



DISCUSSION

The main purpose of this study was to establish and characterize expandable HBCs from hPSCs. First, we identified that human LN111 could support self-renewal and proliferation of hPSC-derived HBCs in the presence of HGF and EGF. Second, we showed that the hPSC-derived HBCs have the potential to segregate into both hepatic and biliary lineages, and to integrate into the mouse liver parenchyma.

We have demonstrated that the hPSC-derived HBCs could be maintained on a human LN111-coated dish in an integrin $\alpha 6$ - and $\beta 1$ -dependent manner (Figure 1). It is known that undifferentiated hPSCs could be maintained on a human LN511-coated dish but not on a human LN111-coated dish (Rodin et al., 2010). This might suggest that human LN111 has the potential not only to selectively maintain HBCs, but also to eliminate residual undifferentiated cells. Our hepatoblast-like cells could efficiently proliferate for more than 3 months on a human LN111-coated dish (Figure 2). In the human liver development (during 5–10 weeks gestation), laminin is observed in both the perisinusoidal space and portal tracts (Couvelard et al., 1998). The expression of laminin is localized around the periportal biliary trees during the later stage of liver development (Couvelard et al., 1998). Hepatic stem cells reside around the hepatic portal area (Clément et al., 1988). It is also known that laminin is accumulated around oval cells although laminin is not expressed around quiescent mature hepatocytes (Paku et al., 2001). These facts suggest that laminin plays an important role in the maintenance and proliferation of hepatoblasts.

The hPSC-derived HBC P10 and clone were positive for hepatoblast markers (AFP, ALB, CYP3A7, and I-CAM), but negative for hepatic stem cell markers (N-CAM and Claudin 3) (Figure 3) (Schmelzer et al., 2007). Although the hPSC-derived HBCs were able to expand on human LN111-coated dish, Schmelzer et al. showed that human hepatoblasts do not proliferate under a monolayer culture condition, but human hepatic stem cells could self-replicate for more than 6 months (Schmelzer et al., 2007). Although further investigations of the hepatoblast characteristics in the hPSC-derived HBCs will be needed in the future, the results in the present study suggest that the characteristics of hPSC-derived HBCs expanded on human LN111-coated

dishes were similar to those in human hepatoblasts isolated from the human liver (Schmelzer et al., 2007; Zhang et al., 2008).

The hPSC-derived HBCs had the ability to integrate into the mouse liver parenchyma (Figure 5), in the manner of human hepatic stem cells or hepatoblasts (Schmelzer et al., 2007). The human ALB serum levels (approximately 20–70 ng/ml) in mice transplanted with the hESC-derived HBC P0 or HBC P10 were comparable to those in the previous paper in which the hESC-derived definitive endoderm cells, hepatoblasts, and hepatocyte-like cells were transplanted into mice (Liu et al., 2011), but were lower than those of human liver chimeric mice (Tateno et al., 2004). Human ALB serum levels would increase if more suitable host mice, such as urokinase plasminogen activator-SCID mice were used (Tateno et al., 2004).

In this study, we have developed a technology for the maintenance and proliferation of hPSC-derived HBCs by using human LN111. To transplant these cells for purposes of regenerative medicine, a xeno-free culture condition for hPSC-derived HBCs must be developed in the future. It is hoped that the hPSC-derived HBCs and their derivatives will be helpful in various medical applications, such as drug screening and regenerative medicine.

EXPERIMENTAL PROCEDURES

hESC and hiPSC Culture

The hESC lines (H1 [WA01] and H9 [WA09] [WiCell Research Institute]) and the hiPSC line, Dotcom (JCRB number: JCRB1327) (Makino et al., 2009; Nagata et al., 2009), were maintained on a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts (Millipore) with ReproStem medium (ReproCELL) supplemented with 5 and 10 ng/ml fibroblast growth factor 2 (FGF2) (Katayama Kagaku Kogyo), respectively. H1 and H9 were used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and, furthermore, the study was approved by an independent ethics committee.

In Vitro Hepatoblast Differentiation

The differentiation protocol for the induction of definitive endoderm cells and hepatoblasts was based on our previous report with some modifications (Inamura et al., 2011; Takayama et al., 2012a, 2012b, 2013). In mesendoderm differentiation, hESCs/iPSCs were

(C–F) The gene expression levels of hepatic stem cell markers (C), pan-hepatoblast markers (D), hepatoblast markers (E), and mature hepatocyte (*CYP3A4*, *7A1*, *2C9*, and *T0*) or cholangiocyte markers (*integrin $\beta 4$* and *SOX9*) (F) were measured in the definitive endoderm cells, HBC P0, HBC P1, or HBC P10 by real-time RT-PCR.

(G) The gene expression levels of *CD13*, *CK7*, *DLK1*, and *PROX1* were measured in the hESC-derived definitive endoderm cells, HBC P0, HBC P1, or HBC P10 by real-time RT-PCR. Data represent the mean \pm SD from three independent experiments. Statistical significance was evaluated by ANOVA followed by Bonferroni post hoc tests to compare four groups (DE cells, HBC P0, HBC P1, and HBC P10). Groups that do not share the same letter are significantly different from each other ($p < 0.05$). DE, definitive endoderm cells.

See also Figures S2 and S3 and Tables S2–S4.

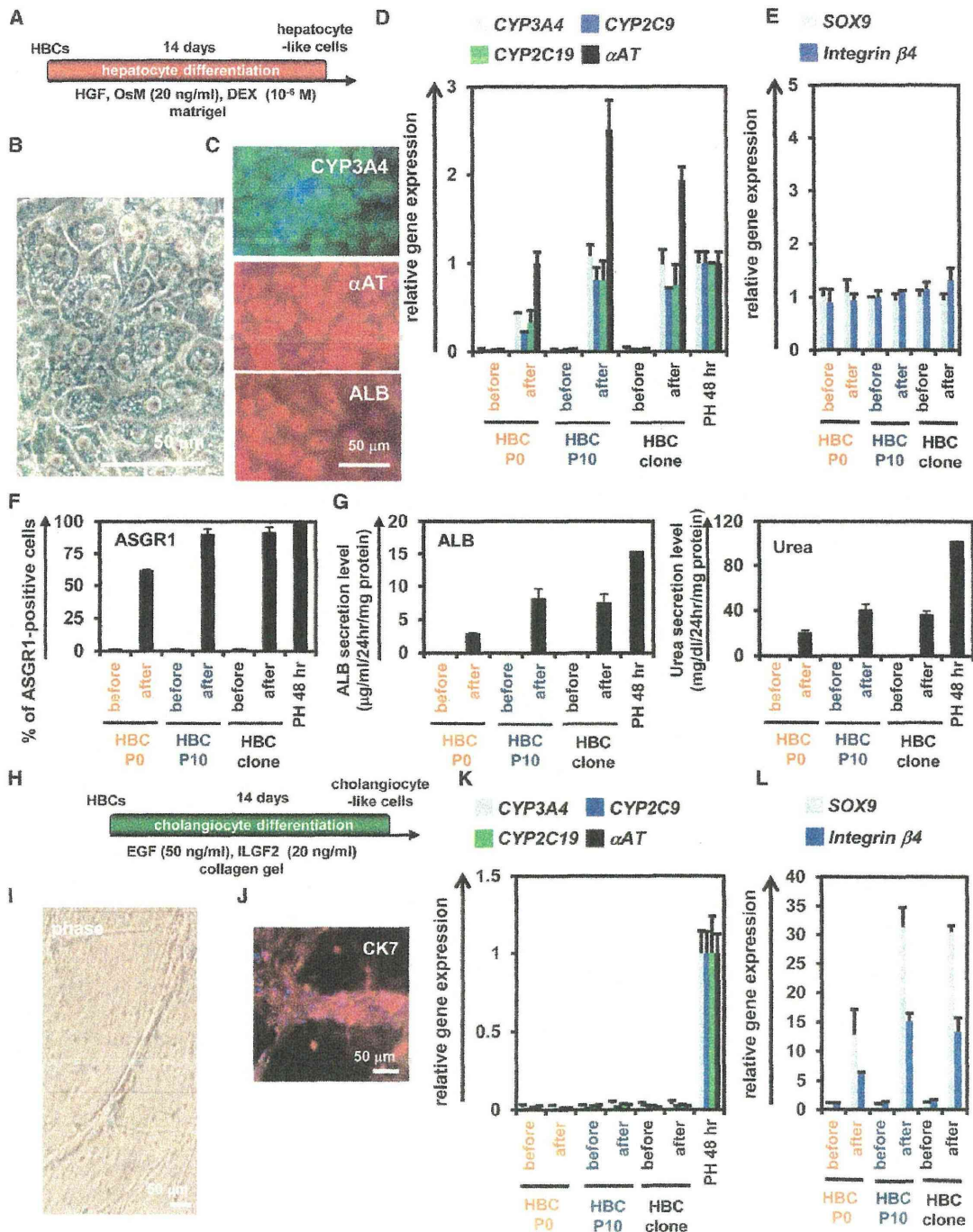


Figure 4. The hESC-Derived HBCs Could Differentiate into Both Hepatic and Biliary Lineages

(A) The procedure for differentiation of the hESC (H9)-derived HBC P0, HBC P10, or HBC clone into the hepatocyte-like cells is presented schematically. Details are described in [Experimental Procedures](#).

(B) Phase-contrast micrographs of the HBC P10-derived hepatocyte-like cells are shown.

(C) The HBC P10-derived hepatocyte-like cells were subjected to immunostaining with anti-CYP3A4 (green), anti- α AT (red), and anti-ALB (red) antibodies.

(D and E) The gene expression levels of hepatocyte (D) or cholangiocyte (E) markers in HBC P0-, HBC P10-, or HBC clone-derived hepatocyte-like cells were measured by real-time RT-PCR after 14 days of hepatocyte differentiation. In (D), the gene expression levels in

(*legend continued on next page*)



cultured for 2 days on Matrigel (BD Biosciences) in differentiation hESF-DIF medium that contains 100 ng/ml Activin A (R&D Systems) (hESF-DIF medium was purchased from Cell Science & Technology Institute; differentiation hESF-DIF medium was supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ M 2-mercaptoethanol, 10 μ M ethanolamine, 10 μ M sodium selenite, and 0.5 mg/ml bovine fatty acid free serum albumin [all from Sigma]). To generate definitive endoderm cells, the mesendoderm cells (day 2) were transduced with 3,000 vector particle (VP)/cell of FOXA2-expressing adenovirus vectors (Ad-FOXA2) for 1.5 hr and cultured until day 6 on Matrigel in differentiation hESF-DIF medium supplemented with 100 ng/ml Activin A. For induction of hepatoblasts, the definitive endoderm cells were cultured for 3 days on a Matrigel in differentiation hESF-DIF medium supplemented with 30 ng/ml bone morphogenetic protein 4 (BMP4) (R&D Systems) and 20 ng/ml FGF4 (R&D Systems).

Establishment and Maintenance of the hPSC-Derived HBCs

The hPSC-derived HBCs were first purified from the hPSC-derived cells (day 9) by selecting attached cells on a human recombinant LN111 (BioLamina)-coated dish at 15 min after plating. The hPSC-derived HBCs were cultured on a human LN111-coated dish (2.0×10^4 cells/cm²) in maintenance DMEM/F12 medium (DMEM/F12 medium [Invitrogen] was supplemented with 10% FBS, 1 \times insulin/transferrin/selenium, 10 mM nicotinamide, 10^{-7} M dexamethasone (DEX) (Sigma), 20 mM HEPES, 25 mM NaHCO₃, 2 mM L-glutamine, penicillin/streptomycin, 40 ng/ml hepatocyte growth factor [HGF] [R&D Systems] and 20 ng/ml epidermal growth factors [EGF] [R&D Systems]). The medium was refreshed every day. The hPSC-derived HBCs were dissociated with Accutase (Millipore) into single cells and subcultured every 6 or 7 days.

Establishment and Maintenance of a Single hPSC-Derived HBC

For single-cell culture, the single HBC was plated to separate well of human LN111-coated 96-well plate in maintenance DMEM/F12

medium supplemented with 25 μ M Y-27632 (ROCK inhibitor) (Millipore), and then colonies derived from a single cell were manually picked up and cultured as well as HBCs (these cells were designated the HBC clone).

In Vitro Hepatocyte and Cholangiocyte Differentiation

To induce hepatocyte differentiation, the hPSC-derived HBC P0, HBC P10, and HBC clone were cultured for 14 days on a Matrigel-coated dish (7.5×10^4 cells/cm²) in HCM (Lonza) supplemented with 20 ng/ml HGF, 20 ng/ml Oncostatin M (OsM) (R&D Systems), and 10^{-6} M DEX. To induce cholangiocyte differentiation, the hPSC-derived HBC P0, HBC P10, and HBC clone were cultured in collagen gel for 14 days. To establish collagen gel plates, 500 μ l collagen gel solution (consisting of 400 μ l type I-A Collagen (Nitta gelatin), 50 μ l 10 \times DMEM, and 50 μ l of 200 mM HEPES buffer containing 2.2% NaHCO₃ and 0.05 M NaOH) was added to each well, and then the plates were incubated at 37°C for 30 min. The hPSC-derived HBC P0, HBC P10, and HBC clone (5×10^4 cells) were resuspended in 500 μ l differentiation DMEM/F12 medium (differentiation DMEM/F12 medium was supplemented with 20 mM HEPES, 2 mM L-glutamine, 100 ng/ml EGF, and 40 ng/ml insulin-like growth factor 2 [ILGF2]), and then mixed with 500 μ l of the collagen gel solution and plated onto the basal layer of collagen. After 30 min, 2 ml of differentiation DMEM/F12 medium was added to the well.

Ad Vectors

Ad vectors were constructed by an improved in vitro ligation method. The human EF-1 α promoter-driven FOXA2-expressing Ad vectors (Ad-FOXA2) were constructed previously (Takayama et al., 2012b). All of Ad vectors contain a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of hESCs, hiPSCs, mesendoderm cells, and definitive endoderm cells, in which transfection efficiency was almost 100%, and purified as described previously (Inamura

PH 48 hr were taken as 1.0. In (E), the gene expression levels in HBC P10 (before differentiation) were taken as 1.0. Data represent the mean \pm SD from three independent experiments. Student's t test indicated that gene expression levels of the hepatocyte markers in "after" were significantly higher than those in "before" ($p < 0.01$).

(F) The efficiency of hepatocyte differentiation was measured by estimating the percentage of ASGR1-positive cells using FACS analysis.

(G) The amounts of ALB (left) and urea (right) secretion were examined. Data represent the mean \pm SD from three independent experiments. Student's t test indicated that the percentage of ASGR1-positive cells, the ALB secretion level, and urea secretion level in "after" were significantly higher than those in "before" ($p < 0.01$).

(H) The procedure for the differentiation of the hESC-derived HBC P0, HBC P10, or HBC clone into cholangiocyte-like cells is presented schematically. Details are described in [Experimental Procedures](#).

(I) Phase-contrast micrographs of the HBC P10-derived cholangiocyte-like cells are shown.

(J) The HBC P10-derived cholangiocyte-like cells were subjected to immunostaining with anti-CK7 (red) antibodies.

(K and L) The gene expression levels of hepatocyte (K) or cholangiocyte (L) markers in the HBC P0-, HBC P10-, or HBC clone-derived cholangiocyte-like cells were measured by real-time RT-PCR after 14 days of cholangiocyte differentiation. In (K), the gene expression levels in PH 48 hr were taken as 1.0. In (L), the gene expression levels in HBC P10 (before cholangiocyte differentiation) were taken as 1.0. Data represent the mean \pm SD from three independent experiments. Student's t test indicated that the gene expression levels of cholangiocyte markers in "after" were significantly higher than those in "before" ($p < 0.01$). "Before" indicates the HBCs before hepatocyte or cholangiocyte differentiation; "After" indicates the HBCs after hepatocyte or cholangiocyte differentiation.

See also [Figures S4](#) and [S5](#) and [Tables S1–S4](#).

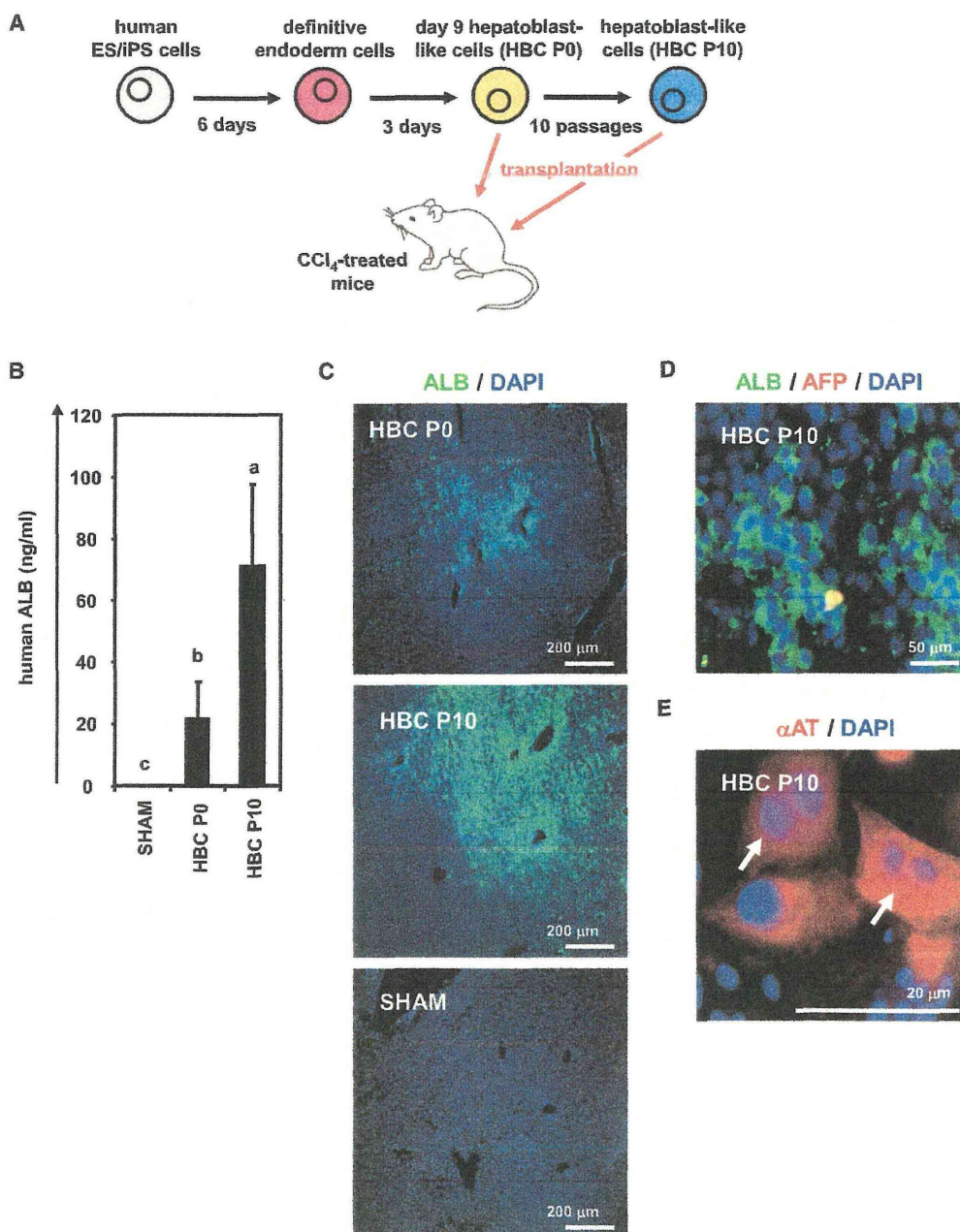


Figure 5. The hESC-Derived HBCs Were Integrated into the Mouse Liver Parenchyma

(A) The procedure for transplantation of the hESC (H9)-derived HBC P0 and HBC P10 into CCl₄ (4 ml/kg)-treated Rag2/IL2 receptor gamma double-knockout mice is presented schematically.

(B) The human ALB level in recipient mouse serum was measured at 2 weeks after transplantation. Data represent the mean ± SD from six to eight mice in each group. Statistical significance was evaluated by ANOVA followed by Bonferroni post hoc tests to compare all groups. Groups that do not share the same letter are significantly different from each other ($p < 0.05$).

(C) Expressions of the ALB (green) in the liver of transplanted mice were examined by immunohistochemistry at 2 weeks after transplantation.

(D and E) The expressions of AFP (red), ALB (green) (D), and αAT (red) (E) were examined by immunohistochemistry at 2 weeks after hESC-derived HBC P10 transplantation. White arrows show transplanted cells, which have double nuclei.

See also Tables S2 and S3.



et al., 2011; Takayama et al., 2011; Tashiro et al., 2010). The VP titer was determined by using a spectrophotometric method.

Flow Cytometry

Single-cell suspensions of the hPSC-derived cells were fixed with 4% paraformaldehyde (PFA) at 4°C for 10 min and then incubated with the primary antibody (described in Table S2), followed by the secondary antibody (described in Table S3). Control cells were incubated with anti-mouse, goat, or rabbit immunoglobulin (Ig) G antibodies (Santa Cruz Biotechnology) and then incubated with the secondary antibody. Flow cytometry analysis was performed using a fluorescence-activated cell sorting (FACS) LSR Fortessa flow cytometer (BD Biosciences). Cell sorting was performed using a FACS Aria (BD Biosciences).

RNA Isolation and RT-PCR

Total RNA was isolated from hPSCs and their derivatives using ISOGENE (Nippon Gene). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed with SYBR green PCR Master Mix (Applied Biosystems) using an Applied Biosystems StemOnePlus real-time PCR systems. Relative quantification was performed against a standard curve, and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase. The primer sequences used in this study are described in Table S4. In addition, we confirmed that every beta integrin primer used in this manuscript showed a similar amplification efficacy (Table S5). The amplification efficiency was calculated from the slope of the standard curve according to the following formula: $e = 10^{(-1/\text{slope})-1}$. Every beta integrin primer used in this manuscript showed a similar amplification efficacy.

Immunohistochemistry

The cells were fixed with 4% PFA for 15 min and then blocked with PBS containing 2% FBS, 2% bovine serum albumin (BSA), and 0.1% Triton X-100 (Wako Pure Chemicals Industries) for 1 hr. The cells were incubated with primary antibody (described in Table S2) at 4°C for overnight, followed by incubation with a secondary antibody (described in Table S3) at room temperature for 1 hr. Nuclei were counterstained with DAPI (blue).

ELISA

The hPSC-derived HBC P0, HBC P10, and HBC clone were differentiated into the hepatocyte-like cells as described in Figure 4A. The culture supernatants, which were incubated for 24 hr after fresh medium was added, were collected and analyzed for the amount of ALB secretion by ELISA. ELISA kits for ALB were purchased from Bethyl Laboratories. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well. The human ALB amount in mice serum was also examined by ELISA.

Transplantation of the hESC-Derived HBCs

The hESC-derived HBCs were dissociated using accutase and then suspended with maintenance DMEM/F12 medium without serum.

Eight- to 10-week-old Rag2/IL2Rg double-knockout mice were prepared. The hESC-derived HBCs (1×10^6 cells) were transplanted 24 hr after administration of CCl₄ (4 ml/kg) by intrasplenic injection. Recipient mouse liver and blood were harvested at 2 weeks after transplantation. The livers were fixed with 4% PFA and processed for immunohistochemistry. Human hepatocytes producing the ALB, AFP, and α AT protein were identified in mouse liver by an antibody specifically recognizing human but not mouse albumin. In addition, serum was extracted and subjected to ELISA analysis. All animal experiments were conducted in accordance with institutional guidelines.

Urea Secretion

The hPSC-derived HBC P0, HBC P10, and HBC clone were differentiated into hepatocyte-like cells as described in Figure 4A. The culture supernatants, which were incubated for 24 hr after fresh medium was added, were collected and analyzed for the amount of urea secretion. The urea measurement kits were purchased from BioAssay Systems. The amount of urea secretion was calculated according to each standard followed by normalization to the protein content per well. In Figure S5B, both the HBC-derived hepatocyte-like cells and primary human hepatocytes (PHs) (three lots of cryopreserved human hepatocytes were used), that were cultured for 48 hr after the cells were plated (PH 48 hr), were cultured in HCM (containing glutamine) or DMEM (not containing glutamine; Wako) in the presence or absence of 1 mM ammonium chloride (NH₄Cl, Wako) for 24 hr, and then the amount of urea secretion was measured.

Primary Human Hepatocytes

Three lots of cryopreserved human hepatocytes (lot Hu8072 [CellzDirect], HC2-14, and HC10-101 [Xenotech]) were used. The vials of hepatocytes were rapidly thawed in a shaking water bath at 37°C; the contents of the vial were emptied into prewarmed Cryopreserved Hepatocyte Recovery Medium (Gibco) and the suspension was centrifuged at 100 g for 10 min at room temperature. The hepatocytes were seeded at 1.25×10^5 cells/cm² in HCM (Lonza) containing 10% fetal calf serum (FCS) (Gibco) onto type I collagen-coated 12-well plates. The medium was replaced with hepatocyte culture medium containing 10% FCS 6 hr after seeding. The hepatocytes, which were cultured 48 hr after plating the cells, were used in the experiments.

Adhesion-Blocking Assay Using Integrin Antibody

Twelve-well plates were coated with human recombinant LN111, 211, 411, or 511 (all from BioLamina) and blocked by 1% heat-denatured BSA containing PBS. The hESC-derived single cells were incubated with function-blocking antibodies to integrin α 6 and β 1 (at the concentrations as recommended by the manufacturer) for 30 min, plated on a human LN111-coated 12-well dish, and allowed to adhere for 1 hr at 37°C. After unattached cells were removed, the remaining adherent cells were fixed for 20 min with 5% glutaraldehyde. The hESC-derived cells that had adhered to the wells were stained with 200 μ l of 0.3% crystal violet (Wako) solution at room temperature for 15 min. Excess crystal violet was then removed, and the wells were washed three times. Fixed crystal violet was solubilized in 200 μ l of 100% ethanol at room



temperature for 15 min. Cell viability was estimated by measuring the absorbance at 595 nm of each well using a microtiter plate reader (Sunrise, Tecan).

CYP Activity

To measure the CYP1A2, 2C9, and 3A4 activity of the cells, we performed lytic assays by using P450-Glo™ CYP1A2, 2C9, and 3A4 Assay Kits (Promega), respectively. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold) according to the manufacturer's instructions. The CYP activity was normalized with the protein content per well.

Karyotyping

This experiment was carried out at Chromosome Science Labo.

Cell Viability Tests

Cell viability was assessed by using a WST-8 assay kit (Dojindo), and the results are presented in Figure S5C. After treatment with test compounds, such as acetaminophen (Wako) and troglitazone (Wako) for 24 hr, the cell viability was measured. The control cells were incubated in the absence of test compounds and were considered to have 100% viability value. Controls were treated with DMSO (final concentration 0.1%).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2013.08.006>.

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REFERENCES

Clément, B., Rescan, P.Y., Baffet, G., Loréal, O., Lehry, D., Campion, J.P., and Guillouzo, A. (1988). Hepatocytes may produce laminin in fibrotic liver and in primary culture. *Hepatology* 8, 794–803.

Couvelard, A., Bringuier, A.F., Dauge, M.C., Nejjar, M., Darai, E., Benifla, J.L., Feldmann, G., Henin, D., and Scoazec, J.Y. (1998). Expression of integrins during liver organogenesis in humans. *Hepatology* 27, 839–847.

Hay, D.C., Zhao, D., Fletcher, J., Hewitt, Z.A., McLean, D., Urruti-coechea-Uriguen, A., Black, J.R., Elcombe, C., Ross, J.A., Wolf, R., and Cui, W. (2008). Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem Cells* 26, 894–902.

Inamura, M., Kawabata, K., Takayama, K., Tashiro, K., Sakurai, F., Katayama, K., Toyoda, M., Akutsu, H., Miyagawa, Y., Okita, H., et al. (2011). Efficient generation of hepatoblasts from human ES cells and iPSC cells by transient overexpression of homeobox gene HEX. *Mol. Ther.* 19, 400–407.

Kamiya, A., Kakinuma, S., Yamazaki, Y., and Nakauchi, H. (2009). Enrichment and clonal culture of progenitor cells during mouse postnatal liver development in mice. *Gastroenterology* 137, 1114–1126.

Liu, H., Kim, Y., Sharkis, S., Marchionni, L., and Jang, Y.Y. (2011). In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. *Sci. Transl. Med.* 3, 82ra39.

Makino, H., Toyoda, M., Matsumoto, K., Saito, H., Nishino, K., Fukawatase, Y., Machida, M., Akutsu, H., Uyama, T., Miyagawa, Y., et al. (2009). Mesenchymal to embryonic incomplete transition of human cells by chimeric OCT4/3 (POU5F1) with physiological co-activator EWS. *Exp. Cell Res.* 315, 2727–2740.

Nagata, S., Toyoda, M., Yamaguchi, S., Hirano, K., Makino, H., Nishino, K., Miyagawa, Y., Okita, H., Kiyokawa, N., Nakagawa, M., et al. (2009). Efficient reprogramming of human and mouse primary extra-embryonic cells to pluripotent stem cells. *Genes Cells* 14, 1395–1404.

Paku, S., Schnur, J., Nagy, P., and Thorgeirsson, S.S. (2001). Origin and structural evolution of the early proliferating oval cells in rat liver. *Am. J. Pathol.* 158, 1313–1323.

Rodin, S., Domogatskaya, A., Ström, S., Hansson, E.M., Chien, K.R., Inzunza, J., Hovatta, O., and Tryggvason, K. (2010). Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat. Biotechnol.* 28, 611–615.

Schmelzer, E., Zhang, L., Bruce, A., Wauthier, E., Ludlow, J., Yao, H.L., Moss, N., Melhem, A., McClelland, R., Turner, W., et al. (2007). Human hepatic stem cells from fetal and postnatal donors. *J. Exp. Med.* 204, 1973–1987.

Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H. (2008). Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. *Development* 135, 2969–2979.

Takayama, K., Inamura, M., Kawabata, K., Tashiro, K., Katayama, K., Sakurai, F., Hayakawa, T., Furue, M.K., and Mizuguchi, H. (2011). Efficient and directive generation of two distinct endoderm lineages from human ESCs and iPSCs by differentiation stage-specific SOX17 transduction. *PLoS ONE* 6, e21780.

Takayama, K., Inamura, M., Kawabata, K., Katayama, K., Higuchi, M., Tashiro, K., Nonaka, A., Sakurai, F., Hayakawa, T., Furue, M.K., and Mizuguchi, H. (2012a). Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4 α transduction. *Mol. Ther.* 20, 127–137.

Takayama, K., Inamura, M., Kawabata, K., Sugawara, M., Kikuchi, K., Higuchi, M., Nagamoto, Y., Watanabe, H., Tashiro, K., Sakurai,



F, et al. (2012b). Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1 α transduction. *J. Hepatol.* *57*, 628–636.

Takayama, K., Kawabata, K., Nagamoto, Y., Kishimoto, K., Tashiro, K., Sakurai, F., Tachibana, M., Kanda, K., Hayakawa, T., Furue, M.K., and Mizuguchi, H. (2013). 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. *Biomaterials* *34*, 1781–1789.

Tanimizu, N., Saito, H., Mostov, K., and Miyajima, A. (2004). Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *J. Cell Sci.* *117*, 6425–6434.

Tashiro, K., Kawabata, K., Inamura, M., Takayama, K., Furukawa, N., Sakurai, F., Katayama, K., Hayakawa, T., Furue, M.K., and Mizuguchi, H. (2010). Adenovirus vector-mediated efficient transduc-

tion into human embryonic and induced pluripotent stem cells. *Cell Reprogram.* *12*, 501–507.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., et al. (2004). Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* *165*, 901–912.

Zhang, L., Theise, N., Chua, M., and Reid, L.M. (2008). The stem cell niche of human livers: symmetry between development and regeneration. *Hepatology* *48*, 1598–1607.

Zhao, D., Chen, S., Cai, J., Guo, Y., Song, Z., Che, J., Liu, C., Wu, C., Ding, M., and Deng, H. (2009). Derivation and characterization of hepatic progenitor cells from human embryonic stem cells. *PLoS ONE* *4*, e6468.

