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LETTER TO THE EDITOR

Promotion of the efficient metabolic maturation of human pluripotent stem cell-derived hepatocytes by correcting specification defects

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Dear Editor,

Promoting the functional maturation of the desired cell types derived from human pluripotent stem cells (hPSCs) remains a major challenge, especially for hepatocytes, as routine access to metabolically functional hepatocytes would enable their use in drug toxicity screening. Although previous attempts to induce hepatic specification from hPSCs yielded cells possessing some hepatic features [1-3], most of these cells showed poor metabolic activities, and the responsible mechanisms are unknown. In this study, we explored the intrinsic defects in hPSC-derived immature hepatocytes and tested whether correcting these defects would promote the metabolic maturation of the differentiated cells.

One possible explanation for the inability to derive mature hepatic cells is incomplete specification of the differentiated cells [4] caused by the lack of some key transcription factors. To test this hypothesis, immunofluorescence staining was carried out to assess the co-expression of a panel of crucial transcription factors, including FOXA2, GATA4, HNF4A, GATA6, PROX1, HNF6 and TBX3, along with the mature hepatocyte markers ALB and CYP3A4 in hepatocyte-like cells differentiated from human embryonic stem cells (hESCs), according to a previously published protocol [5]. Although ALB was efficiently expressed and co-localized with HNF4A in hepatocyte-like cells differentiated from hESCs, CYP3A4 was rarely observed in the differentiated cultures (Supplementary information, Data S1 and Figure S1A-S1B). Surprisingly, the limited expression of CYP3A4 correlated well with the expression of PROX1 and HNF6, which also were rarely observed in the cultures. When cells at the hepatoblast stage were characterized, similar defects were already present (Supplementary information, Figure S1C-S1E). Although FOXA2, GATA4, HNF4A and GATA6 were expressed ubiquitously (> 95%) in hESC-derived AFP-positive cells, PROX1 and HNF6 were barely detectable (< 0.1%). Interestingly, in mouse embryos, a deficiency in PROX1 or HNF6 in hepatoblasts does not disturb the emergence of Alb^+ cells from the endoderm [6, 7]. Therefore, these results suggest that two populations can be distinguished during hepatic differentiation from hESCs based on the expression of PROX1 and HNF6. The cells lacking PROX1 and HNF6 expression at an early hepatoblast stage, although retaining ALB expression, are incompletely programmed and fail to complete late metabolic maturation.

Given the importance of PROX1 and HNF6 in liver organogenesis together with the fact that these proteins were co-expressed with CYP3A4, we tested whether the robust induction of PROX1 and HNF6 expression during hepatic differentiation would promote efficient hepatic metabolic maturation. After the induction of hESCs through definitive endoderm cells [8] to ventral foregut cells (Figure 1A and Supplementary information, Figures S2 and S3), the cells are recruited to the hepatic lineage. Because both BMP and FGF signals were included in our previous protocol [5], we postulated that an additional signal is required for hepatic PROX1 and HNF6 expression. Among the various conditions that we tested, we found that replating dissociated ventral foregut cells at a relatively low density (2×10^5/ml) followed by treatment with induction factors (FGF7, BMP2 and BMP4) for 5 days greatly promoted the emergence of PROX1- and HNF6-expressing hepatoblast-like colonies; these colonies were also positive for AFP, HNF4A and TBX3 (Supplementary information, Figure S4). Intracellular flow cytometric analysis revealed an overall 63.7% efficiency for the correct induction of hepatoblasts, as characterized by the co-expression of HNF4A and PROX1 (Figure 1D). Quantitative PCR analysis confirmed that PROX1 and HNF6 expression could be efficiently induced only when both the induction factors were present and the replating procedure was performed (Figure 1B). In contrast, the expression of AFP, HNF4A and ALB showed no such change under the replating treatment (Supplementary information, Figure S4C). After expo-

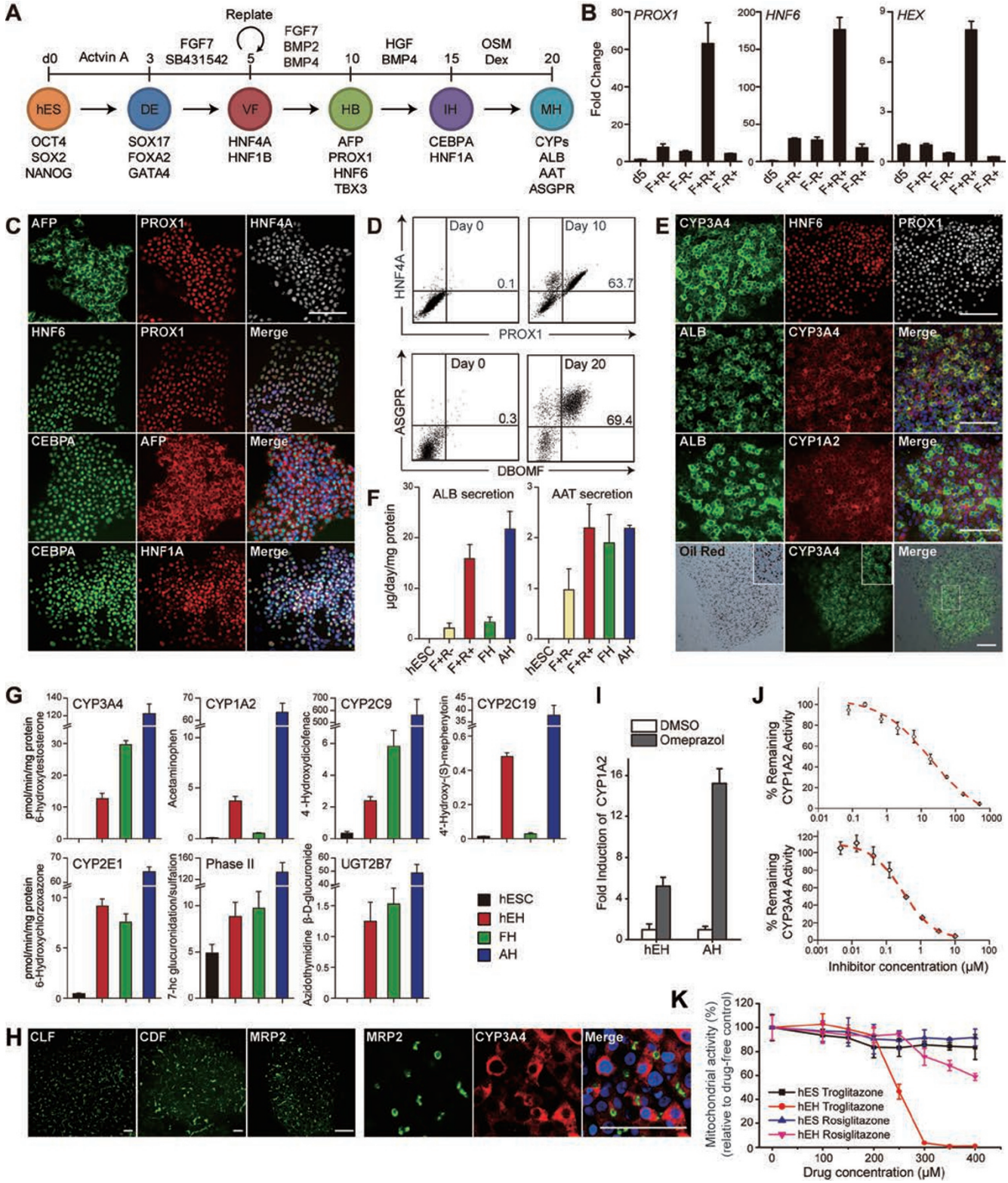

Figure 1 (A) A schematic representation of the five-stage differentiation protocol from hESCs to mature hepatocytes. ES, hESCs; DE, definitive endoderm; VF, ventral foregut; HB, hepatoblasts; IH, immature hepatocytes; MH, mature hepatocytes. **(B)** Quantitative PCR analysis of the mRNA expression of *PROX1*, *HNF6* and *HEX* in day-10 differentiated cells that were generated by treating replated (R+) or unmanipulated (R−) ventral foregut cells with (F+) or without (F−) growth factors for 5 days. *n* = 3. **(C)** Immunofluorescence analysis of immature hepatocytes. The majority of the cells in the colonies maintain

sure to HGF and BMP4 to promote further hepatic commitment, the hepatoblast-like colonies expanded extensively and started expressing HNF1A and CEBPA, which are expressed at later stages of liver development, while retaining the expression of HNF4A, PROX1 and HNF6 (Figure 1C). Interestingly, the hESC-derived hepatoblasts showed differentiation plasticity (Supplementary information, Figure S5) similar to their *in vivo* counterparts.

To determine whether the hepatoblasts would efficiently mature into hepatocytes that express metabolic enzymes now that the specification defects had been corrected, cultures were treated using the further maturation conditions that have been previously reported [5]. At the end of the differentiation period, CYP3A4 was expressed in extensive regions of the hepatic clusters but was confined to PROX1- and HNF6-expressing cells (Figure 1E and Supplementary information, Figures S7A and S10), consistent with our initial findings. Based on flow cytometric analysis, nearly 70% of the cells were double-positive for the hepatic surface marker ASGPR and the fluorescent metabolite of the CYP3A4-specific substrate DBOMF (Figure 1D), which was verified by immunofluorescence in the absence or presence of the CYP3A4-specific inhibitor ketoconazole (Supplementary information, Figure S7D). In addition, the hESC-derived hepatocytes (hEHs) co-expressed the hepatic functional gene ALB along with CYP3A4, CYP1A2, CYP reductase and AAT (Figure 1E and Supplementary information, Figure S7A-S7B). Moreover, these cells co-expressed the transcription factors PROX1, HNF6, HNF1A, CEBPA, HNF4A, FOXA2, HNF1B and CEBPB, all of which are essential for liver morphogenesis [9], whereas TBX3 and GATA4 were downregulated (Supplementary information, Figures S2 and S6), as occurs *in vivo* during the advancement of hepatocyte differentiation. For further functional validation, hEHs were assessed using a panel of functional assays. The secretion of albumin and α1-antitrypsin increased greatly to levels comparable to those of primary human hepatocytes (Figure 1F) and much higher than those in previous reports [2, 5]. Moreover, Oil red O staining demonstrated the presence of lipid droplets in the CYP3A4-expressing hEHs (Figure 1E); periodic acid-Schiff staining showed glycogen synthesis; and incubation with indocyanine green demonstrated the uptake ability of the hEHs. The capacity to engraft into the liver parenchyma *in vivo* was also verified by immunostaining and by the secretion of human albumin into the plasma (Supplementary information, Figure S7E-S7G). When this newly developed protocol (Figure 1A) was applied to the induced pluripotent stem cells (3U1 line) [1], similar results were obtained (Supplementary information, Figure S7C).

To confirm the requirement for PROX1 and HNF6 in regulating the formation of CYP-expressing hepatocytes from hESCs, we attempted to knockdown the expression of PROX1 and HNF6 by lentivirus-mediated shRNA at the hepatoblast stage of the newly developed protocol (Supplementary information, Figure S8). Compared with the control shRNA-treated cells, the PROX1 or HNF6 shRNA-treated cells failed to differentiate into CYP3A4-expressing cells, as indicated by a marked decrease in immunofluorescence-positive cells and by a great reduction in albumin secretion and CYP3A4 metabolic activity. Quantitative PCR analysis showed that the knock-

expression of AFP, HNF4A, and PROX1, as well as HNF6. The onset of CEBPA and HNF1A expression in the hepatoblast-like colonies occurs during the transition to immature hepatocytes. Scale bar, 100 μm. **(D)** Representative flow cytometric analysis shows hepatic differentiation efficiency at day 10 (upper) and day 20 (lower). hESCs (day 0) were used as a control. The cultures on day 10 comprise 63.7% $HNF4A^+PROX1^+$ cells and few (6.9%) $HNF4A^+PROX1^-$ cells are observed. At day 20, 69.4% of the cells expressed ASGPR and possessed CYP activity, as quantified by DBOMF metabolism. **(E)** Characterization of the hEHs. Immunofluorescence analysis showed the co-expression of CYP3A4, HNF6 and PROX1 (first row), ALB and CYP3A4 (second row), and ALB and CYP1A2 (third row). The regions of hEH clusters labeled by Oil red O (fourth row), indicating lipid stores, were well matched with clusters immunostaining positive for CYP3A4. Scale bar, 100 μm. **(F)** The secretion of albumin and α1-antitrypsin, as measured by ELISA. The analysis involved hESCs, hepatocytes differentiated from hESC-derived ventral foregut cells treated with growth factors and with or without replating manipulation (F+R+ or F+R−), 25-week-old fetal hepatocytes (FH), and human adult primary hepatocytes (AH). $n = 8$. **(G)** Activity of phase I (CYP450) and phase II (conjugation) enzymes, as measured via conventional probe substrates. **(H)** Phase III transporter activity in hEHs. CLF and CDF, when incubated with cultures, are transported into the canalicular spaces of these cells (left). MRP2 shows a similar pattern and localizes to the canalicular membrane domain of the cells. The right panel shows the double staining of MRP2 and CYP3A4 at a higher magnification. Scale bar, 50 μm. **(I)** Induction of CYP1A2 activity in the hEHs via omeprazole (50 μM). All data were normalized to vehicle-only controls (fold induction of 1). AH, human adult primary hepatocytes. $n = 3$. **(J)** Effects of model inhibitors on individual P450 activity. The hEHs were incubated with substrates in the presence of different concentrations of furafylline (for CYP1A2, upper) or ketoconazole (for CYP3A4, lower). $n = 3$. **(K)** The utility of hEHs for screening for hepatotoxicity. After incubation with troglitazone or rosiglitazone for 24 h, mitochondrial toxicity was evaluated using the MTT assay. All data were normalized to mitochondrial activity in vehicle-treated cultures (100% activity).

down of either PROX1 or HNF6 impaired the expression of HNF1A in addition to ALB and CYP3A4, and had little or no effect on the expression of FOXA2 and HNF4A, suggesting that the regulation of hepatic differentiation by PROX1/HNF6 may not rely on HNF4A/FOXA2.

To evaluate the suitability of the newly generated hEHs for drug metabolism studies, we systematically characterized the phase I CYP and phase II conjugation activities using the only determination method approved by the FDA (standard chemical substrates by LC/MS/MS analysis). We found that the top five major CYP enzymes, which are responsible for ~85% of known oxidative drug metabolism, showed activity in the hEHs, and the most important phase II glucuronidation enzymes were also active (Figure 1G). Among these enzymes, the expression of CYP1A2 and 2C19 can only be detected well after birth in the human liver [10]. The activities of CYP3A4, 2C9, 2E1 and UGT in the hEHs were comparable to or higher than the activities in 25-week-old fetal hepatocytes. These data suggest that hEHs are differentiated to a state that possesses hepatic metabolic activity that is most likely to be similar to that of hepatocytes at the perinatal stage. Moreover, we observed the phase III transport of carboxy-dichloro-fluorescein diacetate (CDF) and cholyl-lysyl-fluorescein (CLF) into bile canaliculi between the hEHs. Immunostaining also showed that MRP2, which mediates CDF efflux, was co-expressed with CYP3A4 and had a similar pattern of localization in the canalicular space (Figure 1H).

To further determine the utility of hEHs in drug development, we assessed the potency of CYP activity modulation in mediating drug interactions and the ability of the hEHs to predict drug toxicity. When hEHs were incubated with the AhR activator omeprazole, the metabolic activity of CYP1A2 increased by over 5-fold, indicating that hEHs are susceptible to drug induction (Figure 1I). After treatment with ketoconazole and furafylline, model inhibitors of CYP3A4 and CYP1A2, respectively, the hEHs exhibited a dose-dependent reduction in metabolite production (Figure 1J). To evaluate drug-induced hepatotoxicity in the hEHs, we assessed the toxicity of troglitazone, which was withdrawn by the FDA due to hepatotoxicity, and its structural analog rosiglitazone (Supplementary information, Figure S7H). After the hEHs were exposed to the drugs for 24 h, the reduction in the mitochondrial activity of the treated hEHs was quantified to measure the hepatotoxicity of these compounds (Figure 1K). Troglitazone exhibited dose-dependent toxicity, with a TC_{50} value of approximately 0.25 mM. In contrast, rosiglitazone exhibited far less toxicity in the hEHs, and both compounds had little toxic effect on hESCs.

In this study, we efficiently generated a relatively homogenous population of hepatocytes from hPSCs. These hepatocytes expressed the metabolic enzymes responsible for ~85% of known oxidative drug metabolism activities. Utilizing model compounds and common pharmacological assays, we demonstrated that these hEHs can be successfully used to measure metabolic activities, to conduct preliminary screening for the inhibitory effects of new drugs, and to evaluate drug-induced cytotoxicity. In addition, we discovered that the acquisition of metabolic activity by hEHs depends on the activation of PROX1 and HNF6, which are regulated in parallel to HNF4A, FOXA2 and other factors (Supplementary information, Figure S9). Taken together, our findings provide an important insight into the transcriptional regulation of hepatic specification and maturation and represent a critical step toward the generation of hepatocytes for use in drug discovery.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)