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Short Article

Human Hepatocytes with Drug Metabolic Function Induced from Fibroblasts by Lineage Reprogramming

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SUMMARY

Obtaining fully functional cell types is a major challenge for drug discovery and regenerative medicine. Currently, a fundamental solution to this key problem is still lacking. Here, we show that functional human induced hepatocytes (hiHeps) can be generated from fibroblasts by overexpressing the hepatic fate conversion factors *HNF1A*, *HNF4A*, and *HNF6* along with the maturation factors *ATF5*, *PROX1*, and *CEBPA*. hiHeps express a spectrum of phase I and II drug-metabolizing enzymes and phase III drug transporters. Importantly, the metabolic activities of CYP3A4, CYP1A2, CYP2B6, CYP2C9, and CYP2C19 are comparable between hiHeps and freshly isolated primary human hepatocytes. Transplanted hiHeps repopulate up to 30% of the livers of Tet-uPA/Rag2$^{-/-}$/γc$^{-/-}$ mice and secrete more than 300 μg/ml human ALBUMIN in vivo. Our data demonstrate that human hepatocytes with drug metabolic function can be generated by lineage reprogramming, thus providing a cell resource for pharmaceutical applications.

INTRODUCTION

Functional human cell types are in high demand in the field of regenerative medicine and drug development. They show great potential for repairing or replacing diseased and damaged tissues and can be valuable tools for pharmaceutical applications. However, the application of functional human cell types in these areas is limited due to a shortage of donors (Castell et al., 2006). To solve this dilemma, novel strategies for generating functionally mature cells are in high demand. Recently, lineage reprogramming has emerged as an effective method for changing the fate of somatic cells (Vierbuchen and Wernig, 2012). In principle, one cell type can be converted directly to the final mature state of another cell type and can bypass its intermediate states during lineage reprogramming. Consequently, functionally mature cells may be obtained using this strategy and may potentially provide a promising source of functional human cells.

Functional human hepatocytes are the most significant in vitro model for evaluating drug metabolism and are potentially widely applicable in pharmaceutical development. Because unacceptable metabolic and toxicity effects on the liver are largely responsible for the failure of new chemical entities in drug discovery (Baranczewski et al., 2006), it is essential to use human hepatocytes, which serve as the closest in vitro model of human liver in assays of absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox), to identify compounds that display favorable pharmacokinetics (Sahi et al., 2010). Currently, primary human hepatocytes that are derived from individuals with different genetic backgrounds are frequently used in drug development, but the resulting diversity of genetic backgrounds hinders the reproducibility of the results obtained from pharmaceutical studies using these cells. Additionally, the scarcity of human liver donors greatly limits the use of primary human hepatocytes (Castell et al., 2006) and, as a result, alternative resources for human hepatocytes with a high reproducibility are urgently required for use in drug discovery.

Different strategies to generate functional hepatocytes have been studied. Human hepatocytes have been derived from human pluripotent stem cells by directed differentiation (Cai et al., 2007; Ogawa et al., 2013; Takebe et al., 2013; Zhao et al., 2013). This strategy has progressed quickly in recent years, although the immature phenotype of the cells derived from pluripotent stem cells remains a technological obstacle. In principle, fully functional hepatocytes are relatively difficult to obtain using this method, as the whole process involves multiple key steps that affect the final stage of hepatocyte formation. In contrast, lineage reprogramming allows the lineage conversion of a somatic cell without passing through an intermediate state. Although mouse hepatocytes have been

transdifferentiated from fibroblasts (Huang et al., 2011; Sekiya and Suzuki, 2011), these cells still express several hepatoblast markers such as α-fetoprotein (AFP) and lack the expression of several key cytochrome P450 enzymes (CYPs) that are responsible for drug metabolism, suggesting a functionally immature phenotype for these cells (Willenbring, 2011). Most importantly, it is still unknown whether human hepatocytes with metabolic function can be generated by lineage reprogramming.

In this study, we sought to generate functional human hepatocytes from fibroblasts by ectopically expressing hepatic fate conversion factors together with maturation factors. With this strategy, we were able to readily and reproducibly generate human induced hepatocytes (hiHeps) that possess drug metabolic function and are potentially applicable for drug development.

RESULTS

Identification of Factors that Induce Hepatic Fate

To identify the combination of transcription factors that induce human embryonic fibroblasts (HEFs) into hepatocytes, we selected a pool of transcription factors (Table S1 available online) that were previously shown to be expressed in human hepatocytes and are crucial to the determination of hepatic cell fate (Nagaoka and Duncan, 2010; Zaret, 2008). Previous studies also showed that proliferation arrest and cell death are general barriers to cell reprogramming (Huang et al., 2011; Zhao et al., 2008), and therefore *MYC* and *p53* small interfering RNAs (siRNAs) were employed in the reprogramming process (Figure S1A). Briefly, *HNF1A* and *HNF4A* are preferentially considered because of their critical role in both embryonic and adult liver among the 17 transcription factors. Then we screened additional factors using a "2+1" strategy by the addition of one candidate factor at a time to the combination of *HNF1A* and *HNF4A*. We found that *HNF6*, cooperating with *HNF4A* and *HNF1A*, can result in a high percentage of ALBUMIN (ALB)-positive cells within 20 days (Figures S1B and S1C). These three factor-induced hepatocyte-like cells (3H cells) exhibited some hepatic properties, including glycogen synthesis and low-density lipoprotein (LDL) uptake (Figures S1D and S1E). However, the expression level of *ALB* in these cells was extremely low (Figure S1F). Moreover, the expression of the major cytochrome P450 enzymes in hepatocytes was not detected in these cells (Figure S1G). Therefore, the 3H cells appear to be functionally immature, implying that additional factors are required for their full maturation.

Identification of Factors that Generate Mature Hepatocytes

To identify the factors capable of inducing the functional maturation of hepatocyte-like cells, we performed a global gene expression analysis on 3H cells, freshly isolated primary human hepatocytes (F-HEPs), and fetal liver cells. Differential expression of several hepatic transcription factors, including *CEBPA*, *ATF5*, and *PROX1*, were observed among the three samples (data not shown). These three genes were expressed at relatively low levels in the 3H cells and in fetal hepatocytes compared to the levels in adult hepatocytes. This difference was further confirmed by quantitative PCR (Figures 1A and S1H). Among these genes, *PROX1* was shown in our recent study to be a key transcription factor that is critical in the metabolic maturation of hepatocytes (Zhao et al., 2013). *CEBPA* and *ATF5* are highly abundant liver-enriched transcription factors, indicating the importance of transcriptional regulation in hepatic function. Furthermore, a gene expression study showed that these three genes were highly expressed in F-HEPs (Figure 1B). Collectively, these data suggested that overexpressing these factors may lead to the functional maturation of 3H cells.

To generate mature human hepatocytes from fibroblasts, we combined the three factors with *CEBPA*, *PROX1*, and *ATF5*. After overexpressing these factors in HEFs, we observed a dramatic morphological change of fibroblasts into epithelial cells in 1 week. These cells proliferated rapidly in hepatocyte culture medium (HCM), with the doubling time ranging from 9 to 11 hr (Figure S1I). At 2 weeks postinfection, the replated cells showed the typical morphology of primary human hepatocytes (Figures 1C and 1D). At about 25 days postinfection, *p53* siRNA was silenced, as indicated by a GFP reporter (Figure S1J), and the induced cells were transferred to a modified William's E medium (Figures 1C and S1I). Quantitative PCR results showed that the induced hepatocyte-like cells expressed *ALB* at a level that was comparable to that of primary human hepatocytes (Figure 1E), which was significantly higher than that of 3H cells (Figure S1F). We further analyzed the reprogramming efficiency and found that 90% of the induced cells were ALB positive and nearly 100% were α-1 antitrypsin (AAT) positive (Figures 1F and 1G). The secretion of ALB was dramatically enhanced and was comparable to that of primary human hepatocytes (Figure 1H). Furthermore, the four major cytochrome P450 enzymes, CYP3A4, CYP1A2, CYP2C9, and CYP2C19, were also expressed in the induced cells detected by immunostaining (Figure 1I). Removal of any of these six factors would lead to a substantial decrease in the expression of drug metabolic enzymes and transporters (Figure S1K). These results indicate that functional hepatic properties were obtained in these induced hepatocyte-like cells, which we termed hiHeps.

hiHeps Possess the Typical Characteristics of Human Hepatocytes

To evaluate hepatic fate conversion, we first analyzed typical hepatic features. Immunofluorescence microscopy showed that the epithelial marker E-cadherin (ECAD) was coexpressed with ALB in hiHeps (Figure 2A). In addition, the fibroblast marker COL1A1 was not detected (Figure S2A). These results indicate a successful mesenchymal-epithelial transition in hiHeps. Next, we further examined endogenous hepatic transcription network activation in hiHeps. The RT-PCR results showed that the endogenous expression of *FOXA1*, *FOXA2*, and *FOXA3* (Zaret et al., 2008) was activated (Figure 2B). *LRH1*, another core transcription factor involved in the hepatic cross-regulatory network (Nagaoka and Duncan, 2010), was also endogenously expressed in hiHeps (Figure 2B). We confirmed the expression of FOXA2 and LRH1 using immunofluorescence (Figure S2B). Additionally, fibroblast marker genes, including *COL1A1*, *PDGFRB*, and *THY1*, were not detected in hiHeps (Figure 2B). In accordance with *p53* siRNA silencing (Figure S1J), we further found that exogenous expression of *HNF1A*, *HNF6*, *HNF4A*, *ATF5*, *PROX1*, and *CEBPA* was silenced in hiHeps (Figure 2C). In addition, *MYC* was decreased to a level lower than that of freshly isolated primary human hepatocytes, as revealed by

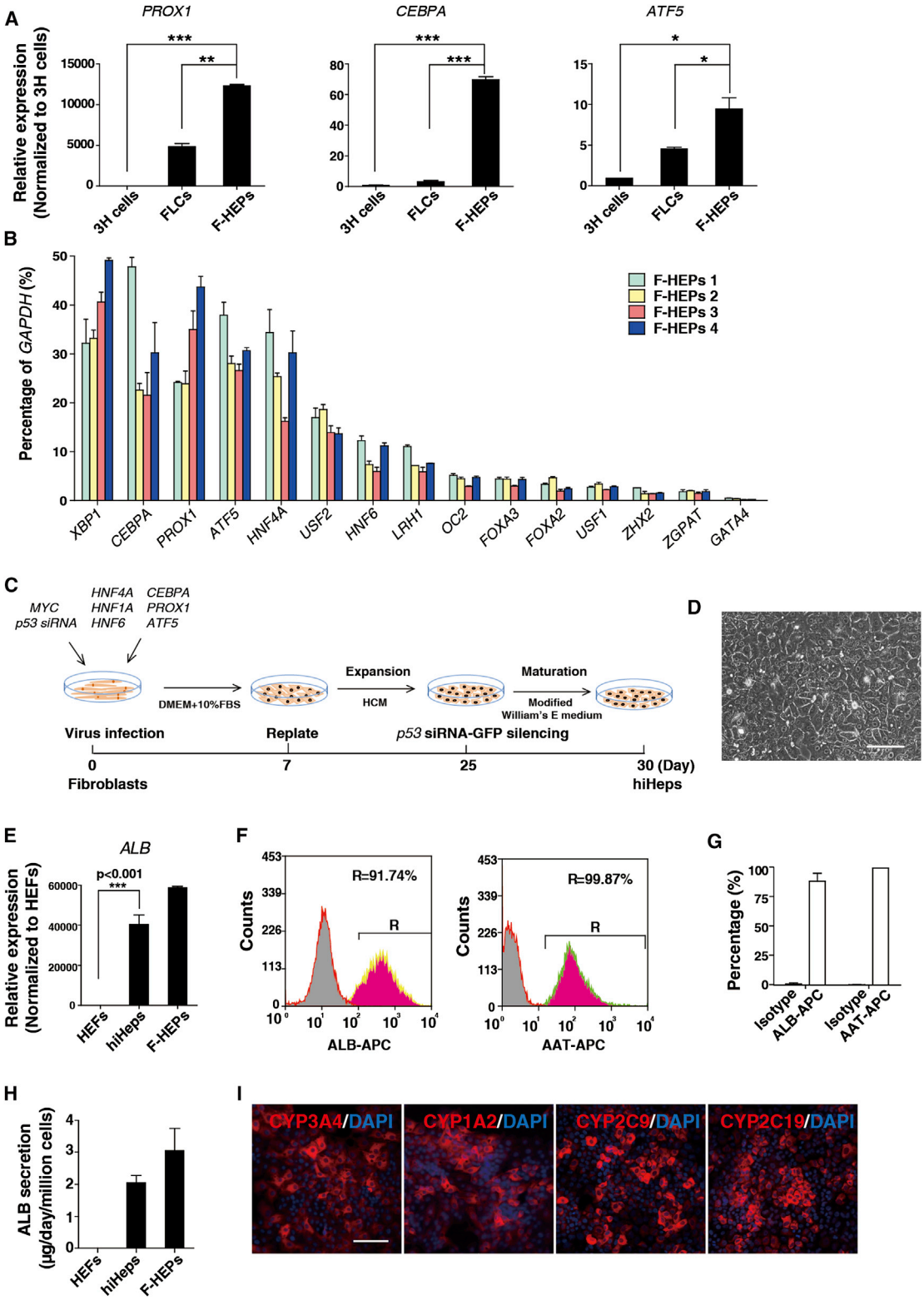

Figure 1. Screening for Hepatic Critical Transcription Factors to Generate hiHeps

(A) Quantitative comparison of the expression of hepatic transcription factors in 3H cells, fetal liver cells (FLCs), and F-HEPs. n = 2. *p < 0.05; **p < 0.01; ***p < 0.001.

(B) Quantitative analysis of the abundance of hepatic transcription factors in four individual F-HEPs. n = 2.

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quantitative RT-PCR (qRT-PCR) (Figure 2D). Collectively, these data indicate that hiHeps gain a hepatic transcription network.

Next, we evaluated hiHeps for functional characteristics of human hepatocytes. hiHeps were competent for LDL uptake (Figure 2E). We also observed hiHeps could incorporate indocyanine green (ICG) from the medium and exclude the absorbed ICG after withdrawal (Figure 2F). Oil red O staining in hiHeps showed an accumulation of fatty droplets, and Periodic Acid-Schiff (PAS) staining indicated glycogen synthesis (Figures 2G and 2H). Similar to human adult hepatocytes, hiHeps were AFP negative (Figure S2C). G banding analysis revealed that hiHeps had a normal karyotype after 7 weeks of culture (Figure 2I). Besides HEFs, we converted human adult foreskin fibroblasts using the same factors and obtained similar results (Figures S2D–S2F). Collectively, these results indicate that hiHeps exhibit typical hepatic functional features.

We also compared the global gene expression patterns between hiHeps and F-HEPs by RNA sequencing. Principle component analysis and hierarchical clustering analysis revealed that hiHeps established from different donors were clustered with human hepatocytes and separated from human fibroblasts, HepG2 cells, and human embryonic stem cell (ESC)-derived hepatocytes (ES-Heps) (Figure 2J). Indeed, hepatic transcription factors were upregulated and the expression of fibroblast signature genes was downregulated in hiHeps (Figure 2K). Additionally, hiHeps displayed the gene expression patterns of hepatocytes in a set of genes involved in lipoprotein, cholesterol, fat, glucose, and drug metabolism (Figure 2K). Altogether, these results indicate that hiHeps show a similar expression profile to primary human hepatocytes.

Establishment of the Central Network of Drug Metabolism in hiHeps

To evaluate whether hiHeps expressed key enzymes in drug metabolism, we quantitatively confirmed the expression in hiHeps of five key CYP enzymes, *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP3A4*. The five key CYPs are major phase I enzymes that account for 60% of human drug oxidation (Zhou et al., 2009). As the positive control, pooled F-HEPs from five individual donors were used. Notably, comparable mRNA levels of these major CYPs could be detected in hiHeps and F-HEPs, in contrast to their expression in hepatocytes derived from human ESCs and HepG2 cells (Figure 3A). Next, we analyzed hiHeps for the presence of phase II enzymes and phase III transporters, which are important for the excretion of xenobiotic drugs. The expression levels of these genes were similar to those in F-HEPs (Figures 3B–3C and S3A). Additionally, hiHeps expressed a broad spectrum of phase I and phase II metabolic enzymes and phase III transporters (Figure S3B). Collectively, these findings suggest that the central network of drug metabolism was successfully established in hiHeps and resembled that of pooled freshly isolated primary human hepatocytes.

Level of Key Drug Metabolic Activities in hiHeps Is Comparable to that in Freshly Isolated Primary Human Hepatocytes

To evaluate the drug metabolic activities of hiHeps, we first focused on CYP3A4. Using ultraperformance liquid chromatography-tandem mass spectrometry technology, we examined the drug metabolic activity of CYP3A4 in hiHeps using two structurally different substrates, testosterone and midazolam. Because of the remarkable interindividual variability in drug clearance, two batches of freshly isolated primary human hepatocytes were used as the positive control. In contrast to the HepG2 cell line, ES-Heps, and HEFs, hiHeps were able to metabolize the two CYP3A4-selective substrates efficiently to a similar degree as F-HEPs (Figure 3D). Furthermore, we found that the metabolic activities of CYP1A2 and CYP2B6 in hiHeps were comparable to that of F-HEPs (Figure 3D). The activities of CYP2C9 and CYP2C19 in hiHeps were approximately 30% of F-HEPs (Figure 3D). The metabolic activities of all these CYP enzymes in hiHeps were at least 100-fold higher than that of ES-Heps. These data indicate that hiHeps exhibit comparable metabolic activities of the key CYP enzymes to that of freshly isolated primary human hepatocytes.

To further evaluate the functional central network of drug metabolism in hiHeps, we compared the expression of nuclear receptors between hiHeps and F-HEPs, which are critical in regulating the expression of metabolizing enzymes. Nuclear receptors that are responsible for the xenobiotic metabolizing system were expressed in hiHeps (Figure S3C). Moreover, hiHeps responded to the standard inducers of *CYP3A4*, *CYP1A2*, and *CYP2B6* at the mRNA level (Figure S3D). Taken together, these data suggest a functional establishment of the nuclear receptor network in hiHeps.

To assess the potential application of hiHeps in studying hepatotoxicity, we quantified the acute toxicity of model hepatotoxins. As hepatotoxicity is the most common adverse event resulting in drug failure (Sahi et al., 2010), the sensitivity of drug toxicity is a key index for the potential application of human hepatocytes in drug discovery. hiHeps showed a level of sensitivity comparable to that of primary human hepatocytes when incubated with a series of model hepatotoxins (Figure S3E), suggesting the potential of using hiHeps for testing drug toxicity.

Repopulation of Tet-uPA/Rag2$^{-/-}$/γc$^{-/-}$ Mouse Liver with hiHeps

To investigate the capacity of hiHeps to repopulate mouse liver, Tet-uPA (urokinase-type plasminogen activator)/Rag2$^{-/-}$/γc$^{-/-}$ mice were injected intrasplenically with hiHeps (Song et al., 2009). The secretion of human ALBUMIN in mouse serum

(C) Schematic view of the hiHep reprogramming diagram.
(D) The hepatic morphology of hiHeps.
(E) Quantitative analysis of *ALBUMIN* expression among hiHeps, HEFs, and F-HEPs.
(F and G) Reprogramming efficiency measured by flow cytometry analysis marked by ALB and AAT. n = 3. APC, allophycocyanin.
(H) Quantitative analysis of ALBUMIN secretion among hiHeps, HEFs, and F-HEPs by ELISA. n = 3.
(I) Immunofluorescence analysis of the expression of CYP3A4, CYP1A2, CYP2C9, and CYP2C19 in hiHeps. DAPI, 4′,6-diamidino-2-phenylindole.
The scale bars represent 100 μm. Data are presented as mean ± SD. See also Figure S1 and Table S1.

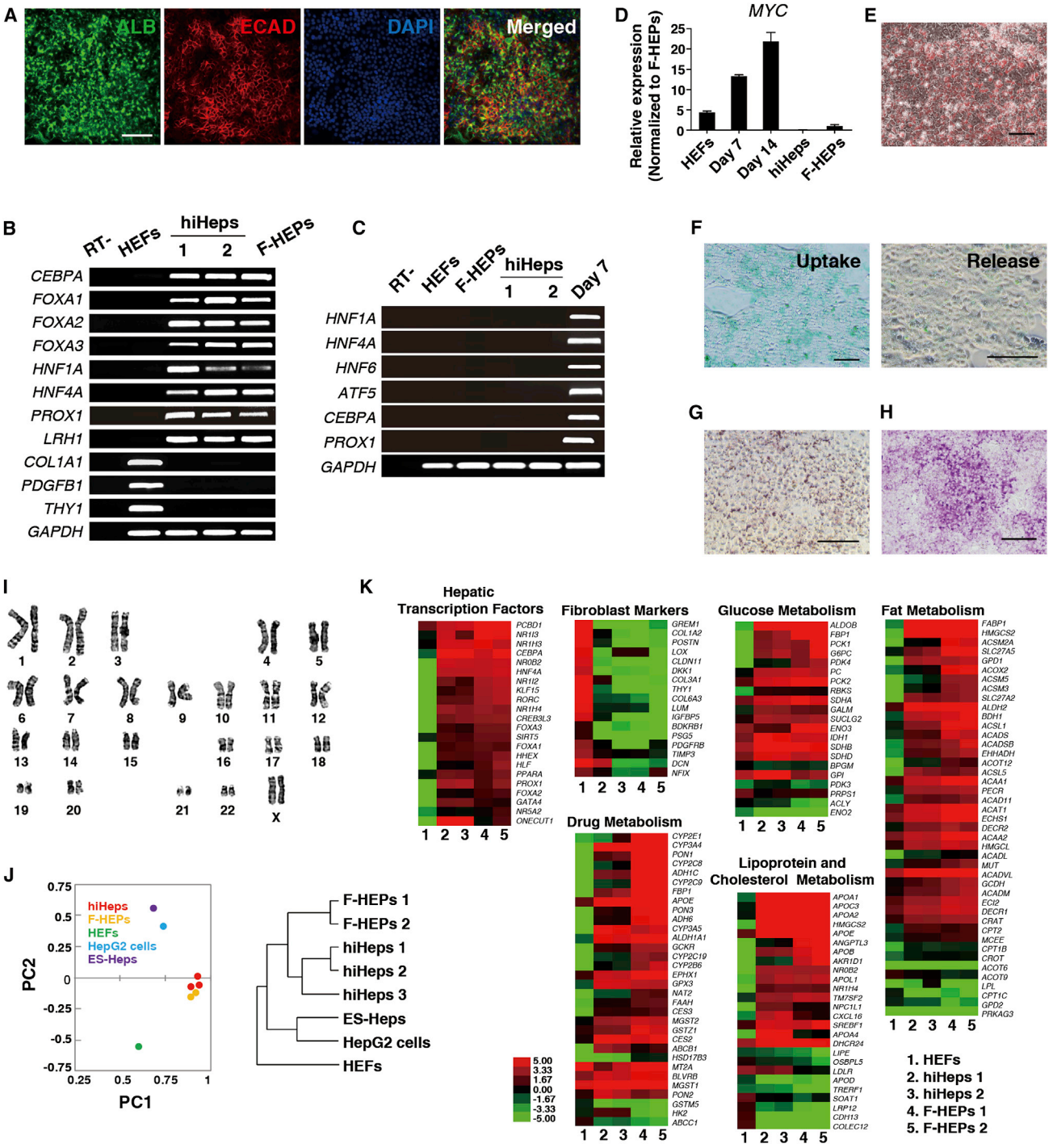

Figure 2. Characterization of hiHeps In Vitro

(A) Immunofluorescence analysis of ALB and ECAD in hiHeps.

(B) Endogenous gene expression analysis of hepatic transcription factors and fibroblast markers in hiHeps by RT-PCR.

(C) The silence of exogenous genes detected by RT-PCR. Day 7, 7 days postinfection.

(D) Relative expression of *MYC* during the hepatic conversion process measured by qRT-PCR. Day 7 and day 14, 7 and 14 days postinfection. n = 2.

(E–H) Analysis of basic hepatic function in hiHeps, including LDL uptake (E), ICG uptake and release (F), oil red O staining (G), and PAS staining (H).

(I) G banding analysis of hiHeps demonstrating a normal human karyotype (44, XX).

(J) Principal component analysis was performed to compare global gene expression profiles in HEFs, HepG2 cells, ES-Heps, hiHeps, and F-HEPs. Genes expressed at least 3-fold differently among HEFs, hiHeps, and F-HEPs were selected for further analysis. Left: a scatter plot of the expression

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increased gradually and the highest level reached was 313 μg/ml at 7 weeks after hiHep transplantation (Figures 4A–4C), which was 1,000-fold higher than ES-Heps and comparable to primary human hepatocytes (Figure 4B). To analyze the engraftment efficiency, hepatocytes were isolated from whole liver of two mice and measured by flow cytometry analysis. The repopulation efficiency was about 30% in the mouse that secreted 313 μg/ml human ALBUMIN (Figure 4C). No tumorigenesis was observed 2 months after hiHep transplantation.

We also analyzed the grafts of hiHeps. Six weeks after transplantation, clusters of cells expressing human ALB were observed in the recipient mice (Figure 4D). To confirm the metabolic function of hiHeps in vivo, CYP expression was analyzed. The expression of major CYPs including CYP3A4, CYP2C9, CYP1A2, CYP2E1, CYP2C19, and CYP2D6 (Figure 4D) indicated that hiHeps were still functional in vivo. Collectively, these results suggest that hiHeps can robustly repopulate the liver of Tet-uPA/Rag2$^{-/-}$/γc$^{-/-}$ mice and were functional in vivo.

DISCUSSION

Here, we show that human hiHeps are readily and reproducibly generated from HEFs using a combination of hepatic fate conversion factors *HNF1A*, *HNF4A*, and *HNF6* together with the maturation factors *ATF5*, *PROX1*, and *CEBPA*. Similar to primary human hepatocytes, hiHeps exhibit many typical hepatic features, including their epithelial morphology, expression of hepatocyte-specific markers, basic functional properties of hepatocytes, and global gene expression patterns. Importantly, an integral spectrum of phase I and phase II drug-metabolizing enzymes and phase III drug transporters is well established in hiHeps. Furthermore, transplanted hiHeps can repopulate up to 30% of the livers of Tet-uPA/Rag2$^{-/-}$/γc$^{-/-}$ mice and secrete more than 300 μg/ml human ALBUMIN in vivo. Therefore, we have strong evidence that human hepatocytes with drug-metabolizing functions can be generated from fibroblasts using lineage reprogramming.

One key question in lineage reprogramming is how to obtain fully functional cells. In hepatic transdifferentiation, through the expression of hepatic fate determination factors in fibroblasts, mouse induced hepatocyte-like cells were generated with several important hepatic characteristics (Huang et al., 2011; Sekiya and Suzuki, 2011). However, incomplete hepatocyte differentiation and expression of certain hepatoblast markers by hiHeps are compatible with an immature or progenitor-like state (Willenbring, 2011). In our study, we also found that certain hepatic fate determination factors could reprogram HEFs into hepatocyte-like cells (Figures S1A–S1E). However, these cells are not functional until the addition of another three factors (Figures 1D–1H). The additional three factors promote the further metabolic maturation of hiHeps (Figures 1I and S1G). This phenomenon suggests that hepatic fate determination and hepatic functional maturation might be governed by different master genes and are somewhat independent of each other. To obtain fully functional cells, the ectopic expression of cell fate determination factors may not be sufficient, and additional functional maturation factors are required to promote this process.

The drug metabolic capacity of human hepatocytes is one of the most important functions that distinguish hepatocytes from other lineages and has broad applications in drug development. Efforts to differentiate human pluripotent stem cells into hepatocytes have resulted in cells that were functionally immature. Our recent study showed that human ES-Heps express CYP1A2 and CYP3A4 (Zhao et al., 2013). However, the activities of these two CYP enzymes were significantly lower than that of primary hepatocytes. In another study, differentiated hepatocytes exhibited CYP3A4 and CYP1A2 activities only comparable to that of cultured primary hepatocytes (Ogawa et al., 2013). However, a number of liver-essential functions are progressively lost with time in cultured primary hepatocytes (Elaut et al., 2006). In our study, the gold standard, freshly isolated primary human hepatocytes, was used as the positive control. Our hiHeps express the key phase I (*CYP3A4*, *CYP2C9*, *CYP2C19*, *CYP2B6*, and *CYP1A2*) and phase II drug-metabolizing enzymes and phase III drug transporters at a level comparable to that of freshly isolated primary human hepatocytes. Importantly, the metabolic activities of the five CYP enzymes in hiHeps were comparable to those in freshly isolated primary human hepatocytes, suggesting the potential application of hiHeps in evaluating drugs metabolized by these CYP enzymes (Figure 3D). We were also able to detect the expression of endogenous nuclear receptors related to xenobiotic metabolizing systems in these cells (Nakata et al., 2006) (Figure S3C). Moreover, the expression of *CYP3A4*, *CYP1A2*, and *CYP2B6* was increased by the standard inducers (Figure S3D). In addition, because integrated metabolism pathways (phase I and phase II enzymes and phase III drug transporters) in hepatocytes are of vital importance for drug discovery (Castell et al., 2006), we closely analyzed the drug metabolic network of hiHeps. The expression pattern of genes encoding the drug-metabolizing markers was similar to that in primary human hepatocytes, implying an upregulation of the drug metabolic network in hiHeps (Figures 3A–3C and S3A–S3C). Collectively, these results indicate the integral establishment of the central network of functional drug metabolism in hiHeps, making these cells a potential alternative for preclinical screening assays.

Another key characteristic of human hepatocytes in drug development is their sensitivity to drug toxicity. Human hepatocytes derived from human pluripotent stem cells have a relatively low sensitivity to drug toxicity (Zhao et al., 2013). In our study, the sensitivity of hiHeps to multiple model hepatotoxins is comparable to that of primary human hepatocytes (Figure S3E), which suggests that hiHeps could be a valuable alternative cell resource in hepatotoxicity assays for new drug discovery. Importantly, our results demonstrate that the induced cells could be expanded at a large scale at an early stage (Figures 1C and S1I), and the function of hiHeps could be maintained for 16 days (Figure S3F). Considering the reprogramming efficiency

profiles on the planes spanned by the first and second principal components (PCs). Right: a dendrogram of the hierarchical clustering of expression profiles.

(K) Genes that exhibited significantly different expression levels among genes involved in lipoprotein, cholesterol, fat, glucose, and drug metabolism and fibroblast markers were extracted.

Data are presented as mean ± SD. See also Figure S2 and Tables S2 and S3.

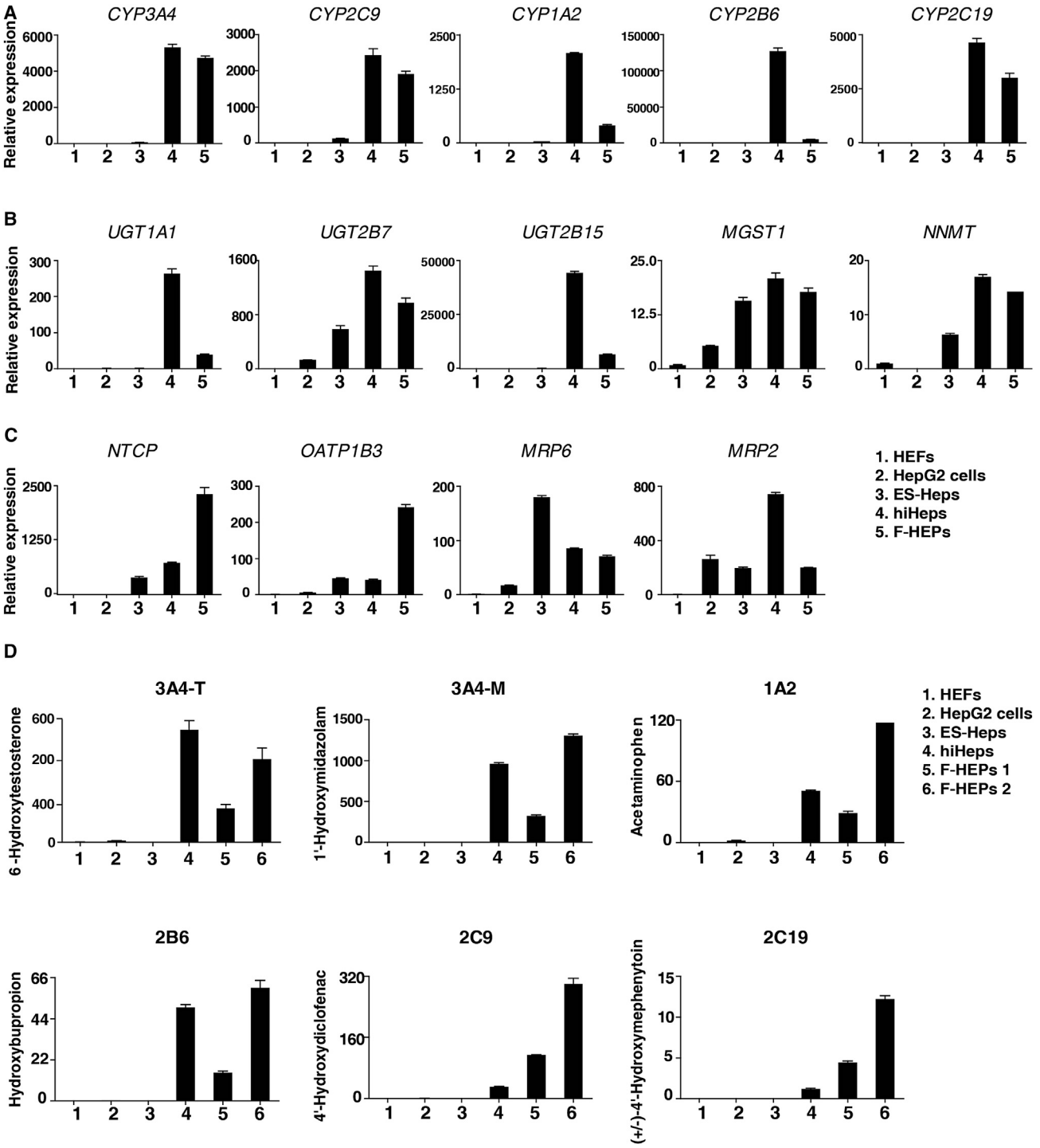
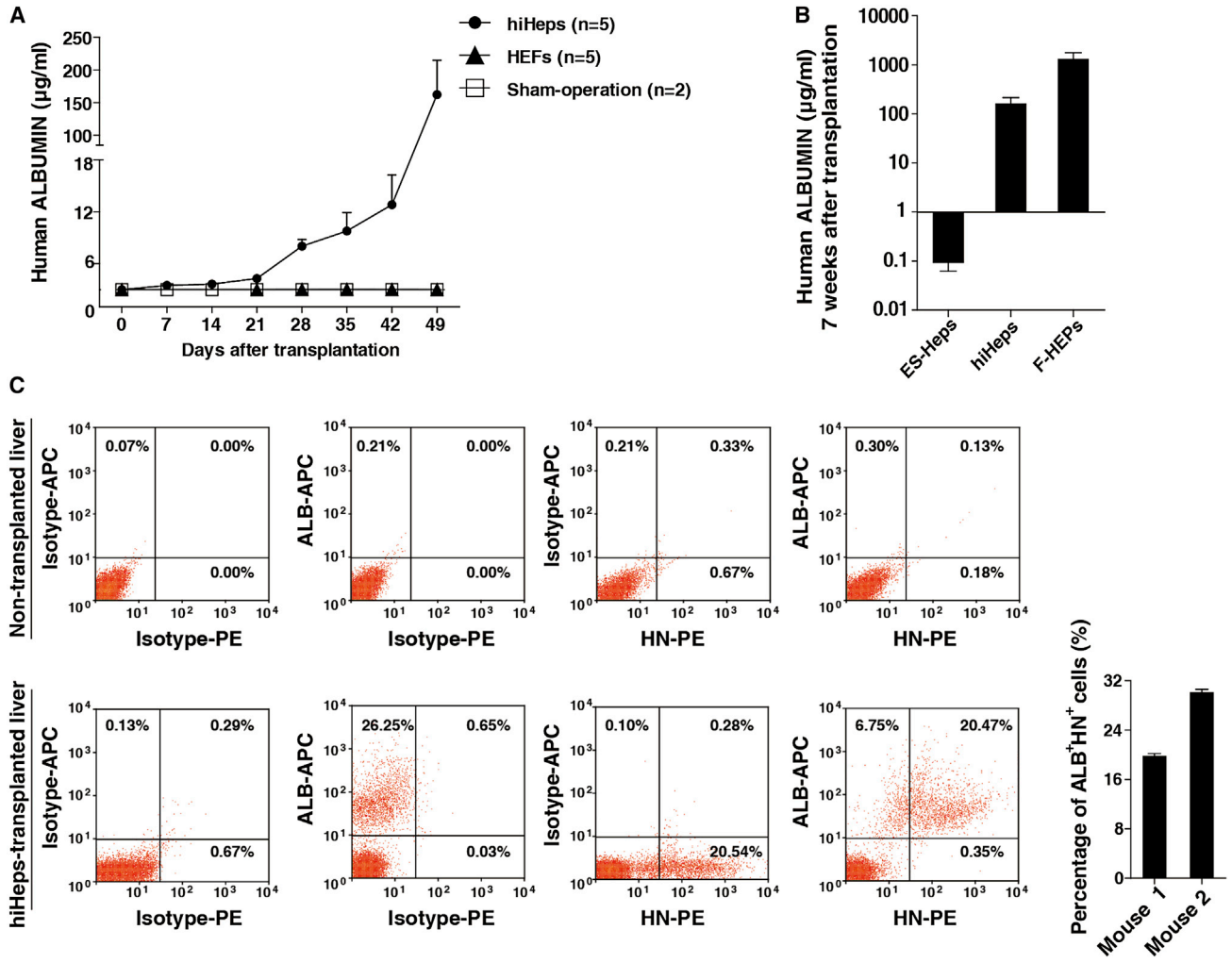


Figure 3. Expression of Drug Metabolic-Associated Genes and Drug Metabolic Activities of hiHeps

(A–C) Quantitative analysis of the expression of drug metabolic phase I (A) and phase II enzymes (B) and phase III transporters (C) in HEFs, HepG2 cells, ES-Heps, hiHeps, and F-HEPs. The relative expression of each gene was normalized to HEFs; if not detected, it was normalized to HepG2 cells. n = 2.

(D) Metabolic activities of CYP3A4 (3A4-T, testosterone; 3A4-M, midazolam), CYP1A2 (phenacetin), CYP2B6 (bupropion), CYP2C9 (diclofenac), and CYP2C19 [(S)-mephenytoin] in hiHeps, ES-Heps, F-HEPs1, F-HEPs2, HepG2 cells, and HEFs were determined by HPLC-MS. n = 3. Two batches of freshly isolated primary human hepatocytes (F-HEPs1 and F-HEPs2) were applied as the positive control. The results are presented as pmol/min per million cells.

Data are presented as mean ± SD. See also Figure S3 and Table S4.



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(Figures 1F and 1G), we can obtain more than 10^{11} functional hiHeps starting from 10^4 of fibroblasts (Figure 1I). These results suggested that hiHeps could be used in a practical manner for pharmaceutical development.

Hepatocyte transplantation is a promising alternative to orthotopic liver transplantation (Dhawan et al., 2010). However, the limited supply of donor organs that can provide good-quality cells remains a major challenge. In our study, hiHeps were able to repopulate mouse liver robustly and secreted up to 313 μg/ml human ALBUMIN, which is two orders of magnitude higher than recent studies using human hepatocytes derived from human embryonic stem cells (Figures 4A and 4B) (Takebe et al., 2013; Woo et al., 2012). Furthermore, transplanted hiHeps expressed major CYP enzymes (Figure 4D), suggesting that hiHeps retained drug metabolic function in vivo. Collectively, hiHeps could be a potential cell source for the establishment of a humanized mouse model and hepatocyte transplantation.

In conclusion, we generated human hepatocytes with drug-metabolizing functions using the combined expression of cell fate determination factors and cell maturation factors. This strategy could potentially facilitate the generation of a fundamental solution for creating various functional cell types. Further optimization of the combination of hepatic maturation factors would probably lead to the fully functional maturation of hiHeps in the future. The generation of functional human hepatocytes with lineage reprogramming provides a way to obtain well-characterized, reproducible, and functional human hepatocytes for pharmaceutical applications.

EXPERIMENTAL PROCEDURES

Generation of hiHeps

This study was approved by the Clinical Research Ethics Committee of the China-Japan Friendship Hospital (ethical approval 2009-50) and Stem Cell Research Oversight of Peking University (SCRO201103-03), and conducted according to the principles of the Declaration of Helsinki. Human fibroblasts were infected overnight and cultured in DMEM plus 10% fetal bovine serum for 1 week before transfer into HCM (Lonza) for expansion. After 3 weeks of culture, HCM was replaced by modified William's E medium (Beijing Vitalstar Biotechnology).

CYP Metabolism Assay

Drug metabolic activity was evaluated using the traditional suspension method as previously described (Gebhardt et al., 2003). hiHeps were cultured in the medium with 50 μM rifampicin, 50 μM β-naphthoflavone, and 1 mM phenobarbital for 72 hr and refreshed every 24 hr. Cell viability of dissociated hiHeps, HepG2 cells, ES-Heps, fibroblasts, and freshly isolated primary human hepatocytes was measured by trypan blue. One milliliter of prewarmed incubation medium (William's E medium, 10 mM HEPES [pH 7.4], 2 mM GlutaMAX) was added per 1×10^6 total cells (cell suspension). The substrate solutions were prepared with the same incubation medium [400 μM testosterone, 10 μM midazolam, 200 μM phenacetin, 1 mM bupropion, 500 μM (S)-mephenytoin, 50 μM diclofenac]. The reactions were started by mixing 250 μl of the substrate solution with 250 μl of cell suspension in a 5 ml polystyrene round-bottom tube (BD Falcon). The tubes were put in an orbital shaker in the incubator and the shaker speed was adjusted to 210 rpm. After a 15–240 min incubation at 37°C, the tubes were centrifuged at room temperature to collect the supernatant. The reactions were stopped by addition of sample aliquots to tubes containing triple the volume of quenching solvent (methanol) and frozen at −80°C. Isotope-labeled reference metabolites were used as internal standards. Internal reference metabolites for testosterone, midazolam, (S)-mephenytoin, diclofenac, bupropion, and phenacetin are 6β-hydroxytestosterone-[D7], hydroxymidazolam-[13C3], 4′-hydroxymephenytoin-[D3], 4′-hydroxydiclofenac-[13C6], hydroxybupropion-[D6], and acetomidophenol-[13C2, 15N], respectively. The metabolites were used to make standard curves for the metabolite analyses. Standard metabolites were 6β-hydroxytestosterone, 1′-hydroxymidazolam, hydroxybupropion, 4′-hydroxydiclofenac, (+/−)-4′-hydroxymephenytoin, and acetaminophen. The metabolites were quantified by Pharmaron using validated traditional LC-MS methods. The results are expressed as picomoles of metabolite formed per minute and per million cells. Chemicals were purchased from Sigma including β-naphthoflavone, rifampicin, testosterone, midazolam, diclofenac, and phenacetin. Standard metabolites and internal reference metabolites were purchased from BD Biosciences. Phenobarbital was a kind gift from Jinning Lou.

Animals and Transplantation

Tet-uPA/Rag2$^{-/-}$/γc$^{-/-}$ mice on a BALB/c background were purchased from Beijing Vitalstar Biotechnology. hiHeps, ES-Heps, and primary human hepatocytes (2×10^6 cells/animal) were injected into the spleens of the mice. Blood samples were collected and human ALBUMIN was quantified using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories). Livers of recipient mice were embedded in OCT compound (Sakura) and then frozen in liquid nitrogen. Cryostat sections (10 μm) were stained.

Statistical Analysis

For statistical analysis, a two-tailed unpaired t test was used. Results are expressed as mean ± SD. p values are as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

ACCESSION NUMBERS

RNA-sequencing data have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE54066.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.01.008.

AUTHOR CONTRIBUTIONS

Y.D., J.W., J.J., N.S., and C.X. conducted most of the experiments. S.L., Y. Shi, and H.D. conceived and supervised the study. J.X., Z.H., X.S., B.L., T.J., Q.G., M.Y., and D.S. contributed to some of the experiments. D.Z., Y. Sun, and J.S. contributed to data analysis.

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Figure 4. Repopulation of Tet-uPA/Rag2$^{-/-}$/γc$^{-/-}$ Mouse Liver with hiHeps

(A) Human ALBUMIN level in mouse serum was monitored by ELISA.

(B) Comparison of human ALB secretion in mouse serum among ES-Heps (n = 16), hiHeps (n = 5), and F-HEPs (n = 6).

(C) Flow cytometry analysis of the engraftment efficiencies of hiHeps. Mouse 1 and mouse 2 secreted human ALB at 267 and 313 μg/ml, respectively. HN, human nuclei; PE, phycoerythrin.

(D) Expression of human ALBUMIN and CYPs in engrafted hiHeps revealed by immunofluorescence. The ALBUMIN antibody is human specific and the CYPs antibody reacts with both human and mouse.

The scale bar represents 100 μm. Data are presented as mean ± SD.

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