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Infectious Diseases & Therapy

Malick et al., J Infect Dis Ther 2013, 1:1
http://dx.doi.org/10.4172/2165-7866.1000e101

Editorial Open Access

Start or End? One of the Biggest Mysteries is Finally Solved?

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Hepatitis B virus (HBV) is a small DNA virus, which was found a half century ago. No useful and convenient *in vitro* infection system for HBV has hampered detailed analysis of HBV life cycle, which leads to development of HBV related diseases. Here, Yan et al. [1] reported this November, 2012 that a sodium-taurocholate cotransporting polypeptide also called NTCP was a receptor for HBV and established an HBV *in vitro* infection system using ordinary hepatocellular carcinoma derived cell lines. This finding will shed light on total elucidation of the HBV life cycle and lead to development of new anti-HBV drugs.

It is now a half century since hepatitis B virus (HBV) was identified by Blumberg et al. [2]. This virus is a small DNA virus whose genome is partially double stranded circular DNA and is only 3.2 kb in size. The viral genes are compactly organized and four genes; core, S, polymerase and X are identified [3].

There are about 360 million people infected with the virus worldwide, forming one of the biggest infectious diseases and one of the most serious health programs of human being [4]. HBV infection causes not only severe acute hepatitis including fulminant hepatitis that is highly lethal, but also chronic hepatitis leading liver cirrhosis and hepatocellular carcinoma especially through vertical infection. Treatment against HBV infection consists of interferon therapy and anti-viral drugs. The interferon therapy is, however, very limited and not as effective as that against hepatitis C virus (HCV) infection [5]. And furthermore, anti-HBV drugs, all of which are nucleoside analogues, were developed as anti-human immunodeficiency virus (HIV) drugs not as anti-HBV ones and the HBV polymerase specificity and peculiarity were not considered. Needless to say, both viruses have a reverse transcription process of their replication cycles and thus reverse transcriptase activity work in a similar way, i.e., their activity center is composed of an MYDD motif [3]. These kinds of anti-viral drugs always lead to generation and expansion of mutants that escape their effectiveness.

As mentioned, exploitation and development of new really effective drugs against HBV considering its viral life cycle and gene functions is one of the most important issues for human health. But we have not had very useful HBV infection system *in vitro* and *in vivo* to study detail viral life cycle and its pathogenesis. Hepadnaviruses are highly species specific and tissue specific and HBV never infects present hepatocyte-originated human hepatocellular carcinoma cell lines such as HepG2. Although there are several animal hepadnaviruses; avians (duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHV)), rodents (woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV)) and primates for HBV [6,7], it is not easy to hatch and raise these kinds of animal and to prepare primary hepatocytes from these animals.

As for *in vitro* infection systems, two systems are available at present; one is primary human hepatocytes (PHH) or tree shrew (*Tupaia belangen*) [8] which is also susceptible to HBV as human and chimpanzee and another is HepaRG [9], a cultured human hepatocellular carcinoma (HCC) cell line established from HCV caused HCC. These *in vitro* systems facilitate our knowledge about how HBV infects hepatocytes, but still not good enough because of their commercialism, invariability is dependent on the lot and takes cost for preparation and time to induce infectivity.

A recent splendid report by Yan et al. may improve such inconvenience to study HBV [1]. For long time, we have been searching for HBV receptors and several molecules including those from the DHBV system were nominated as HBV receptors but they had never contributed establishment for HBV infection systems *in vitro*, though many data have suggested that the ligand on the HBV side for the receptor must be in the preS1 region [10-12]. This time, Yan et al. [1] successfully pulled down an HBV receptor, a sodium taurocholate cotransporter polypeptide called also NTCP or NTCP-1, with a photo-activating preS1 peptide using Primary Tupaia Hepatocytes (PTH). Many researchers had been trying to elucidate HBV receptors with the same kind methods including phage libraries screening with the preS1 region; synthetic peptides or the protein purified from *E. coli*, insect cells and yeast expression/purification system etc., and wonder why such a molecule has not been picked up until now.

Yan et al. [1] showed that NTCP expression was low in common HCC cell lines but high in PHH and PTH, and that introduction of NTCP expression raised the competency of HBV in these unsusceptible cell lines and knockdown of NTCP in PTH lowered the susceptibility.

Thus, a tip of the mystery has been finally solved by Yan et al. [1]. Though this must be re-evaluated by the other researchers, the finding will shed light on total elucidation of the HBV life cycle and lead to development of new anti-HBV drugs.

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Received December 20, 2012; **Accepted** December 22, 2012; **Published** December 29, 2012

Citation: Ueda K (2013) Start or End? One of the Biggest Mysteries is Finally Solved? J Infect Dis Ther 1: e101. doi:10.4172/jidt.1000e101

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Citation: Ueda K (2013) Start or End? One of the Biggest Mysteries is Finally Solved? J Infect Dis Ther 1: e101. doi:10.4172/jidt.1000e101

Stem Cell Reports
Resource
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Long-Term Self-Renewal of Human ES/iPS-Derived Hepatoblast-like Cells on Human Laminin 111-Coated Dishes

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http://dx.doi.org/10.1016/j.stemcr.2013.08.006

SUMMARY

The establishment of self-renewing hepatoblast-like cells (HBCs) from human pluripotent stem cells (PSCs) would realize a stable supply of hepatocyte-like cells for medical applications. However, the functional characterization of human PSC-derived HBCs was not enough. To purify and expand human PSC-derived HBCs, human PSC-derived HBCs were cultured on dishes coated with various types of human recombinant laminins (LN). Human PSC-derived HBCs attached to human laminin-111 (LN111)-coated dish via integrin alpha 6 and beta 1 and were purified and expanded by culturing on the LN111-coated dish, but not by culturing on dishes coated with other laminin isoforms. By culturing on the LN111-coated dish, human PSC-derived HBCs were maintained for more than 3 months and had the ability to differentiate into both hepatocyte-like cells and cholangiocyte-like cells. These expandable human PSC-derived HBCs would be manageable tools for drug screening, experimental platforms to elucidate mechanisms of hepatoblasts, and cell sources for hepatic regenerative therapy.

INTRODUCTION

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have the ability to self-replicate and to differentiate into all types of body cells including hepatoblasts and hepatocytes. Although cryopreserved primary human hepatocytes are useful in drug screening and liver cell transplantation, they rapidly lose their functions (such as drug metabolism capacity) and hardly proliferate in in vitro culture systems. On the other hand, human hepatic stem cells from fetal and postnatal human liver are able to self-replicate and able to differentiate into hepatocytes (Schmelzer et al., 2007; Zhang et al., 2008). However, the source of human hepatic stem cells is limited, and these cells are not available commercially. Therefore, the human pluripotent stem cell (hPSC)-derived hepatoblast-like cells (HBCs), which have potential to differentiate into the hepatocyte-like cells, would be an attractive cell source to provide abundant hepatocyte-like cells for drug screening and liver cell transplantation.

Because expandable and multipotent hepatoblasts or hepatic stem cells are of value, suitable culture conditions for the maintenance of hepatoblasts or hepatic stem cells obtained from fetal or adult mouse liver were developed (Kamiya et al., 2009; Tanimizu et al., 2004). Soluble factors, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF), are known to support the proliferation of mouse hepatic stem cells and hepatoblast (Kamiya et al., 2009; Tanimizu et al., 2004). Extracellular matrix (ECM) also affects the maintenance of hepatoblasts or hepatic stem cells. Laminin can maintain the character of mouse hepatoblasts (Dlk1-positive cells) (Tanimizu et al., 2004). However, the methodology for maintaining HBCs differentiated from hPSCs has not been well investigated. Zhao et al. (2009) have reported that hESC-derived hepatoblast-like cells (sorted N-cadherin-positive cells were used) could be maintained on STO feeder cells. Although a culture system using STO feeder cells for the maintenance of hepatoblast-like cells might be useful, there are two problems. The first problem is that N-cadherin is not a specific marker for human hepatoblasts. N-cadherin is also expressed in hESC-derived mesendoderm cells and definitive endoderm (DE) cells (Sumi et al., 2008). The second problem is that residual undifferentiated cells could be maintained on STO feeder cells. Therefore, their culture condition cannot rule out the possibility of the proliferation of residual undifferentiated cells. Because it is known that hPSC-derived cells have the potential to form teratomas in the host, the production of safer hepatocyte-like cells or hepatoblast-like cells has been required. Therefore, we decided to purify hPSC-derived HBCs, which can differentiate into mature hepatocyte-like cells, and then expand these cells.

In this study, we attempt to determine a suitable culture condition for the extensive expansion of HBCs derived

from hPSCs. We found that the HBCs derived from hPSCs can be maintained and proliferated on human laminin-111 (LN111)-coated dishes. To demonstrate that expandable, multipotent, and safe (i.e., devoid of residual undifferentiated cells) hPSC-derived HBCs could be maintained under our culture condition, the hPSC-derived HBCs were used for hepatic and biliary differentiation, colony assay, and transplantation into immunodeficient mice.

RESULTS

Human PSC-Derived Hepatoblast-like Cells Could Adhere onto Human LN111 via Integrin α6 and β1

The HBCs were generated from hPSCs (hESCs and hiPSCs) as described in Figure 1A (details of the characterization of hPSC-derived HBCs are described in Figure 3). Definitive endoderm differentiation of hPSCs was promoted by stage-specific transient transduction of FOXA2 in addition to the treatment with appropriate soluble factors (such as Activin A). Overexpression of FOXA2 is not necessary for establishing the hPSC-derived HBCs, but it is helpful for efficient generation of the hPSC-derived HBCs. On day 9, these hESC-derived populations contained two cell populations with distinct morphology (Figure 1B). One population resembled human hepatic stem cells that were isolated from human fetal liver (shown in red) (Schmelzer et al., 2007), whereas the other population resembled definitive endoderm cells (shown in green) (Hay et al., 2008). The population that resembled human hepatic stem cells was alpha-1-fetoprotein (AFP) positive, whereas the other population was AFP negative (Figure 1C, left). On day 9, the percentage of AFP-positive cells was approximately 80% (Figure 1C, right). To characterize these two cell populations (hESC-derived HBC and non-HBC [NHBC] populations), the colonies were manually isolated by using a pipette, and then the gene expression analysis was performed. The gene expression levels of *AFP*, *CD133*, *EpCAM*, *CK8*, and *CK18* in the hESC-derived HBCs were higher than those in the bulk population containing both hESC-derived HBCs and NHBCs (*CD133*, *EpCAM*, *CK8*, and *CK18* were named as pan-hepatoblast markers and are known to be strongly expressed in both human hepatic stem cells and hepatoblasts [Schmelzer et al., 2007; Zhang et al., 2008]) (Figure 1D). On the other hand, the gene expressions of *AFP*, *CD133*, *EpCAM*, *CK8*, and *CK18* in the hESC-derived NHBCs were hardly detected. The gene expression levels of DE, mesendoderm, and pluripotent markers in the hESC-derived NHBCs were higher than those in the hESC-derived HBCs, indicating that the hESC-derived NHBCs could remain in a more undifferentiated state than the hESC-derived HBCs (Figures S1A–S1C available online). These results suggest that hepatoblast-like cells could be differentiated from hPSCs.

To purify the hESC-derived HBCs, these cells were plated onto dishes coated with various laminins. There are 15 different laminin isoforms in human tissues. Although laminin is known to be useful to sustain mouse hepatoblasts (Tanimizu et al., 2004), it remains unknown which human laminin isoform has the potential to purify and expand the HBCs. To identify a human laminin isoform that would be useful for purifying hESC-HBCs, the hESC-HBCs and -NHBCs were plated onto dishes coated with various types of commercially available human laminins (Figure 1E). The hESC-derived HBCs could more efficiently adhere onto the human LN111-coated dish compared with hESC-derived NHBCs or unseparated populations (containing both HBCs and NBCs). These data suggest that a hESC-derived HBC population can be purified from the unseparated populations by culturing on human LN111-coated dishes. Because integrins are known to be important molecules for cell adhesion to the ECM including laminins, we expected that certain types of integrins would allow selective adhesion of the hESC-derived HBCs to human LN111-coated dish. The gene expression levels of various integrins were examined (Figure 1F). Among the integrin α subunits, the gene expression level of *integrin α6* in the hESC-derived HBCs was significantly higher than that in the hESC-derived NHBCs. In contrast, among the integrin β subunits, the gene expression level of *integrin β1* was higher than those of *integrin β2* and *β3* in all cell populations. The hESC-derived HBCs, but not NHBCs, expressed both integrin α6 and β1 (Figure S1D). Almost all adhesion of the hESC-derived HBCs to a human LN111-coated dish was inhibited by both function-blocking antibodies to integrin α6 and β1 (Figure 1G). These results indicated that the hESC-derived HBCs could attach to a human LN111-coated dish via integrin α6 and β1.

The hPSC-Derived HBCs Could Be Proliferated and Maintained on a Human LN111-Coated Dish

To obtain the purified hESC-derived HBC population, the hESC-derived cells (day 9) were plated onto a human LN111-coated dish, and then unattached cells were removed at 15 min after plating (Figure 2A). Among various laminins, only human LN111 could proliferate (Figure 2B) and purify (Figure 2C) the AFP-positive population in the presence of HGF and EGF. During culture on the human LN111-coated dish, the morphology of the hESC-derived HBCs gradually changed into that of human hepatoblasts (Figure S1E) (Schmelzer et al., 2007). Therefore, the characteristics of hESC-derived HBCs might be changed by culturing on a human LN111-coated dish (details of the characterization of the hESC-derived HBCs are described in Figure 3). After culturing on a human LN111-coated

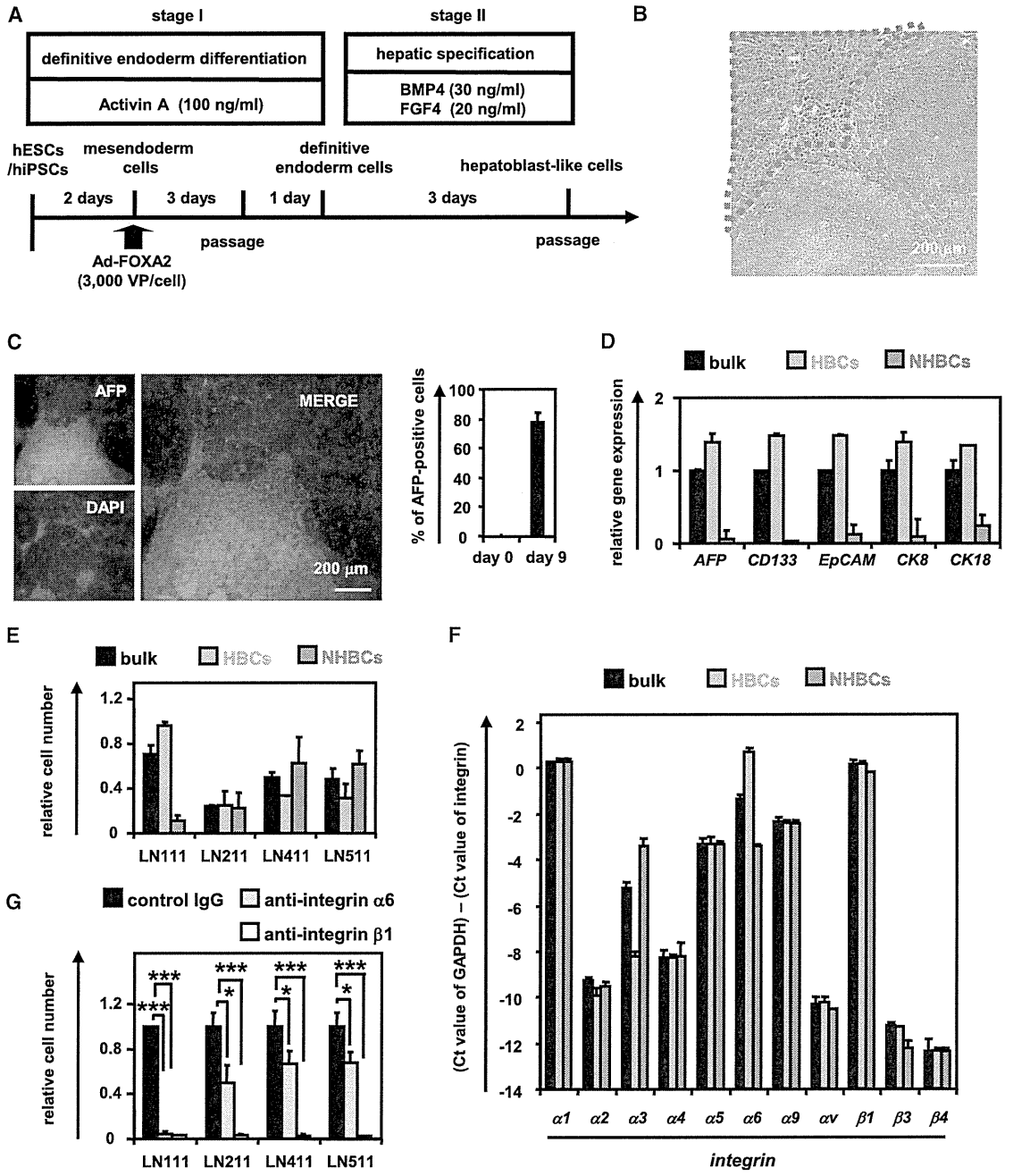

Figure 1. The Human ESC-Derived HBCs Selectively Attached to a Human LN111-Coated Dish via Integrin α6 and β1

(A) The procedure for the differentiation of hESCs (H9) into hepatoblast-like cells (HBCs) is presented schematically. Details are described in the Experimental Procedures.

(B) Phase-contrast micrographs of the hESC-derived HBCs (red) and non-HBCs (NHBCs) (green) are shown.

(C) The hESC-derived cells (day 9) were subjected to immunostaining with anti-AFP (red) antibodies. The percentage of AFP-positive cells was examined on day 0 or 9 by using FACS analysis. Data represent the mean ± SD from ten independent experiments. Cells on "day 0" and "day 9" were compared using Student's t test ($p < 0.01$).

(D) On day 9, the hESC-derived HBCs and NHBCs were manually picked, and the gene expression levels of *AFP* and pan-hepatoblast markers (*CD133*, *EpCAM*, *CK8*, and *CK18*) were measured by real-time RT-PCR. The gene expression levels of *AFP* and pan-hepatoblast markers in the hESC-derived cells (day 9; bulk) were taken as 1.0. Data represent the mean ± SD from four independent experiments. The gene expression levels in the HBCs were significantly different among the three groups (bulk, HBCs, and NHBCs) based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ($p < 0.05$).

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dish for a week, almost all of the cells were still AFP positive (Figures 2C and 2D). To characterize the cells cultured on various types of human laminins for 7 days, the gene expression levels of *AFP* and pan-hepatoblast (*CD133*, *CK8*, *CK18*, and *EpCAM*) markers were examined on day 16 (Figure 2E). The gene expression levels of *AFP* and pan-hepatoblast markers in the hESC-derived HBCs P1 (HBCs passaged once) did not change as compared with those of the hESC-derived HBCs (day 9; HBC P0) (the definitions of HBC P0, P1, P10, and clone in the present study are shown in Figure S3). The gene expression levels of mature hepatocyte and cholangiocyte markers in the hESC-derived HBC P1 did not change as compared with those of the hESC-derived HBC P0 (day 9) (Figure S1F). These results suggest that the characteristics of the hESC-derived HBC P1 are similar to those of the hESC-derived HBC P0, although their morphologies are quite different from each other. Interestingly, the gene expression levels of mature cholangiocyte markers in the cells cultured on human LN411- or 511-coated dishes were upregulated as compared with those of the hESC-derived HBC P0 (day 9) (Figure S1F), suggesting that human LN411 and 511 might promote biliary differentiation. Importantly, both hESC-derived HBCs and hiPSC-derived HBCs could extensively proliferate on a human LN111-coated dish for more than 15 passages (Figure 2F) in the presence of HGF and EGF. Doubling times of hESC (H9)-derived HBCs and hiPSC (Dotcom)-derived HBCs were approximately 78 and 67 hr, respectively. Almost all of the populations cultured on a human LN111-coated dish were AFP positive (Figure 2G). Taken together, these results suggested that the hPSC-derived HBCs would proliferate and be maintained on a human LN111-coated dish.

Characterization of the hESC-Derived HBCs

To characterize the hESC-derived HBCs, the gene expression profiles in the hESC-derived purified HBCs (HBC P0), short-term cultured HBCs (HBCs passaged once [HBC P1]), and long-term cultured HBC (HBCs passaged ten times [HBC P10]) were examined. The hESC-derived HBCs were AFP positive (Figure 3A). Although the hESC-derived HBC P0 were negative for ALB, CK7, and CK19, the hESC-derived HBC P1 and P10 were positive for these genes (Figure 3A). Both integrin α6 and β1 (receptors of LN111) were strongly expressed in the hESC-derived HBC P0, P1, and P10 (Figure 3B). The gene expression levels of human hepatic stem cell markers (*N-CAM* and *Claudin 3* [Schmelzer et al., 2007]; these are not expressed in human hepatoblasts) in the hESC-derived HBC P0 were higher than those of the hESC-derived HBC P1 and P10 (Figure 3C). However, the gene expression level of *CK19* in the hESC-derived HBC P0 was lower than that of the hESC-derived HBC P1 and P10. The gene expression levels of pan-hepatoblast markers in the hESC-derived HBC P0 were similar to those of the hESC-derived HBC P1 and P10 (Figure 3D). The gene expression levels of human hepatoblast markers (*ALB*, *CYP3A7*, and *I-CAM* (Schmelzer et al., 2007), none of which were expressed in human hepatic stem cells) in the hESC-derived HBC P1 and P10 were higher than those of the hESC-derived HBC P0 (Figure 3E). However, the AFP expression level in the hESC-derived HBC P0 was similar to that of the hESC-derived HBC P1 and P10. Because the gene expression levels of mature hepatocyte and cholangiocyte markers in the hESC-derived HBC P1 and P10 were not increased as compared with those in the hESC-derived HBC P0 (Figure 3F), the hESC-derived HBC P1 and P10 were not segregated into either of the hepatic and biliary lineages. We also examined the gene expression levels of hepatoblast markers, which have been reported only in mice and not in humans (Figure 3G). The characteristics of the hPSC-derived HBCs are summarized in Figure S3. In addition, hESC-derived HBC P0 and HBC P10 showed normal karyotypes (Figure S2A). Therefore, the genetic stability of the HBCs was confirmed throughout the maintenance period. Taken together, these results suggest that the hESC-derived HBC P0 resemble human hepatic stem cells and the hESC-derived HBC P1 and P10 resemble human hepatoblasts, although some gene expression patterns in the hESC-derived HBCs differ from those in human hepatic stem cells and human hepatoblasts, respectively.

(E) The hESC-derived cells (day 9; bulk), HBCs, and NHBCs were plated onto human LN111-, 211-, 411-, or 511-coated dishes, and the attached cells were counted at 60 min after plating. The cell number that was initially plated was taken as 1.0. Data represent the mean ± SD from four independent experiments. The number of attached HBCs on LN111-coated dishes were significantly different among three groups (bulk, HBCs, and NHBCs) based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ($p < 0.05$).

(F) The gene expression levels of the indicated integrins were measured in the hESC-derived cells (day 9; bulk), HBCs, and NHBCs by real-time RT-PCR. Data represent the mean ± SD from four independent experiments. The gene expression levels of *integrin α3* and *α6* in the HBCs were significantly different among three groups (bulk, HBCs, and NHBCs) based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ($p < 0.05$).

(G) The adhesion of the hESC-derived HBCs to human LN111-, 211-, 411-, or 511-coated dishes was examined by using the indicated integrin antibodies. IgG antibodies were used as a control for uninhibited cell adhesion. The number of attached cells was estimated at 60 min after plating. The cell number in the control IgG-treated group was taken as 1.0. Data represent the mean ± SD from three independent experiments. "Control IgG" and "anti-integrin α6 or integrin β1" were compared using Student's t test. *$p < 0.05$; ***$p < 0.001$. See also Figure S1 and Tables S2–S5.

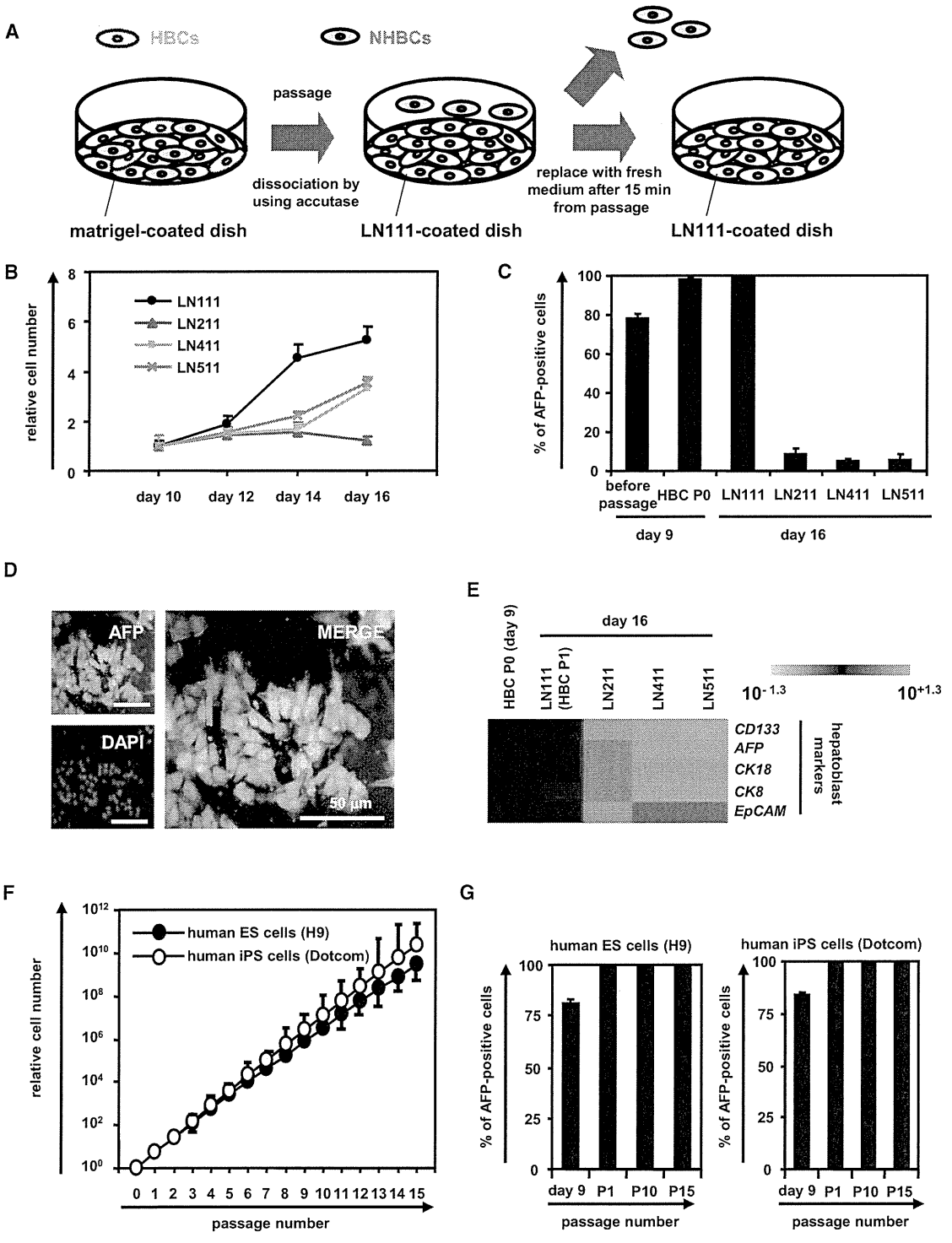

Figure 2. The hESC-Derived HBCs Could Be Proliferated and Maintained on a Human LN111-Coated Dish

(A) The hESC (H9)-derived cells (day 9) were plated onto a human LN111-coated dish. At 15 min after plating, the unattached cells were removed.

(B) The hESC (H9)-derived cells (day 9) were plated onto a human LN111, 211, 411, or 511-coated dish, and then the cell number were counted on days 10, 12, 14, and 16. The cell number on day 10 was taken as 1.0. Data represent the mean ± SD from three independent experiments. "LN111" was significantly different among four groups (LN111, 211, 411, and 511) on day 14 and 16 based on analysis with one-way ANOVA followed by Bonferroni post hoc tests ($p < 0.05$).

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In order to examine whether the hESC-derived HBC P0 have the potential to proliferate clonally on various types of human laminins, single HBCs were plated on separate wells of a human LN111-coated 96-well plate at a low density (one cell per one well) (Table S1). Single cells that attached to the human LN111-coated dish were AFP positive and HNF4α positive (Figure S2B). At 7 days after plating, the hESC-derived HBC colonies (albumin [ALB]- and cytokeratin 7 [CK7] double positive) (a representative colony is shown in Figure S2C) were efficiently generated from the hESC-derived HBC P0 on a LN111-coated dish. Taken together, these results showed that the hESC-derived HBCs could be generated from both the hESC-derived HBC P0 population and the single hESC-derived HBC P0.

The hPSC-Derived HBCs Could Differentiate into Both Hepatic and Biliary Lineages In Vitro

To examine whether the hESC-derived HBCs have the potential to differentiate into both hepatic and biliary lineages, first, these cells were differentiated into hepatocyte-like cells as described in Figure 4A. After 2 weeks of hepatic differentiation, almost all of the cells were polygonal in shape (Figure 4B) and were CYP3A4, αAT, and ALB positive (Figure 4C). The gene expression levels of mature hepatocyte markers in the HBC P0-, HBC P10-, or HBC clone-derived hepatocyte-like cells were higher than those in the cells that had not undergone hepatic differentiation (Figure 4D), although the gene expression levels of mature cholangiocyte markers in these cells did not change (Figure 4E). The ASGR1-positive cells in the HBC P0-, HBC P10-, and HBC clone-derived population accounted for approximately 60%, 90%, and 90% of the total, respectively (Figure 4F). The HBC P0-, HBC P10-, or HBC clone-derived hepatocyte-like cells had the ability to produce ALB (Figure 4G, left) and urea (Figure 4G, right). Next, the hESC-derived HBCs were differentiated into cholangiocyte-like cells as described in Figure 4H. After 2 weeks of biliary differentiation, tubular structures (Figure 4I) that were CK7 positive (Figure 4J) were observed. Although the gene expression levels of mature hepatocyte markers (Figure 4K) in the HBC P0-, HBC P10-, or HBC clone-derived cholangiocyte-like cells did not change, the gene expression levels of mature cholangiocyte markers (Figure 4L) in these cells were higher than those in the cells that had not undergone differentiation. Similar results were obtained by using another hESC line (H1) and hiPSC line (Dotcom) (Figure S4). Moreover, HBC-derived hepatocyte-like cells exhibited CYP metabolism capacity (Figure S5A) and a functional urea cycle that could response to ammonia (Figure S5B) and were considered to have potential to be applied in the prediction of drug-induced hepatotoxicity (Figure S5C). Taken together, these results indicated that the hPSC-derived HBCs have the ability to differentiate into both hepatic and biliary lineages in vitro.

In Vivo Cell Transplantation Assays of the hPSC-Derived HBCs

To examine whether the hESC-derived HBCs could be used for hepatocyte transplantation, these cells were transplanted into CCl_4-treated immunodeficient mice as shown in Figure 5A. The hepatocyte functionality of the hESC-derived HBC P0 or HBC P10 was assessed by measuring secreted human ALB levels in the recipient mice (Figure 5B). Although human ALB was detected in the mice that were transplanted with the hESC-derived HBC P0 or HBC P10, it was not detected in the mice that were not transplanted with these cells. The ALB-positive cells were observed in mice transplanted with the hESC-derived HBC P0 or HBC P10 (Figure 5C). Most of the ALB-positive cells in mice transplanted with the hESC-derived HBC P10 were AFP negative (Figure 5D), indicating that transplanted hESC-derived HBCs were differentiated into mature hepatocyte-like cells (some of them were binuclear [Figure 5E, white arrows]). These results demonstrated that hESC-derived HBCs have the potential to be applied for hepatocyte transplantation.

(C) The hESC-derived cells (day 9) were plated onto a human LN111, 211, 411, or 511-coated dish. The percentage of AFP-positive cells was examined by using FACS analysis on day 9 (before passage and after passage [HBC P0]) or day 16. Data represent the mean ± SD from three independent experiments.

(D) The hESC-derived cells cultured on a human LN111-coated dish for 7 days were subjected to immunostaining with anti-AFP (green) antibodies.

(E) The hESC-derived cells (day 9) were plated onto human LN111, 211, 411, or 511-coated dishes. The gene expression levels of *AFP* and pan-hepatoblast markers (*CD133*, *EpCAM*, *CK8*, and, *CK18*) were measured by real-time RT-PCR on day 16. The gene expression levels in the hESC-derived HBCs (the LN111-attached cells were collected at 15 min after plating) were taken as 1.0.

(F) The HBCs derived from hESCs (H9) or hiPSCs (Dotcom) were cultured and cell growth was analyzed by obtaining a cell count at each passage. Data represent the mean ± SD from three independent experiments.

(G) The percentage of AFP-positive cells was examined by using FACS analysis on day 9 (before passage), P1 (HBCs passaged once), P10 (HBCs passaged ten times), and P15 (HBCs passaged 15 times). Data represent the mean ± SD from seven independent experiments.

See also Tables S2 and S3.

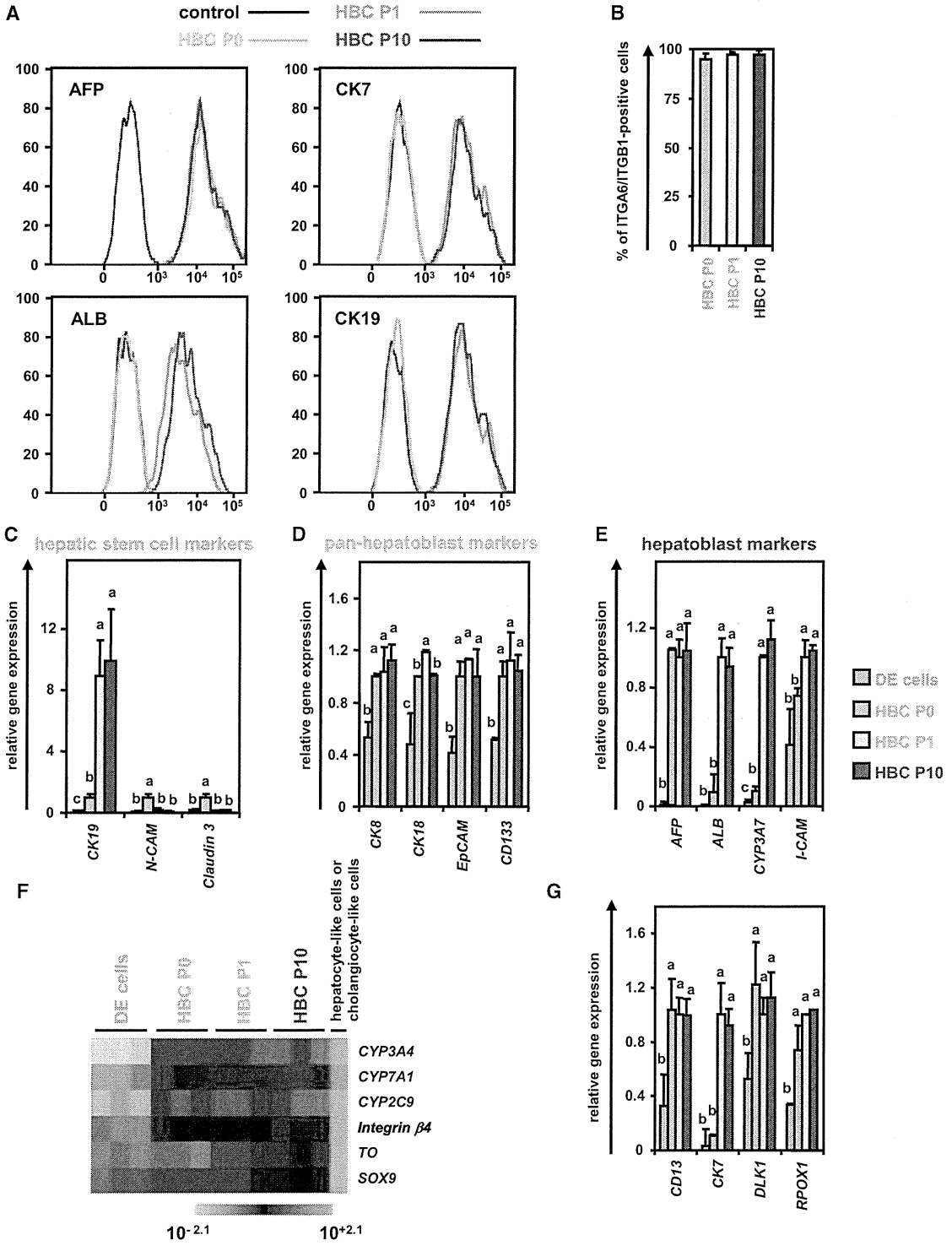

Figure 3. The hESC-Derived HBCs Were Characterized

(A and B) The hESCs (H9) were differentiated according to Figure 1A and then passaged onto a human LN111-coated dish. The attached cells (hESC-derived HBCs [HBC P0]) were collected at 15 min after plating. The percentage of AFP-positive, ALB-positive, CK7-positive, CK19-positive (A), and integrin α6- and integrin β1-double positive (B) cells in the hESC-derived HBC P0, HBC P1 (HBCs passaged once), and HBC P10 (HBCs passaged ten times) populations was estimated by using FACS analysis. Data represent the mean ± SD from seven independent experiments.

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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 α6- and β1-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 *TO*) or cholangiocyte markers (*integrin β4* 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 ± 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.

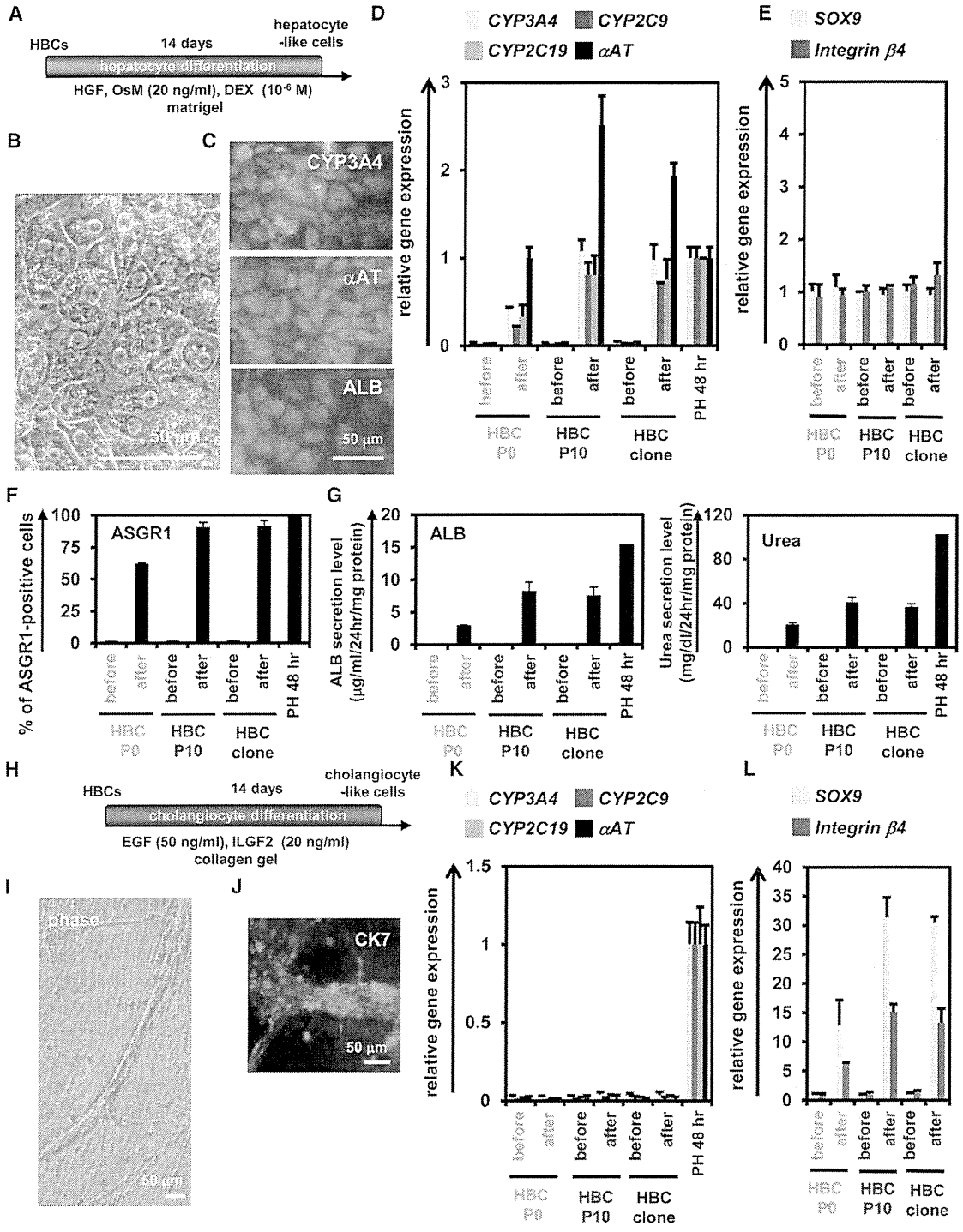

Figure 4. The hESC-Derived HBCs Could Differentiate into Both Hepatic and Biliary Lineages

(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-αAT (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 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM 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/cm^2) in maintenance DMEM/F12 medium (DMEM/F12 medium [Invitrogen] was supplemented with 10% FBS, 1 × insulin/transferrin/selenium, 10 mM nicotinamide, 10^{-7} M dexamethasone (DEX) (Sigma), 20 mM HEPES, 25 mM $NaHCO_3$, 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 μM 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/cm^2) 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 μl collagen gel solution (consisting of 400 μl type I-A Collagen (Nitta gelatin), 50 μl 10 × DMEM, and 50 μl of 200 mM HEPES buffer containing 2.2% $NaHCO_3$ 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 μl 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 μl 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-1α 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 ± 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 ± 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 ± 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.

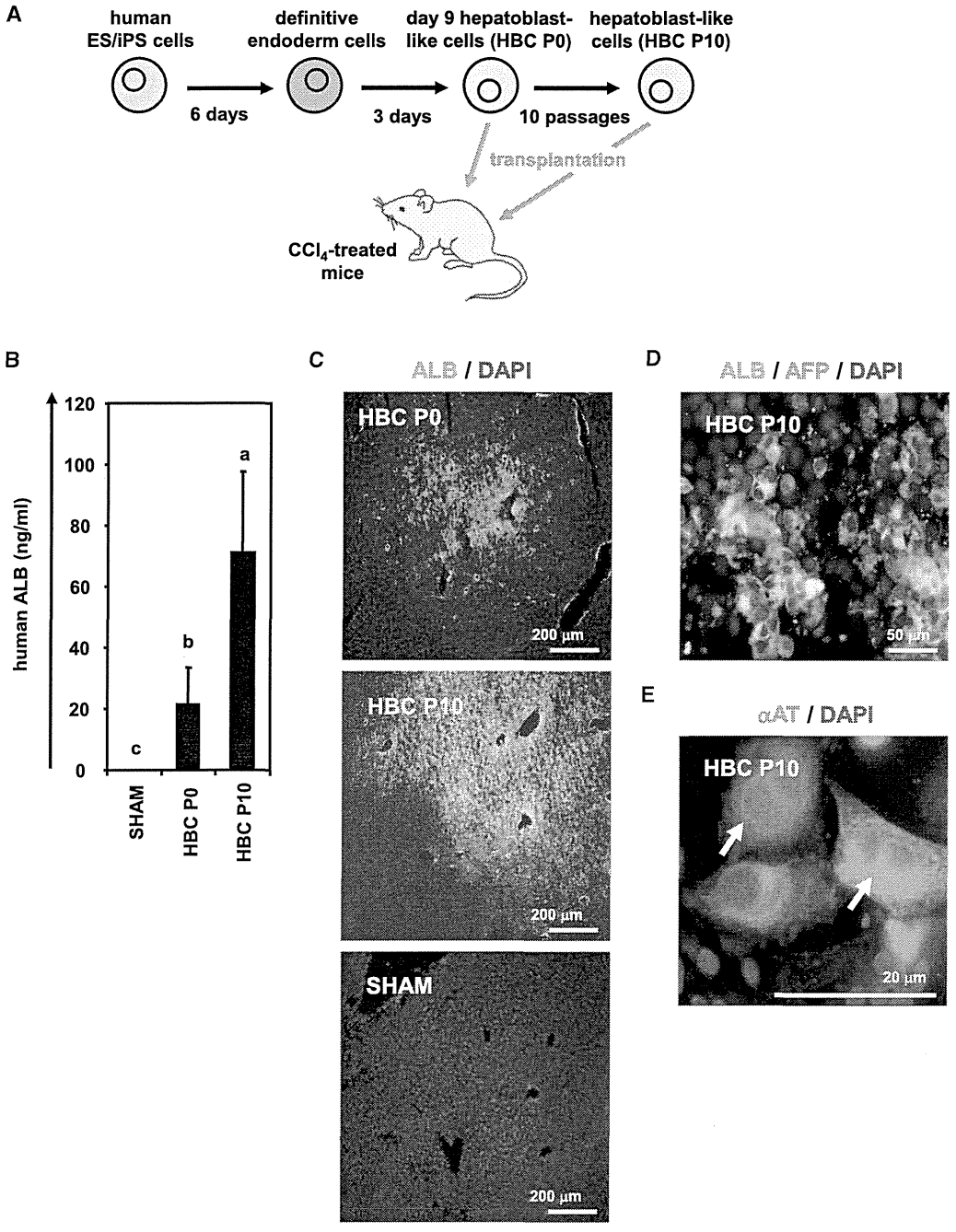

Figure 5. The hESC-Derived HBCs Were Integrated into the Mouse Liver Parenchyma

(A) The procedure for transplantation of the hESC (H9)-derived HBC P0 and HBC P10 into CCl_4 (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/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 CCl_4 (4 ml/kg) by intrasplenical 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 αAT 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 (NH_4Cl, 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/cm^2 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 α6 and β1 (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 μl 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 μl 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-GloTM 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.

ACKNOWLEDGMENTS

We thank Yasuko Hagihara for her excellent technical support. H.M., K.K., and T.H. were supported by grants from the Ministry of Health, Labor, and Welfare of Japan. H.M. was also supported by the Project for Technological Development of the Japan Science and Technology Agency (JST) and by the Uehara Memorial Foundation. F.S. was supported by Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. K.T. and Y.N. are Research Fellows of the Japan Society for the Promotion of Science.

Received: June 6, 2013
Revised: August 27, 2013
Accepted: August 27, 2013
Published: October 3, 2013

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Tiss. Cult. Res. Commun. 32: 183–187 (2013)

ヒト多能性幹細胞から肝細胞への分化誘導法の開発とその毒性評価系への応用

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要約　肝臓は薬物代謝に関与する主要な臓器であり、ヒト胚性幹細胞（human embryonic stem cells；ヒトES細胞）やヒト人工多能性幹細胞（human induced pluripotent stem cells；ヒトiPS細胞）からヒト初代培養肝細胞に類似した肝細胞を分化誘導できれば、創薬過程における医薬品候補化合物の肝毒性評価などに使用できる。ヒトES/iPS細胞を肝細胞へ分化させるためには、生体内での肝発生・分化の環境を模倣してサイトカインや増殖因子などの各種液性因子を作用させる方法が汎用されている。しかしながら、その肝分化誘導効率は不十分であり、さらなる分化効率の向上が要求されている。そこで、筆者らは肝発生に必要な転写因子をヒトES/iPS細胞から分化させた細胞に遺伝子導入することによって、肝分化誘導効率を改善させることを試みた。本稿では、筆者らの最近の研究成果を紹介しつつ、ヒトES/iPS細胞から肝細胞への分化誘導の現状と毒性評価系への応用の可能性について概説する。

キーワード：　肝細胞、ヒトES細胞、ヒトiPS細胞、毒性スクリーニング

序　　文

薬物誘発性肝障害（drug-induced liver injury：DILI）は医薬品の市場撤退原因の主要なものであり、米国においては急性肝障害の50%以上を占める。これまでに600種類以上の薬剤がDILIの起因薬剤として報告されている。現在、ヒト初代培養肝細胞（本稿では、凍結ヒト肝細胞を含めてヒト初代培養肝細胞とする）を用いて、医薬品開発の初期段階にDILIを予測することで、安全性の高い医薬品を開発することが試みられている[1]。しかしながら、ヒト初代培養肝細胞は非常に高価であり、ロット差が大きいこと、培養することで薬物代謝能などの肝機能が急速に減弱すること、高い肝機能を有したヒト肝細胞ロットの安定供給が難しいといった問題を有している[2,3]。そこで、無限に増殖するヒトES/iPS細胞から分化誘導した肝細胞（分化誘導肝細胞）を、薬物の毒性評価系などへ応用することが期待されている[4,5]。

ヒトES/iPS細胞は中内胚葉、内胚葉、肝幹前駆細胞を通して肝細胞へ分化する。そこで、各分化過程で、生体内での肝発生の環境を模倣してサイ

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トカインや増殖因子などの各種液性因子を作用させる肝分化誘導法が開発された（図1）。しかしながら、サイトカインや増殖因子などの各種液性因子の作用のみからなる分化誘導法では、肝分化誘導効率は不十分であり、分化効率の向上が必要であった。そこで、著者らは各々の分化段階の細胞に肝分化に関連する転写因子を順次遺伝子導入することで、肝細胞への分化を飛躍的に促進させる方法を開発した。

本稿では、ヒトES/iPS細胞から肝細胞への分化誘導の現状について、著者らの最新の研究成果を紹介するとともに、分化誘導肝細胞の毒性評価系への応用の可能性について解説する。

1．分化誘導肝細胞の肝機能評価

肝臓は、生体内に投与された薬物を代謝する主要な臓器であるだけでなく、脂質代謝や糖代謝、胆汁（酸）やアルブミン（ALB）の生産・分泌等、多彩な機能を有する。それゆえ、ヒトES/iPS細胞から分化誘導した肝細胞の機能を評価するにあたっては、様々な機能を検討する必要がある。そこでまず、ヒトES/iPS細胞から肝細胞への分化誘導と毒性評価系への応用について紹介する前に、肝細胞としての機能を評価する場合に必要とされる検討項目について解説する。

分化誘導肝細胞の評価としては、肝細胞の形状、肝関連遺伝子・タンパク質の発現、ALB産生能をはじめとする肝機能の評価が必要である[6-9]。具体的には、分化誘導肝細胞の形状として、肝細胞に特徴的な構造（グリコーゲン顆粒、ゴルジ体の豊富な細胞質、微細胆管構造、頂端側の微絨毛、よく発達した密着結合など）の形成度合が評価される[6]。また、alpha-1-antitrypsin（αAT）、ALB、各種cytochrome P450（CYP）などの遺伝子発現量や、ALB産生、尿素産生[8]、グリコーゲン貯蔵[8,10]、低密度リポタンパク（LDL）の取り込み能[9,10]、インドシアニングリーンの取り込み・排泄能[8,10]の検討も必要であり、これらの機能をヒト初代培養肝細胞と比較検討することが好ましい。これらの評価項目については、これまで発表されてきた論文等で多く実施されている（ヒト初代培養肝細胞と比較検討した例は極めて限られるが）。しかしながら、DILIを引き起こす薬物の多くは、肝臓で薬物代謝酵素により代謝されて反応性代謝物を生じ、肝障害を引き起こすことが知られるため[11]、分化誘導肝細胞を毒性評価に応用する場合には、上述の評価項目に加えて、ヒト初代培養肝細胞に類似

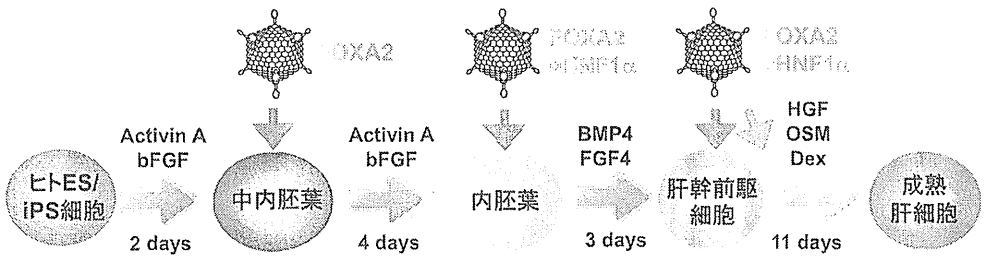

図1　FOXA2、HNF1α遺伝子の導入によるヒトES/iPS細胞から肝細胞への効率の良い分化誘導
ヒトES/iPS細胞は中内胚葉、内胚葉、肝幹前駆細胞を介して、肝細胞へと分化する。ヒトES/iPS細胞から内胚葉への分化にはactivin Aが使用される。内胚葉から肝幹前駆細胞への分化にはBMPおよびFGFが汎用される。肝幹前駆細胞から肝細胞への分化にはHGF、OsM、DEXが使用される。内胚葉分化を促進するために、分化誘導された中内胚葉にFOXA2遺伝子を導入した。肝幹前駆細胞への分化を促進するために、分化誘導された内胚葉にFOXA2、HNF1α遺伝子を導入した。肝細胞への分化を促進するために、肝幹前駆細胞にFOXA2、HNF1α遺伝子を導入した。このように肝細胞分化への適切な時期に適切な転写因子を過剰発現させることで、ヒトES/iPS細胞から肝細胞への効率の良い分化誘導が可能となった。

した薬剤代謝酵素活性を有するか否かの確認が最重要である。

2．ヒトES/iPS細胞から肝細胞への分化誘導法

増殖因子やサイトカインの添加だけからなる従来の肝分化誘導法は、肝細胞への分化誘導効率が不十分であり、さらなる分化効率の向上が必要である[12,13]。そこで、筆者らは増殖因子やサイトカインを作用させる従来の分化誘導法で細胞の外部環境を分化に適した状態にした上に、細胞内から強制的に分化を進行させるように肝分化に関連した転写因子を過剰発現させることで、肝分化誘導効率を向上させる方法を開発した[10,14-16]。即ち、ヒトES/iPS細胞から分化誘導した中内胚葉にsry-related HMG box 17（SOX17）遺伝子を[14]、内胚葉から肝幹前駆細胞への分化ステップではhematopoietically expressed homeobox（HEX）遺伝子を[15]、肝幹前駆細胞から肝細胞への分化ステップではhepatocyte nuclear factor 4α（HNF4α）遺伝子を導入することで[10]、高いアルブミン産生能、グリコーゲン貯蔵能、LDL取り込み能、インドシアニングリーン取り込み能等を有した肝細胞を分化誘導することに成功した。さらに最近では、ヒトES/iPS細胞から肝細胞への各分化ステップにおいて7種類の肝関連転写因子（FOXA2、SOX17、HEX、HNF1α、HNF1β、HNF4α、HNF6）を導入し、最も効率良く肝分化を促進できる転写因子を探索した結果、forkhead box protein A2（FOXA2）およびhepatocyte nuclear factor 1homeobox A（HNF1α）遺伝子を組み合わせて発現させることにより、さらに効率良く成熟肝細胞を分化誘導することに成功した[16]（図1）。このようにして作製した分化誘導肝細胞は、80～90%以上の細胞がアシアロ糖タンパク質受容体（成熟した肝細胞のマーカー）陽性であり、ヒト初代培養肝細胞に匹敵する薬物代謝酵素の遺伝子発現レベルを示した。また、CYPなどで代謝される9種類の薬物の代謝プロファイルを調べたところ、分化誘導肝細胞の薬物代謝能はヒト初代培養肝細胞より低いものの（ヒト初代培養肝細胞の1～40%程度の活性）、いずれの薬物に対しても代謝能を有していることが確認された[16]。今後は、より高いCYP活性を有した分化誘導細胞を作製することが必要となる。

3．分化誘導肝細胞の創薬応用の可能性について

筆者らは分化誘導肝細胞を薬剤に対する毒性評価に応用できるか検討した。トログリタゾン、アセトアミノフェンといった肝毒性を起こす薬物を、上述の遺伝子導入を組み合わせた分化誘導法で作製した分化誘導肝細胞に作用させたところ、細胞毒性を生じることを確認した[10]。また、肝毒性を引き起こす20種類以上の薬剤について、筆者らの分化誘導肝細胞を用いて細胞毒性評価試験を実施したところ、*in vitro*肝毒性評価系に汎用されるHepG2細胞（肝がん細胞由来細胞株）を用いた場合に比べ、より感度良く細胞毒性を示し、かつその細胞毒性はCYPの阻害剤を作用させることで部分的に消失した[17]。したがって、薬剤がCYPで代謝されることで生じた反応性代謝物によって生じた細胞傷害性を、分化誘導肝細胞が検出できることが示唆された[17]。

一般に、薬剤がDILIを引き起こすメカニズムを解明することは困難であるが、DILIによって、肝細胞の細胞小器官（ミトコンドリア[18,19]、小胞体[20]）に障害が生じることが知られている。今後は、薬剤がミトコンドリア毒性や小胞体ストレスに及ぼす影響についても調べることによって、分化誘導肝細胞のより広範な毒性評価への応用が期待される。