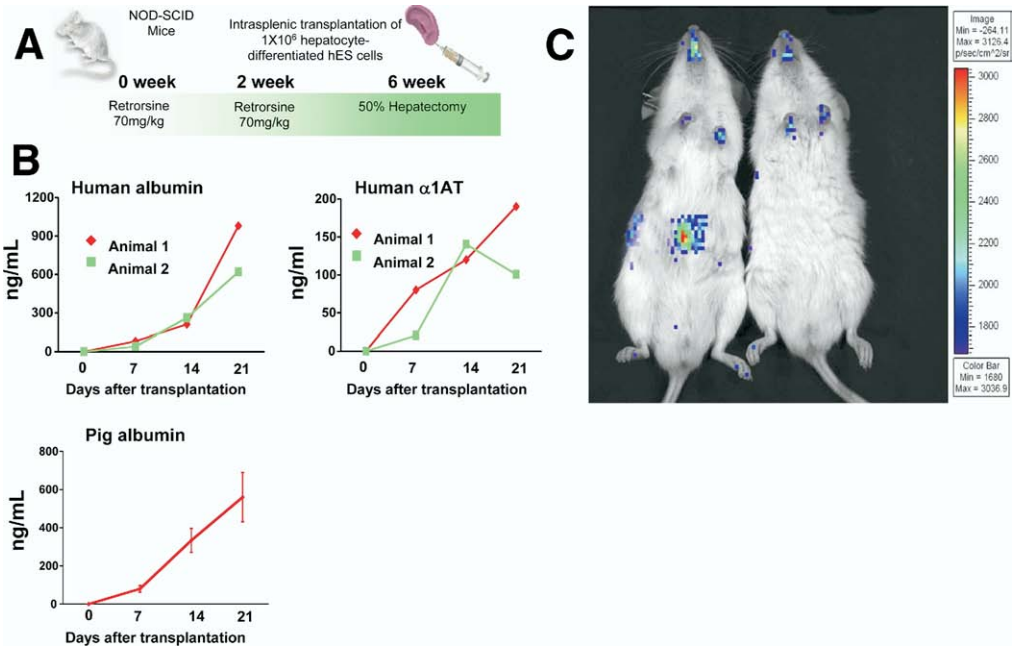

Figure 3. Transplantation of differentiated cells and characterization of cells sorted for surface ASGPR expression. (*A*) One million hepatocyte-differentiated hES cells were transplanted into retrorsine-treated immune-deficient NOD/SCID mice after 50% partial hepatectomy. After transplantation, (*B*) human albumin and human α1AT levels were measured by enzyme-linked immunosorbent assay. Pig albumin levels in control mice transplanted with primary porcine hepatocytes are shown for comparison. (*C*) Imaging for luciferase-expressing cells was performed 3 weeks after transplantation. Before transplantation, differentiated hES cells were transduced to express firefly luciferase. Differentiated cells containing an active albumin promoter, and thus expressing luciferase, appear to have traveled through the portal circulation after introduction into the spleen to engraft in the livers of transplanted mice. There was no evidence of luciferase-expressing cells in the spleen.

Transplant studies then were performed to determine whether differentiated hES cells would, similar to primary hepatocytes, home to and engraft in the liver after injection in the spleen. To allow noninvasive tracking of cells after transplantation, differentiated hES cells were transduced with a recombinant lentivirus containing the gene encoding firefly luciferase under the control of the human albumin promoter.[25] One million cells were transplanted into the spleens of immune-deficient NOD/SCID mice. Before transplantation, recipient mice were injected twice with 70 mg/kg retrorsine, and underwent a 50% partial hepatectomy at the time of transplantation (Figure 3*A*). Under these conditions, the transplanted cells have a selective growth advantage compared with the native liver cells, whose proliferation is blocked by retrorsine.[17,18] After transplantation, the serum of NOD/SCID mouse recipients of differentiated hES contained 500–1000 ng/mL human albumin and 100–200 ng/mL human AAT (Figure 3*B*). Control mice transplanted with primary porcine hepatocytes contained approximately 500 ng/mL porcine albumin (Figure 3*B*). As shown in Figure 3*C*, whole mouse IVIS (Caliper Life Sciences, Hopkinton, MA) imaging performed 21 days after transplantation revealed that differentiated cells containing an active albumin promoter, and thus expressing luciferase, had traveled through the portal circulation after introduction into the spleen to engraft in the livers of transplanted mice. No luciferase-expressing ES-derived cells appeared in the location of the spleen. At death on posttransplant day 21, however, gross and histologic examination revealed teratomas in the liver, spleen, and peritoneal cavity containing poorly differentiated cells.

Enrichment for hES-Derived Hepatocytes Based on ASGPR Expression

To isolate the population of cells with a differentiated hepatocyte phenotype and reduce or eliminate the undifferentiated or poorly differentiated cells, or cells that might have differentiated toward a different cell lineage, differentiated hES cells were sorted by flow cytometry for expression of the ASGPR, a definitive feature of hepatocytes. As shown in Figure 4*A*, approximately 18%–26% of hES-derived cells expressed ASGPR. Nucleic acids from the sorted population, from undifferentiated hES cells, and from hES cells during the various stages of the differentiation process then were assessed by RT-PCR and real-time analysis to determine the extent to which enrichment toward a hepatocyte phenotype resulted from sorting (Figure 4*B* and *C*). Real-time PCR showed that the sorted cells had a gene expression profile near that of primary adult human hepatocytes. Cells also expressed the genes encoding bilirubin-UDP glucuronosyl transferase, ornithine transcarbamylase, and the bile salt export

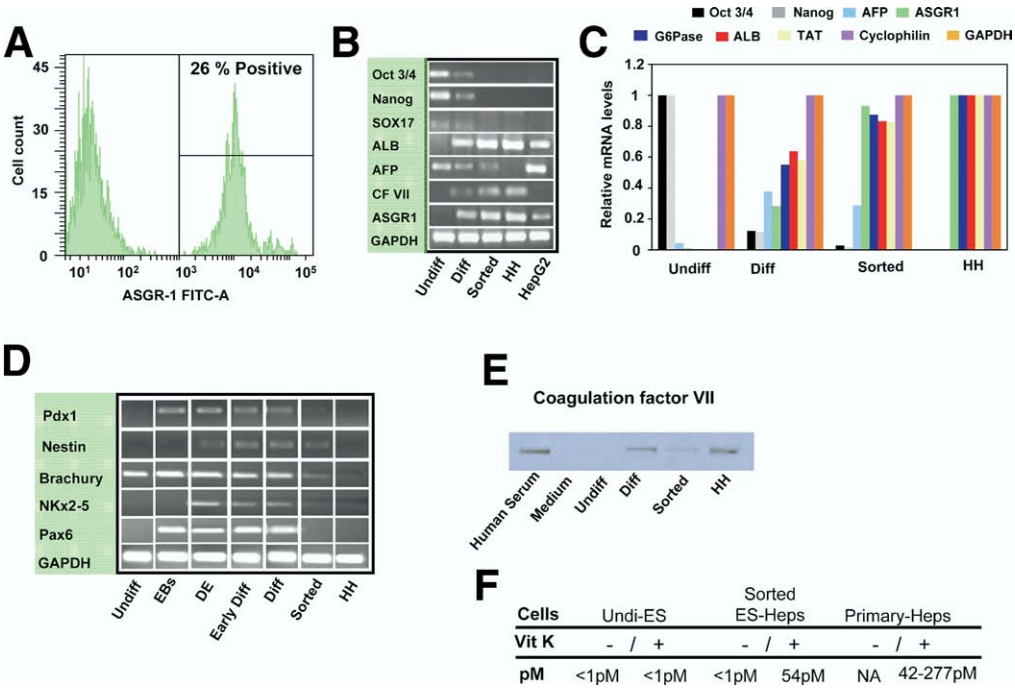

Figure 4. Enrichment of hES differentiated cells by ASGPR expression and characterization of sorted cells. (*A*) Flow cytometry showed that 18%–26% of differentiated cells expressed the ASGPR, a definitive feature of differentiated hepatocytes. (*B*) RT-PCR and (*C*) real-time analysis was performed to determine gene expression levels during the various stages of differentiation and after sorting relative to levels in primary human hepatocytes (HH). Expression levels of lineage-specific hepatic markers (albumin [ALB], tyrosine aminotransferase [TAT], glucose-6-phosphatase [G6Pase], CF VII, and ASGPR1), endoderm (Sox17) and the gestational hepatocyte marker AFP, and markers for undifferentiated cells (Pou5f1 and Nanog) were examined and messenger RNA expression levels were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, and primary human hepatocytes. (*D*) To assess the lineage specificity of the differentiation program, mRNA was analyzed further by RT-PCR for Pdx-1 (a pancreatic marker gene), Nestin (ectoderm), Brachury (mesendoderm), NKx2.5 (mesoderm), and Pax-6 (ectoderm). Undifferentiated hES (*lane 1*), after EB formation (*lane 2*), hES differentiated to DE (*lane 3*), after culture in HGF and dimethyl sulfoxide (Early Diff; *lane 4*), after culture in dexamethasone (Diff; *lane 5*), and Diff stage cells after enrichment for ASGPR surface expression (sorted; *lane 6*). Primary human hepatocytes (*lane 7*) were used as control cells. (*E*) Production of CF VII protein by unsorted and ASGPR-sorted hES-derived cells was confirmed by immunoblot, and (*F*) functional CF VII activity in culture supernatants supplemented with vitamin K was shown to be similar to that produced by cultured primary human hepatocytes.

pump (supplementary Figure 2*A*; see supplementary material online at www.gastrojournal.org). RT-PCR performed for Pdx-1 (a pancreatic marker gene), Nestin (ectoderm), Brachury (mesendoderm), NKx2.5 (mesoderm), and Pax-6 (ectoderm) showed that the sorting process significantly enriched for cells that did not express genes representing other lineages (Figure 4*D*). Complete loss of Pou5f1 and Nanog indicated elimination of less differentiated cells, and loss of Sox17 indicated enrichment of cells beyond the endoderm stage of development. A small amount of AFP expression remained present in sorted cells, indicating the presence of cells that could represent late-gestation fetal hepatocytes.

Characterization and Transplantation of ASGPR-Enriched ES-Derived Cells

To determine the capacity of differentiated hES to function as primary hepatocytes we examined production of CF VII, a gene product specific to hepatocytes. Western blot (Figure 4*E*) and functional assay of vitamin K–supplemented cell supernatants from sorted ES-derived hepatocytes showed production of functionally active CF VII on the order of that produced by primary human hepatocytes cultured under the same conditions (Figure 4*F*). Cells also were assessed for their ability to generate human albumin, AAT, and urea in culture. Cells enriched by sorting for ASGPR expression generated approximately 75% of the albumin, 65% of the AAT, and 65% of the urea generated by primary human hepatocytes (Figure 5*A*). ES-derived cells also were assessed for expression of human hepatocyte–specific cytochrome P450 metabolizing genes and P450 metabolic activity. ASGPR-sorted differentiated hES cells expressed CYP 1A1, 1A2, 2B6, 3A4, and 7A1 by real-time PCR (Figure 5*B*), converted testosterone to 6-β-hydroxytestosterone, a specific measure of CYP 3A4–mediated metabolism, and showed inducible hepatic CYP 1A1/1A2–mediated ethoxyresorufin–O-deethylase activity (Figure 5*C*).

A total of 100,000–200,000 ASGPR-sorted differentiated cells then were transplanted into each of 5 Alb-uPA SCID mice. The Alb-uPA SCID mouse is a T- and B-cell–deficient mouse that carries a tandem array of murine

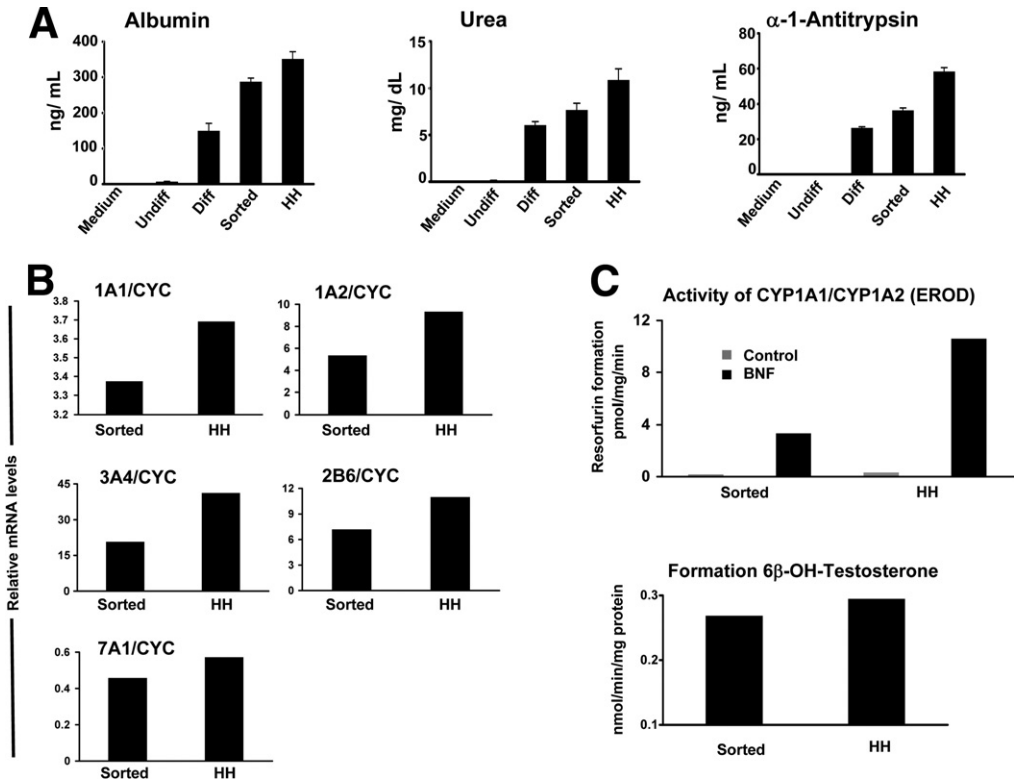

Figure 5. Functional analyses of differentiated hES cells enriched toward a hepatocyte phenotype. (*A*) Albumin, urea, and AAT secretion was determined in vitro by primary human hepatocytes (HH) and hES cells during differentiation (n = 5). Analysis involved undifferentiated hES cells (Undiff), hES cells after culture in dexamethasone (Diff), and hES cells after the Diff stage and after enrichment for ASGPR surface expression (sorted). Culture medium used control. (*B*) Real-time analysis showed expression of cytochrome P450 1A1 (CYP1A1), 1A2, 3A4, 2B6, and 7A1 at levels similar to that derived from fresh primary human hepatocytes. (*C*) To assess human liver–specific cytochrome P450 metabolic activity, ES-derived or normal human hepatocytes were cultured in the presence of phenobarbital or 25 μmol/L β-naphthoflavone (BNF). For measurement of CYP 1A activity, cells were exposed to media containing 20 μmol/L 7-ethoxyresorufin and conversion to 7-hydroxyresorufin in the media was quantified by the fluorescence of the 7-hydroxy metabolite measured at 535 nm (Ex) and 581 nm (Em). Analysis of CYP3A activity was measured by conversion of testosterone to 6β-hydroxytestosterone by high-pressure liquid chromatography. Studies showed BNF-inducible ethoxyresorufin–O-deethylase (EROD) activity at approximately 25%–30% of that generated by primary human hepatocytes, and baseline formation of testosterone by differentiated ASGPR-enriched cells near that produced by cultured primary human hepatocytes.

urokinase genes under the control of an albumin promoter.[15,26] The transgene is toxic to the host liver, and prevents native hepatocytes from responding with regeneration. At 75 days after transplantation, the serum of these animals contained 1000–2000 ng/mL human albumin and AAT (Figure 6*A*). Serum albumin levels from control animals transplanted with 1,000,000 primary human hepatocytes derived from fresh liver resection specimens (supplementary Table 2; see supplementary material online at www.gastrojournal.org) or cadaver donor livers not used for transplantation range from less than 1000 ng/mL to greater than 1 mg/mL depending on donor age and source.[27] At death, examination of liver specimens from transplanted animals revealed small human cytokeratin-18 staining clusters of engrafted cells throughout the liver (Figure 6*B*). No teratomas or tumors were identified in these animals by gross or histologic examination. One million sorted cells also were transplanted into the spleen of 1 FK506-immune suppressed Nagase analbuminemic rat. Before transplantation, the recipient animal was injected with retrorsine and underwent a 70% partial hepatectomy at the time of transplantation, similar to what had been performed previously in NOD-SCID mice, to create an environment in which there was a selective growth advantage to the transplanted cells. At 28 days after transplantation the blood of this recipient contained 2000 ng/mL (0.002 mg/mL) human albumin. For comparison, 28 days after transplantation with 5,000,000 primary pig hepatocytes, serum porcine albumin levels of approximately 5000 ng/mL (0.005 mg/mL) were obtained (Figure 6*C*). At 55 days after transplantation the blood of the animal that received ASGPR-sorted differentiated cells contained 20,000 ng/mL (0.02 mg/mL) human albumin (Figure 6*C*). Examination of liver specimens from this animal revealed large human albumin staining clusters of engrafted cells present throughout the liver (Figure 6*D*). Thus, ES-derived human hepatocytes were capable of homing to and engrafting normally in the liver, expanding in response to a physiologic proliferation signal, and secreting

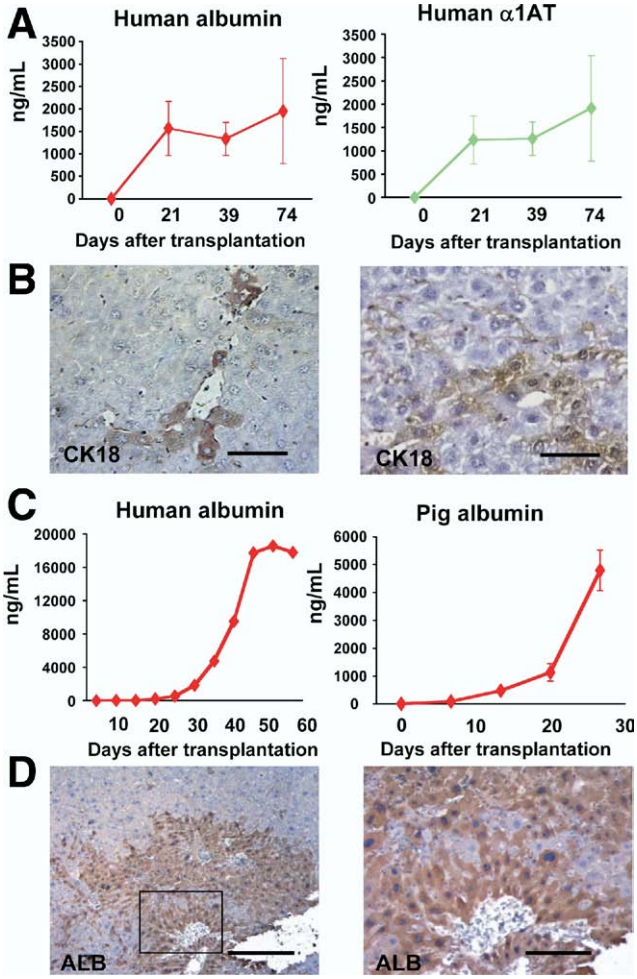

Figure 6. Transplantation of ASGPR-enriched hES-derived hepatocytes in uPA-SCID mice and an immune-suppressed Nagase analbuminemic rat. A total of 100,000–200,000 hES-derived cells, sorted based on surface ASGPR expression, were transplanted in immune-deficient Alb-uPA SCID mice. (*A*) After transplantation, human albumin and human α1AT levels were measured by enzyme-linked immunosorbent assay. At 75 days after transplantation, the serum of these animals contained 1000–2000 ng/mL human albumin and AAT. (*B*) Immunohistochemistry was performed on liver sections of transplanted animals, and small clusters of human cytokeratin-18 (CK18)-staining engrafted cells, which stain dark brown, with hepatocyte morphology, were present throughout the liver. (*C*) One million ASGPR-sorted differentiated hES cells also were transplanted into the spleen of a retrorsine-treated FK506 immune-suppressed Nagase analbuminemic rat that underwent 70% partial hepatectomy at the time of transplantation. After transplantation, human albumin levels were measured at 20,000 ng/mL (0.2 mg/mL) by enzyme-linked immunosorbent assay 55 days after transplantation. Comparable serum porcine albumin levels were measured in control Alb-uPA SCID mice up to 28 days after transplantation with 4°C 24-hour, University of Wisconsin–preserved primary pig hepatocytes. (*D*) At that time, immunohistochemistry showed large colonies of human albumin–expressing cells. ALB, albumin.

functional liver-specific proteins after engraftment. Unfortunately, although there were no intrahepatic tumors, the peritoneum contained tumors histologically consistent with well-differentiated adenocarcinoma (supplementary Figure 2*B*; see supplementary material online at www.gastrojournal.org).

Discussion

In these studies, we present a simple and reproducible method for generating functional human hepatocytes from pluripotent ES cells. Although successful methods for hepatic differentiation of hES cells have been described, none have generated cells with function adequate for clinical use.[8,12,28–30] We have not determined whether co-culture with liver nonparenchymal cells might increase the efficiency of hepatic differentiation, as has been described for mouse ES cell differentiation.[31] Hepatic differentiation, greater than the 18%–26% described here, would be desirable, but the simplicity of the protocol may facilitate clinical application and the eventual scaling up that will be required to generate ES-derived hepatocytes in numbers that could be used in patients.

The protocol we describe incorporates a step involving generating EBs. However, based on the work of D'Amour et al,[32] we have performed a number of additional real-time PCR and albumin and urea production experiments that show essentially identical hepatocyte-specific differentiation whether or not the EB formation step is included in the differentiation program (supplementary Methods; see supplementary material online at www.gastrojournal.org). In fact, there is more extensive and earlier loss of Octamer 3/4, Nanog, Sox7, and AFP when the EB step is removed (supplementary Figure 3; see supplementary material online at www.gastrojournal.org). In addition, our ability to induce differentiation using KSR instead of serum allows removal of animal products from the process, an important consideration for clinical application.

A critically important component of the differentiation protocol relates to enrichment for ES-derived hepatocytes. ASGPR expression is unique for liver cells.[33] Although 55% of differentiated cells expressed albumin by immunohistochemistry, significantly fewer cells expressed ASGPR, indicating that enrichment based on ASGPR expression may be more selective than sorting based on previously described gene transfer techniques using reporter genes driven from liver-specific promoters. The present strategy does not depend on transduction efficiency for selecting a relatively homogeneous population of cells. Although we used flow sorting for enrichment, this technique is relatively time consuming and can lead to significant cell injury. In addition, its efficacy can be affected by sorting speed, which can seriously affect the yield and viability of the differentiated ES cells recovered and allow recovery of unwanted cells. The ASGPR-based sorting approach outlined, however, also would be amenable to enrichment by magnetic sorting[34] or panning on antibody-coated plates. Such strategies might be more efficient than flow sorting because the

steps can be performed repeatedly for enhanced selection, and would result in significantly less damage to the recovered cells. In addition, they might be faster and more appropriate for large-scale, high-throughput enrichment than flow cytometry.

As shown in our studies, tumor risk remains an issue that must continue to be addressed. It appears that neither the number of cells that can be transplanted into immune-deficient mice nor the length of time transplanted rodents can be followed up will be adequate to unequivocally determine whether cell preparations are safe for clinical use. Large-scale studies, performed in nonhuman primates using frozen stocks of differentiated cells, when possible, may be needed for such an analysis. Although ES cell–derived hepatocytes may not be immediately useful for transplantation therapies, they are likely to find early application for the study of human drug metabolism and drug discovery. Mature human livers express important drug-metabolizing enzymes,[35] some of which are inducible after exposure to phenobarbital or β-naphthoflavone.[23,24] Our studies indicate that ES-derived hepatocytes express human CYP genes at levels near those of adult human hepatocytes, and that prior exposure to β-naphthoflavone results in robust induction in the metabolism of ethoxyresorufin, a known substrate for CYP 1A1/2, by both ES-derived hepatocytes and normal human liver cells. Our studies also show that ES-derived hepatocytes and normal human hepatocytes convert testosterone to 6-β-hydroxytestosterone, a specific measure of CYP 3A4–mediated metabolism, to a similar degree in culture. Although prior exposure to phenobarbital did not increase the metabolism of testosterone in ES-derived cells, as occurs with normal human hepatocytes (data not shown), the CYP metabolic activity shown by ES-derived cells is substantial. It is possible that further maturation of CYP functional activity may require differentiation on a more physiologic extracellular matrix or interaction with other cells.

Although we have examined the use of hES cells in this series of experiments, successful generation of hepatocytes from precursors derived from individual patients[36] could lead to the development of individualized patient-specific drug regimens and eventually might be used to circumvent the need for life-long immune suppression after hepatocyte transplantation. In summary, these studies provide a foundation for efficient development of functional human hepatocytes from hES cells. Further studies will be needed to determine whether the differentiation protocol and enrichment strategy outlined can be scaled for use in patients and can be modified to eliminate the risk of contaminating cells and the risk of tumor formation after transplantation. Application of ES-derived hepatocytes for the study of human drug metabolism and drug discovery, however, may soon be possible.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.10.047.

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Conflict of interest

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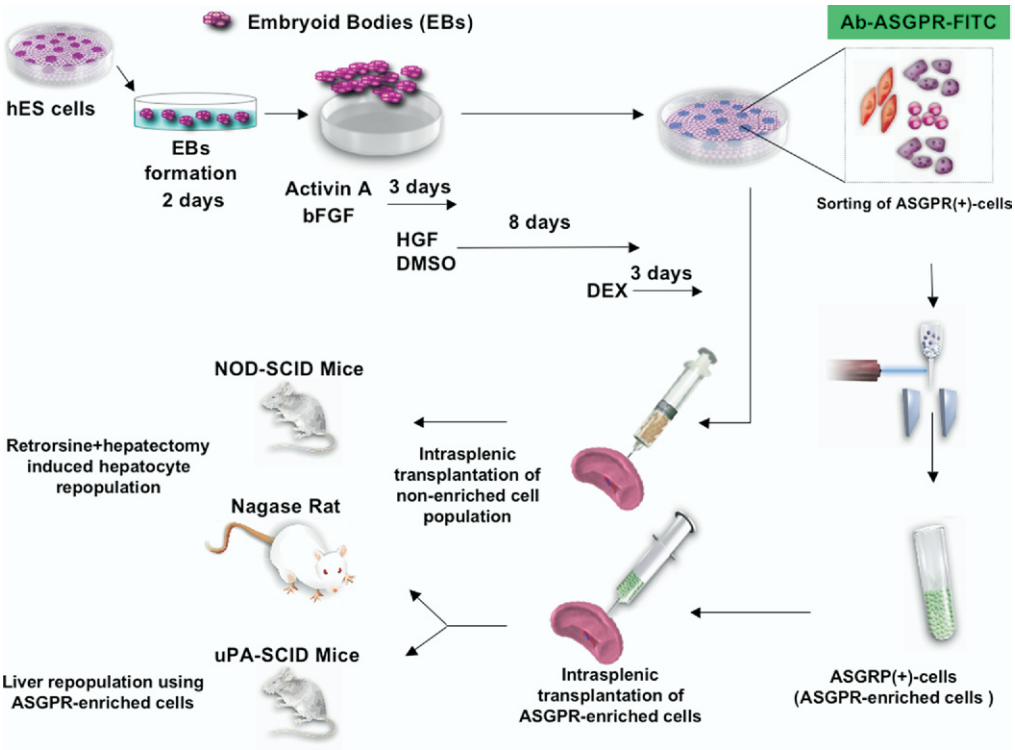
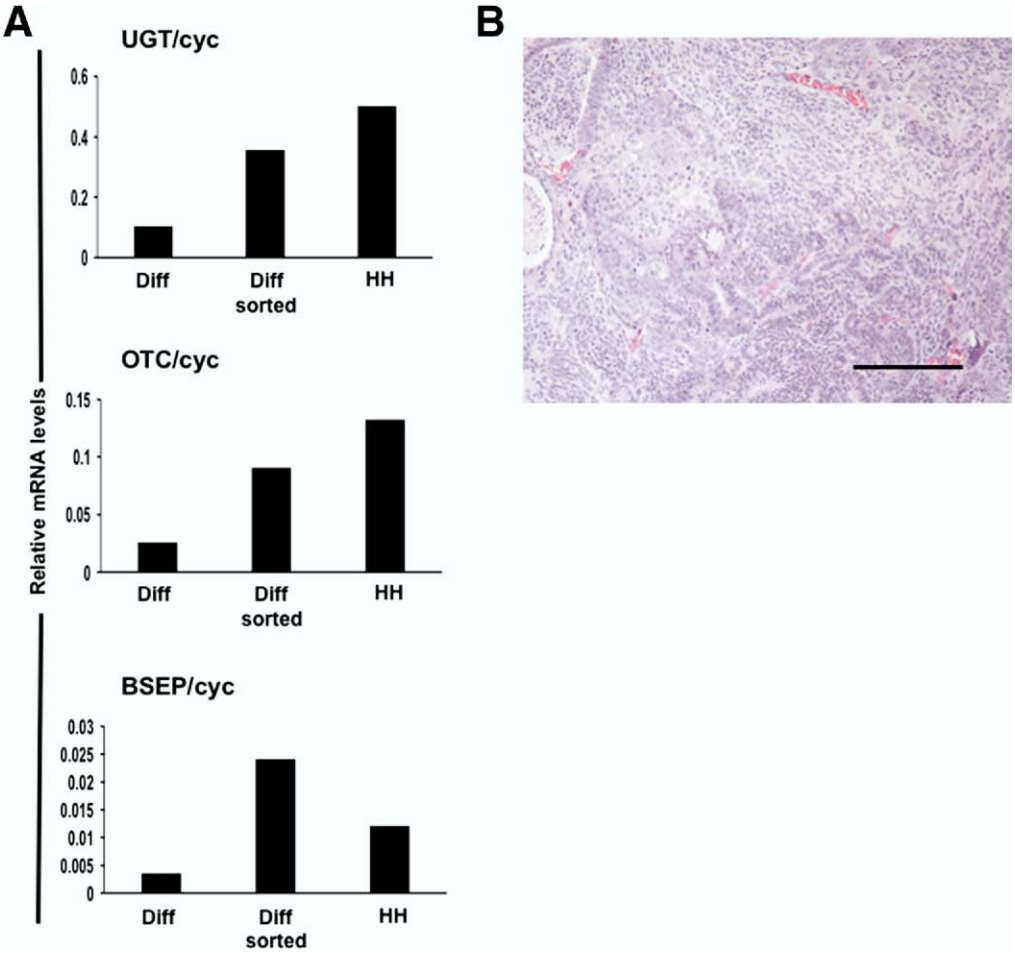
Supplementary Table 1. Specific Primers Used for RT-PCR

Genes	Primer sequence	Product size
Oct3/4	5′-TGGGCTCGAGAAGGATGTG-3′ 5′-GCATAGTCGCTGCTTGATCG-3′	150
Nanog	5′-TGAACCTCAGCTACAAACAG-3′ 5′-AACTGCATGCAGGACTGCA-3′	350
Sox17	5′-GGCGCAGCAGAATCCAGA-3′ 5′-CCACGACTTGCCCAGCAT-3′	300
Albumin	5′-ACTTTTATGCCCCGGAACTC-3′ 5′-AGCAGCAGCACGACAGAGTA-3′	150
AFP	5′-ACTGAATCCAGAACACTGCA-3′ 5′-TGCAGTCAATGCATCTTTCA-3′	170
CF VII	5′-GGGAGACTCCCCAAATATCA-3′ 5′-ACGCAGCCTTGGCTTTCTCT-3′	580
ASGR1	5′-GCTCCACGTGAAGCAGTT-3′ 5′-AACTGCAGAAAGCGCCAC-3′	670
PDX1	5′-TGGGCTCGAGAAGGATGTG-3′ 5′-GCATAGTCGCTGCTTGATCG-3′	320
Nestin	5′-TGGGCTCGAGAAGGATGTG-3′ 5′-GCATAGTCGCTGCTTGATCG-3′	380
Brachury	5′-TGGGCTCGAGAAGGATGTG-3′ 5′-GCATAGTCGCTGCTTGATCG-3′	240
Pax6	5′-TGGGCTCGAGAAGGATGTG-3′ 5′-GCATAGTCGCTGCTTGATCG-3′	330
Nkx 2-5	5′-TGGGCTCGAGAAGGATGTG-3′ 5′-GCATAGTCGCTGCTTGATCG-3′	160
GAPDH	5′-GTTGCCATCAATGACCCCTTCATTG-3′ 5′-GCTTCACCACCTTCTTGATGTCATC-3′	680

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



Supplementary Figure 1. Experimental design. FITC, fluorescein isothiocyanate.

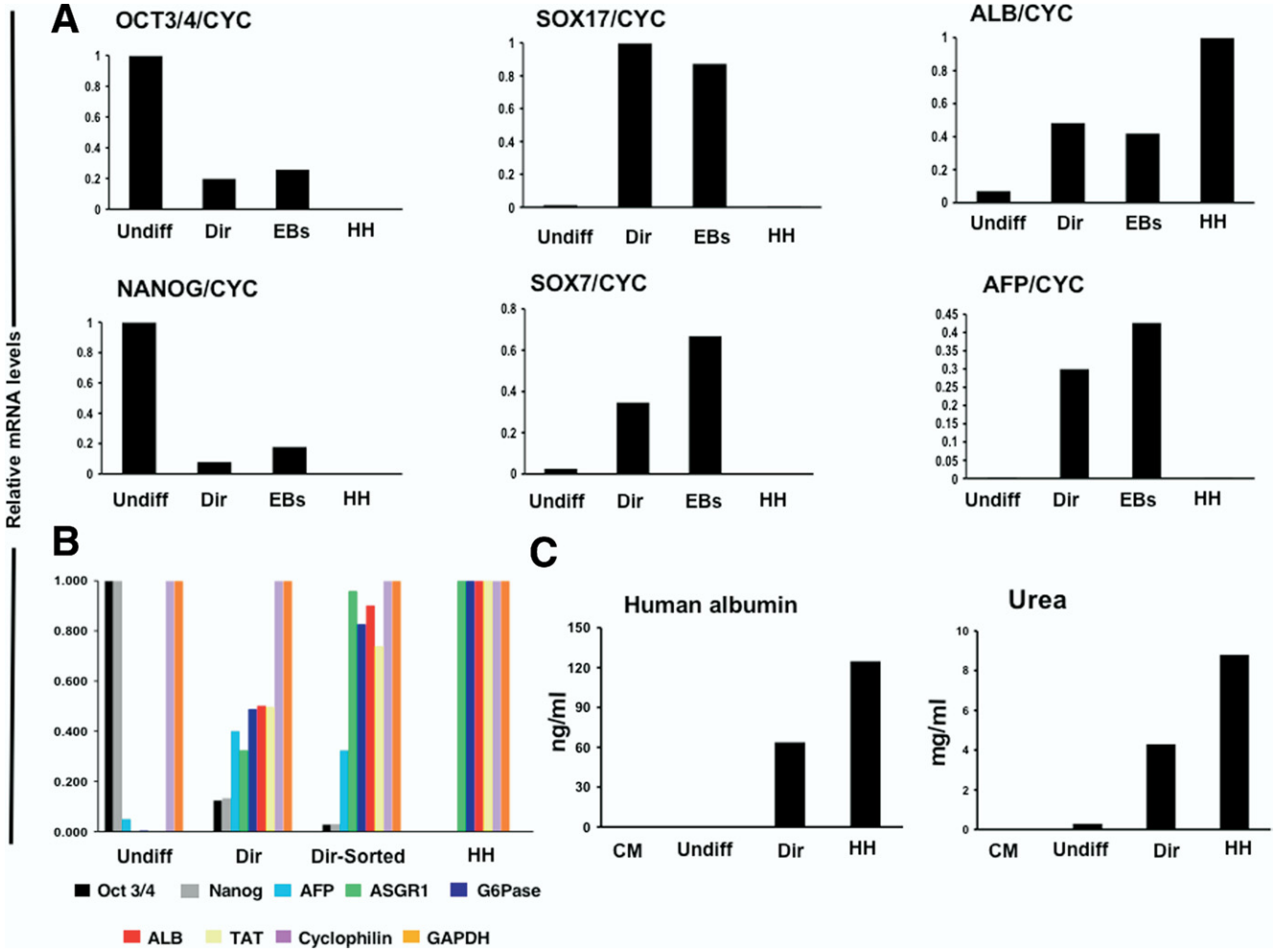


Supplementary Figure 2. (*A*) Real-time analysis showing expression of the genes encoding bilirubin-UDP glucuronosyl transferase, ornithine transcarbamylase (OTC), and bile salt export pump at levels similar to that attained from fresh primary human hepatocytes. Diff, hES cells differentiated toward a hepatocyte phenotype, but not enriched by sorting for ASGPR; Diff sorted, hES cells differentiated toward a hepatocyte phenotype and enriched by sorting for ASGPR; HH, control fresh primary human hepatocytes. (*B*) Photomicrograph of H&E-stained tissue section from a peritoneal implant obtained from a Nagase rat transplanted with hES-derived hepatocytes enriched based on surface ASGPR expression showing characteristics of a well-differentiated adenocarcinoma.

Table 2. Transplantation of Control Primary Hepatocytes in uPA-SCID Mice

		Maximum human albumin level			
Hepatocyte donor group	Transplanted animals (*n*)	None	1000–50,000 ng/mL	50,000 ng/mL to 1 mg/mL	≥1 mg/mL
Age <20 y, fresh	15	3 (20%)	7 (47%)	3 (20%)	2 (13%)
Age <20 y, cryopreserved	18	8 (44%)	1 (6%)	5 (28%)	4 (22%)
Age ≥20 y, cryopreserved	37	15 (41%)	10 (27%)	12 (32%)	0 (0%)

NOTE. Albumin levels from immune-deficient Alb-uPA SCID mice transplanted with 1,000,000 fresh or cryopreserved primary human hepatocytes recovered from liver.



Supplementary Figure 3. Differentiation of human ES cells using a program that does not contain an EB formation step. (*A*) Real-time PCR analysis of Oct 3/4, Nanog, Sox17, Sox7, albumin, and AFP expression indicates that there is more extensive and earlier loss of Oct 3/4, Nanog, Sox7, and AFP when the EB step is removed. Analysis of cyclophilin expression was used as the positive control. Undiff, undifferentiated human ES cells; Dir, hepatocyte-directed differentiation without the EB formation step; EBs, hepatocyte-directed differentiation containing the EB formation step; HH, expression levels for control primary human hepatocytes. (*B*) Real-time PCR analysis for expression of lineage-specific hepatic markers (albumin [ALB], ASGPR 1, glucose-6-phosphatase [G6Pase], and tyrosine aminotransferase [TAT]), an endoderm marker (AFP), and markers for undifferentiated cells (Pou5f1 and Nanog) using a hepatocyte-directed differentiation program that does not involve formation of EBs. *Lanes*: (1) undifferentiated hES cells (Undiff); (2) after treatment with fibroblast growth factor-2 and activin-A, HGF and dimethyl sulfoxide, and then dexamethasone (Dir); and (3) after differentiation and the sorting for ASGPR expression (Dir-Sorted). Primary human hepatocytes (HH) served as the positive control. (*C*) Determination of albumin and urea secretion in vitro by primary human hepatocytes (HH) and hES cells after differentiation when the EB formation step is not included. Protein secretion was assessed in control medium (CM) only; medium from 5×10^5 undifferentiated hES cells (Undiff), hES cells after treatment with fibroblast growth factor-2 and activin-A, HGF, and dimethyl sulfoxide, and then dexamethasone (Dir); and medium from cultured fresh primary human hepatocytes (HH). Twenty-four hours after culture, the amount of human albumin in the media was measured by enzyme-linked immunosorbent assay, and urea was assessed using a urease colorimetric method.