

Fig. 1. Experimental protocol and schematic illustration of the procedure to stratify Swiss 3T3 cells on hepatocyte-like cells. (A) The procedure for hepatic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) using stratification of the Swiss 3T3 cell sheet. Details of the hepatic differentiation procedure are described in the Materials and methods section. (B) The stratifying protocol was performed by using gelatin-coated manipulator. Details of the stratifying procedure are described in the Materials and methods section. (C) Phase-contrast micrographs of the vertical sections with monolayer hESC (H9)-derived hepatocyte-like cells (hEHs-mono) or hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss) on day 15. Scale bars represent 25 μ m.

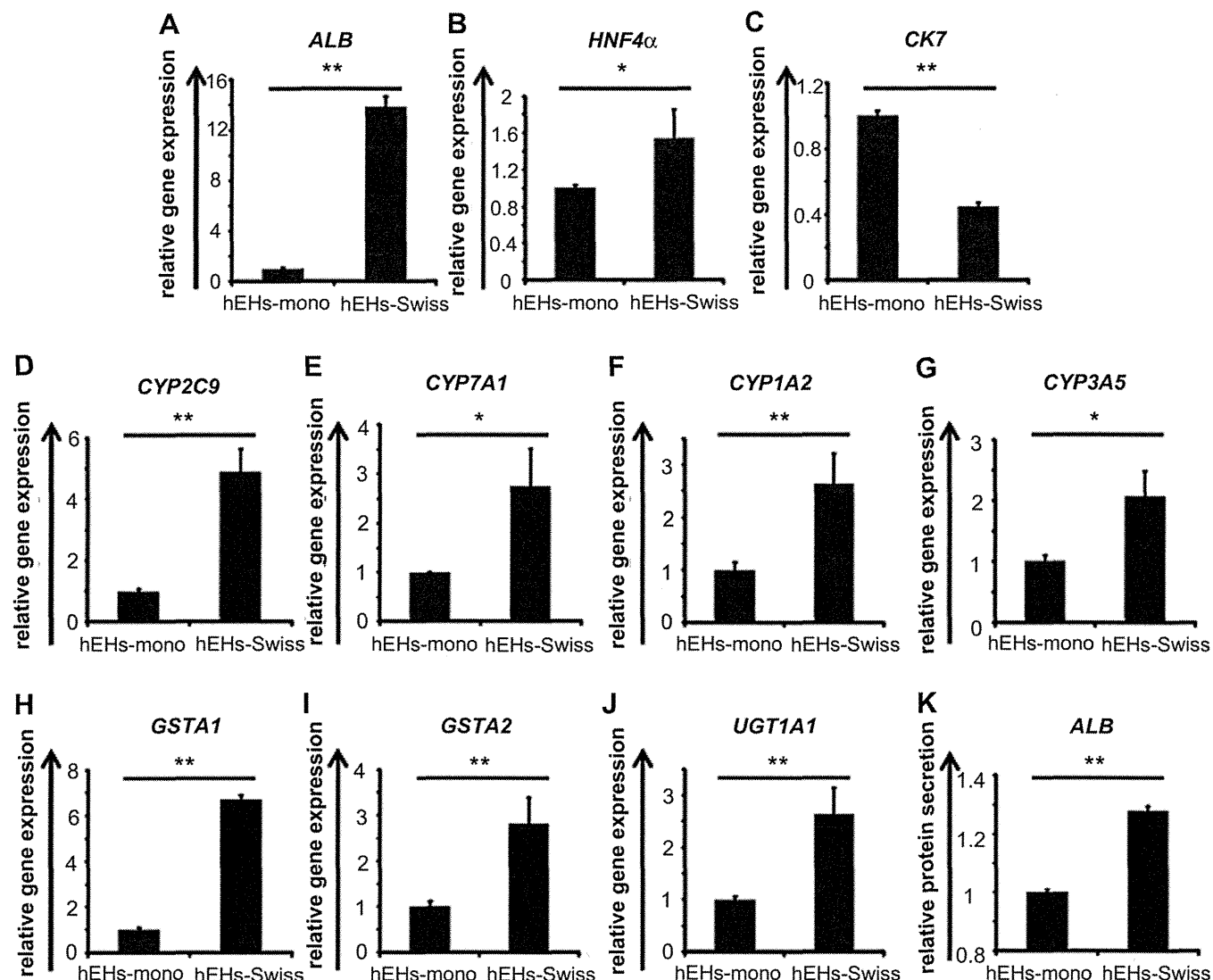


Fig. 2. Stratification of Swiss 3T3 cell sheet on hEHs promotes hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–K): On day 25, the gene expression levels of *ALB* (A), *HNF4 α* (B), *CK7* (C), *CYP2C9* (D), *CYP7A1* (E), *CYP1A2* (F), *CYP3A5* (G), *GSTA1* (H), *GSTA2* (I), and *UGT1A1* (J) were examined in monolayer hESC-derived hepatocyte-like cells (hEHs-mono) and hESC-derived hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss) by real-time RT-PCR. The values were graphed as the fold-changes relative to hEHs-mono. (K) On day 25, the amounts of ALB secretion were examined in hEHs-mono or hEHs-Swiss by ELISA. The values were graphed as the fold-changes relative to hEHs-mono. All data are represented as means \pm Standard Deviation (SD) ($n = 3$). * $P < 0.05$ ** $P < 0.01$.

those in the co-cultured hEHs (Fig. 4A and B). Furthermore, a significant elevation of *CYP1A2* and *CYP3A5* gene expression was observed only in the co-cultured hEHs (Fig. 4C and D). Therefore, these data indicate that physical contacts between hEHs and Swiss 3T3 cells play an important role in hepatic maturation of the hEHs, although Swiss 3T3 cell-derived soluble factors also played a small role in the hepatic maturation.

Because ECMs are important factors in hepatic differentiation [36], we examined the effect of Swiss 3T3 cell-derived ECMs on hepatic maturation of the hEHs. Swiss 3T3 cells abundantly synthesize collagen and almost all of the synthesized collagen is type I collagen [37]. To mimic 3D co-culture with Swiss 3T3 cell sheet, type I collagen gel was stratified onto the hEHs. As a control, Matrigel, which contains abundant type IV collagen but not type I collagen, was stratified onto the hEHs. As with the case of the Swiss 3T3 cell sheet stratification, the hEHs-mono stratified with type I collagen gel showed an elevation of hepatocyte-related marker, but a reduction of cholangiocyte marker (Fig. 5A and B, hEHs-mono). In addition, stratification of type I collagen augmented the hepatic maturation of the Swiss 3T3 cell sheet-stratified hEHs (Fig. 5A and

B, hEHs-Swiss). We further examined the role of Swiss 3T3 cell-derived type I collagen on hepatic maturation using 2,2'-Bipyridyl, an inhibitor of collagen synthesis. The collagen synthesis in Swiss 3T3 cells could be efficiently inhibited by treatment with 2,2'-Bipyridyl, as determined by Western blotting analysis (Fig. 5C). Quantitative RT-PCR analysis revealed that the gene expression level of *ALB* was significantly down-regulated, but that of *CK7* was up-regulated in the hEHs-Swiss cultured in the presence of 2,2'-Bipyridyl (Fig. 5D and E). Taken together, our findings indicated that type I collagen, which was synthesized from Swiss 3T3 cells, was indispensable for the maturation of the hEHs by Swiss 3T3 cell sheet.

4. Discussion

Our main purpose in the current study was to develop a more efficient method for hepatic maturation of the hEHs and hiPHs, because such a method will be needed to generate more mature hepatocyte-like cells, which have potent activity to metabolize drugs, for wide-spread use of drug screening. Therefore, we

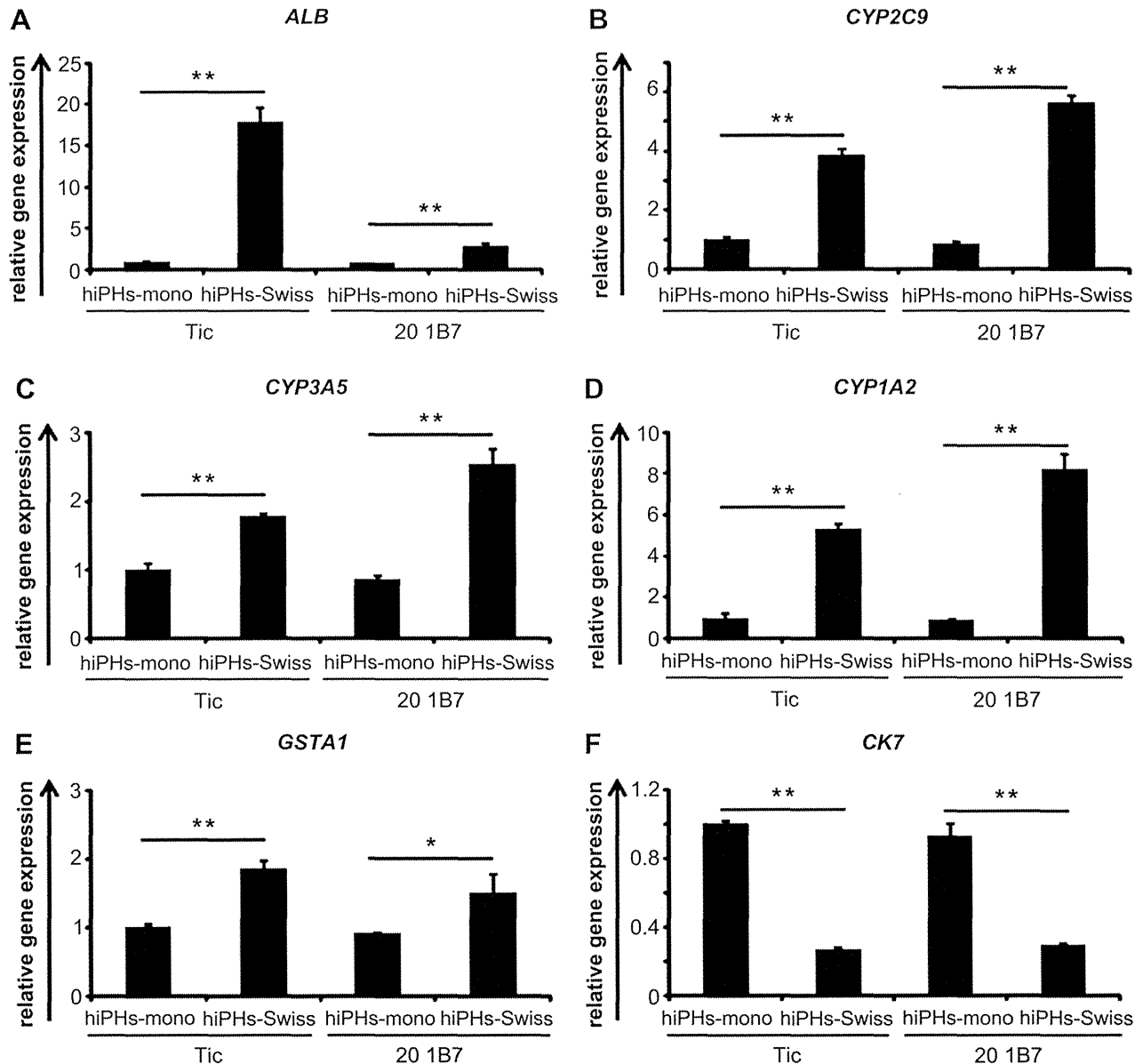


Fig. 3. Stratification of Swiss 3T3 cell sheet on hiPHs promotes hepatic maturation. Human induced pluripotent stem cells (hiPSCs) (Tic and 201B7) were differentiated into hepatocyte-like cells as described in Fig. 1A. (A–F): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), *CYP3A5* (C), *CYP1A2* (D), *GSTA1* (E), and *CK7* (F) were examined in monolayer hiPSC-derived hepatocyte-like cells (hiPHs-mono) and hiPSC-derived hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hiPHs-Swiss) by real-time RT-PCR. The values were graphed as the fold-changes relative to hiPHs-mono differentiated from Tic. All data are represented as means \pm SD ($n = 3$). * $P < 0.05$ ** $P < 0.01$.

attempted to employ a cell sheet engineering technology to further induce maturation of the hEHs and hiPHs.

We observed a significant increase in the expression of hepatocyte-related genes in the hEHs- and hiPHs-Swiss as compared with those in the hEHs- and hiPHs-mono, respectively (Figs. 2 and 3), indicating that 3D co-culture with the Swiss 3T3 cell sheet was effective to promote hepatic maturation of the hEHs and hiPHs. On the other hand, Han et al. have recently shown that hESC-derived DE cells cannot be promoted to differentiate into hepatoblasts by co-culture of mouse fibroblast 3T3 cells [38]. Considering that primary rat hepatocytes are also able to grow and retain their functions for a long period of time in the presence of Swiss 3T3 cells [19,20], Swiss 3T3 cells would probably have the capacity to support the functions of freshly isolated mature hepatocytes and hESC- or hiPSC-derived hepatocyte-like cells, but not DE cells. Besides Swiss 3T3 cells, we attempted to mature the hEHs using

3D co-culture with the bovine carotid artery endothelial cell sheet, because Kim et al. recently succeeded in creating a functional hepatocyte culture system by stacking bovine carotid artery endothelial cell sheets on primary rat hepatocytes [25]. However, our preliminary data showed that Swiss 3T3 cell sheets were superior to the bovine carotid artery endothelial cell sheets in terms of hepatic maturation of hEHs (data not shown). Thus, we conducted the present experiments to facilitate hepatic differentiation of human pluripotent stem cells using Swiss 3T3 cell sheets.

Interestingly, we found a difference in hepatic differentiation efficiency among hiPSC lines (Fig. 3). This might have been due to epigenetic memory of the hiPSC line, because several studies showed that the epigenetic memory of iPSCs affected the differentiation capacity [39,40]. Kleger et al. showed that iPSCs generated from mouse liver progenitor cells, could be more effectively differentiated into hepatocyte-like cells in comparison with iPSCs

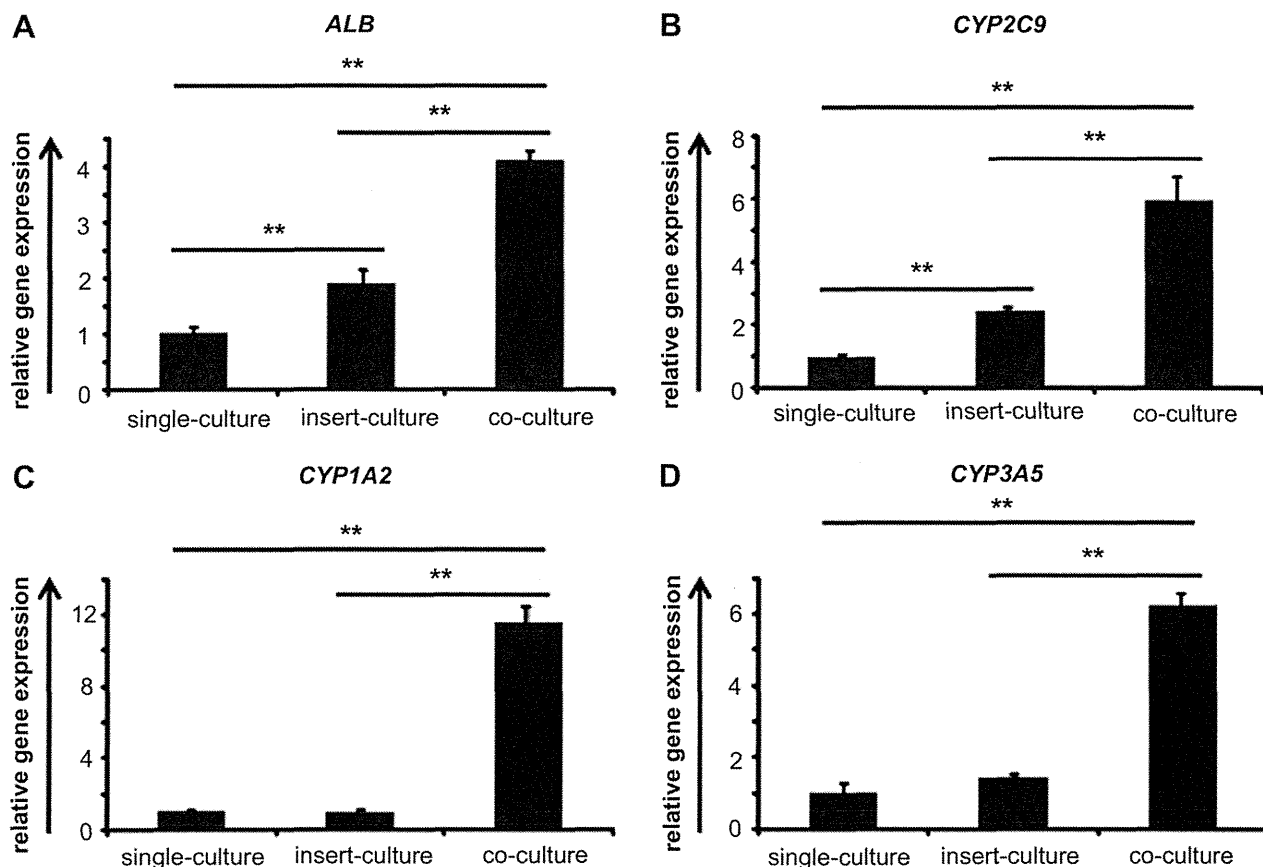


Fig. 4. Physical contacts between hESC-derived hepatocyte-like cells and Swiss 3T3 cells promote hepatic maturation. hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then the cells were differentiated into hepatocyte-like cells by single-culture, insert-culture, or co-culture with Swiss 3T3 cells. (A–D): On day 25, the gene expression levels of *ALB* (A), *CYP2C9* (B), *CYP1A2* (C) and *CYP3A5* (D) were examined in hESC-derived hepatocyte-like cells (hEHs) differentiated by single-culture, insert-culture, or co-culture with Swiss 3T3 cells by real-time RT-PCR. The values were graphed as the fold-changes relative to hEHs by single-culture. All data are represented as means \pm SD ($n = 3$). $^{**}P < 0.01$.

generated from mouse embryo fibroblasts [41]. Thus, to more efficiently differentiate into hepatocyte-like cells from hiPSCs, it might be valuable to employ hiPSCs generated from freshly isolated human hepatocytes. Moreover, by using our 3D co-culture system, such hiPSCs would be differentiated into more mature hepatocyte-like cells.

We investigated the Swiss 3T3 cell-derived hepatic maturation factors by using cell culture inserts, and found that the physical contacts between Swiss 3T3 cells and the hEHs were the major factors contributing to the hepatic maturation of hEHs (Fig. 4). Because Swiss 3T3 cell-derived soluble factors partially induce maturation of hEHs (Fig. 4A and B), it would also be interesting to search for hepatic maturation factors secreted from Swiss 3T3 cells.

To further investigate the maturation factors, we examined whether type I collagen, which is abundantly synthesized by Swiss 3T3 cells, could promote hepatic maturation. Stratification of type I collagen gel could lead to a promotion of hepatic maturation of hEHs-mono as well as hEHs-Swiss (Fig. 5A). We also found that hepatic maturation by 3D co-culture with the Swiss 3T3 cell sheet was suppressed by inhibition of collagen synthesis (Fig. 5D). Taken together, these results show that type I collagen is one of the key molecules in promotion of hepatic maturation by stratification of Swiss 3T3 cells. It is known that the space of Disse, which faces hepatocytes directly, contains various kinds of ECM proteins, including type I collagen [42]. Because the conditions in 3D co-culture, which contains type I collagen synthesized from Swiss 3T3 cells, can mimic the *in vivo* liver microstructure, including the space of Disse, the hepatic maturation from hEHs and hiPHs might

be efficiently promoted. Furthermore, it was also reported that, by the stratification of type I collagen gel in primary rat hepatocyte culture, the cytoskeletal organizations, such as actin localization, in primary rat hepatocytes were changed and stress fibers were obliterated just as in the *in vivo* state [43]. They also showed that the stratification of type I collagen gel in primary rat hepatocyte culture maintained ALB secretion in primary rat hepatocyte. Thus, the alteration of the cytoskeletal organization might also be changed in the hEHs and hiPHs by 3D co-culture with the Swiss 3T3 cell sheet. For these reasons, it could be speculated that stratification of Swiss 3T3 cell sheets positively affects the maturation process of hEHs and hiPHs mediated by cell-to-cell and cell-type I collagen–cell interactions. The expression level of the *CK7* gene in the hEHs was down-regulated by stratification of the Swiss 3T3 cell sheet or type I collagen gel (Figs. 2C and 5B). Although Matrigel, which contains large amount of type IV collagen, is widely used to differentiate hESCs and hiPSCs into hepatocyte-like cells, it is reported that type IV collagen promotes cholangiocyte differentiation [44]. Therefore, it would be important to note that stratification of Swiss 3T3 cell sheet inhibits the cholangiocyte differentiation and thereby allows the cells to drive the way to hepatic differentiation. Although we showed that a Swiss 3T3 cell-derived type I collagen plays an important role in hepatic maturation, it was likely that the other soluble factors would also be involved in the promotion of hepatic maturation.

We employed Swiss 3T3 cells for 3D co-culture with the hEHs and hiPHs. However, it would be an attractive study to employ other kinds of cells such as liver sinusoidal endothelial cells, stellate

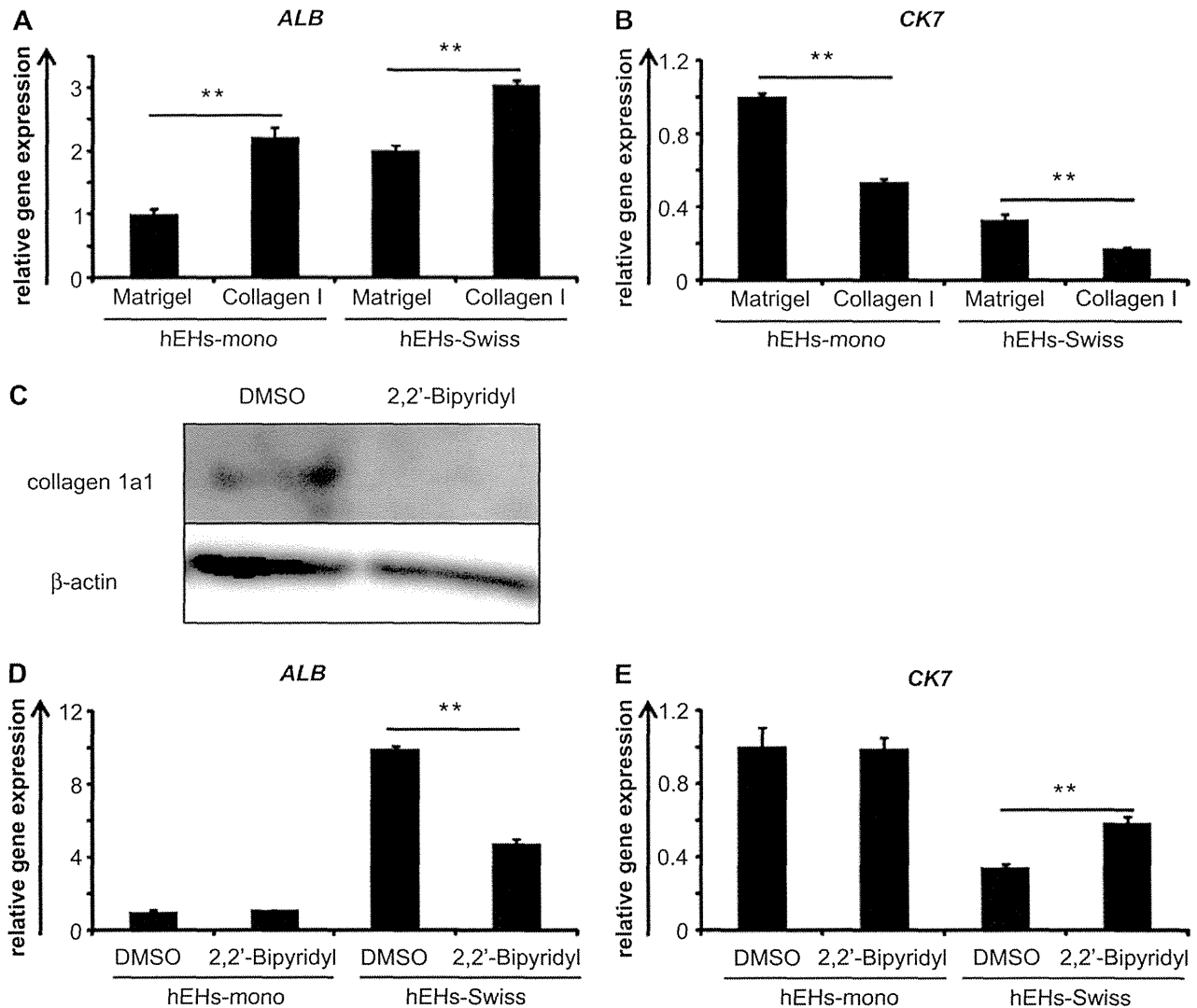


Fig. 5. Stratification of type I collagen gel promotes hepatic maturation. (A and B) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A until day 14, and then type I collagen gel (collagen I) or Matrigel are stratified on monolayer hESC-derived hepatocyte-like cells (hEHs-mono) and hESC-derived hepatocyte-like cells stratified with Swiss 3T3 cell sheet (hEHs-Swiss). On day 25, the gene expression levels of ALB (A) and CK7 (B) were examined in hEHs-mono and hEHs-Swiss cultured with Matrigel or type I collagen gel by real-time RT-PCR. (C) Swiss 3T3 cells were cultured with 2,2'-Bipyridyl or solvent (0.1% DMSO) for 3 days, and then the expression of type I collagen precursor, col1a1, in these cells were detected by Western blot analysis. (D and E) hESCs (H9) were differentiated into hepatocyte-like cells as described in Fig. 1A. After stratification of Swiss 3T3 cells on day 14, these cells were treated with 2,2'-Bipyridyl or solvent (0.1% DMSO). On day 25, the gene expression levels of ALB (D) and CK7 (E) were examined in hEHs-mono and hEHs-Swiss treated with 2,2'-Bipyridyl or solvent (0.1% DMSO) by real-time RT-PCR. The values were graphed as the fold-changes relative to hEHs-mono cultured with Matrigel. All data are represented as means \pm SD ($n = 3$). $^{**}P < 0.01$.

cells, and Kupffer cells, to mimic the *in vivo* liver microstructure. By mimicking the *in vivo* liver microstructure, basic molecular mechanisms, including cell–cell interactions, in liver development would be clarified. Moreover, because our cell sheet technology allows us to stratify the multiple cell sheets and create layered 3D tissue constructs, combinations with multiple layers consisting of various types of cells might be able to develop an efficient method for hepatic maturation of the hEHs and hiPHs. In addition, by using new biomaterials with cell patterning techniques, more mature hepatocyte-like cells would be probably generated from human pluripotent stem cells, and thereby accelerate the research into tissue generation.

5. Conclusions

We succeeded in promoting the hepatic maturation of both the hEHs and hiPHs by stratification of the Swiss 3T3 cell sheet using

a cell sheet engineering technology. We also determined that type I collagen, which is synthesized in Swiss 3T3 cells, plays an important role in hepatic maturation. Since our cell sheet engineering technology enables us to stratify multiple cell sheets, this technology would have the potential to mimic the *in vivo* liver microstructure and to generate hepatocyte-like cells, which have functions similar to primary hepatocytes. Our methods would be powerful tools for *in vitro* applications, such as drug toxicity screening in the early phase of pharmaceutical development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.03.011.

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Efficient Generation of Functional Hepatocytes From Human Embryonic Stem Cells and Induced Pluripotent Stem Cells by HNF4 α Transduction

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Hepatocyte-like cells from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are expected to be a useful source of cells drug discovery. Although we recently reported that hepatic commitment is promoted by transduction of SOX17 and HEX into human ESC- and iPSC-derived cells, these hepatocyte-like cells were not sufficiently mature for drug screening. To promote hepatic maturation, we utilized transduction of the hepatocyte nuclear factor 4 α (HNF4 α) gene, which is known as a master regulator of liver-specific gene expression. Adenovirus vector-mediated overexpression of HNF4 α in hepatoblasts induced by SOX17 and HEX transduction led to upregulation of epithelial and mature hepatic markers such as cytochrome P450 (CYP) enzymes, and promoted hepatic maturation by activating the mesenchymal-to-epithelial transition (MET). Thus HNF4 α might play an important role in the hepatic differentiation from human ESC-derived hepatoblasts by activating the MET. Furthermore, the hepatocyte like-cells could catalyze the toxication of several compounds. Our method would be a valuable tool for the efficient generation of functional hepatocytes derived from human ESCs and iPSCs, and the hepatocyte-like cells could be used for predicting drug toxicity.

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INTRODUCTION

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most of the body's cell types.^{1,2} They could provide an unlimited source of cells for various applications. Hepatocyte-like cells, which are differentiated from human ESCs and iPSCs,

would be useful for basic research, regenerative medicine, and drug discovery.³ In particular, it is expected that hepatocyte-like cells will be utilized as a tool for cytotoxicity screening in the early phase of pharmaceutical development. To catalyze the toxication of several compounds, hepatocyte-like cells need to be mature enough to exhibit hepatic functions, including high activity levels of the cytochrome P450 (CYP) enzymes. Because the present technology for the generation of hepatocyte-like cells from human ESCs and iPSCs, which is expected to be utilized for drug discovery, is not refined enough for this application, it is necessary to improve the efficiency of hepatic differentiation. Although conventional methods such as growth factor-mediated hepatic differentiation are useful to recapitulate liver development, they lead to only a heterogeneous hepatocyte population.^{4–6} Recently, we showed that transcription factors are transiently transduced to promote hepatic differentiation in addition to the conventional differentiation method which uses only growth factors.⁷ Ectopic expression of Sry-related HMG box 17 (SOX17) or hematopoietically expressed homeobox (HEX) by adenovirus (Ad) vectors in human ESC-derived mesendoderm or definitive endoderm (DE) cells markedly enhances the endoderm differentiation or hepatic commitment, respectively.^{7,8} However, further hepatic maturation is required for drug screening.

The transcription factor hepatocyte nuclear factor 4 α (HNF4 α) is initially expressed in the developing hepatic diverticulum on E8.75,^{9,10} and its expression is elevated as the liver develops. A previous loss-of-function study showed that HNF4 α plays a critical role in liver development; conditional deletion of HNF4 α in fetal hepatocytes results in the faint expression of many mature hepatic enzymes and the impairment of normal liver morphology.¹¹ The genome-scale chromatin immunoprecipitation assay showed that HNF4 α binds to the promoters of nearly half of the genes expressed in the mouse liver,¹² including cell adhesion and junctional proteins,¹³ which are important in

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the hepatocyte epithelial structure.¹⁴ In addition, HNF4 α plays a critical role in hepatic differentiation and in a wide variety of liver functions, including lipid and glucose metabolism.^{15,16} Although HNF4 α could promote transdifferentiation into hepatic lineage from hematopoietic cells,¹⁷ the function of HNF4 α in hepatic differentiation from human ESCs and iPSCs remains unknown. A previous study showed that hepatic differentiation from mouse hepatic progenitor cells is promoted by HNF4 α , although many of the hepatic markers that they examined were target genes of HNF4 α .¹⁸ They transplanted the HNF4 α -overexpressed mouse hepatic progenitor cells to promote hepatic differentiation, but they did not examine the markers that relate to hepatic maturation such as CYP enzymes, conjugating enzymes, and hepatic transporters.

In this study, we examined the role of HNF4 α in hepatic differentiation from human ESCs and iPSCs. The human ESC- and iPSC-derived hepatoblasts, which were efficiently generated by sequential transduction of SOX17 and HEX, were transduced with HNF4 α -expressing Ad vector (Ad-HNF4 α), and then the expression of hepatic markers of the hepatocyte-like cells were assessed. In addition, we examined whether or not the hepatocyte-like cells, which were generated by sequential transduction of SOX17, HEX, and HNF4 α , were able to predict the toxicity of several compounds.

RESULTS

Stage-specific HNF4 α transduction in hepatoblasts selectively promotes hepatic differentiation

The transcription factor HNF4 α plays an important role in both liver generation¹¹ and hepatic differentiation from human ESCs and iPSCs (Supplementary Figure S1). We expected that hepatic differentiation could be accelerated by HNF4 α transduction. To examine the effect of forced expression of HNF4 α in the hepatic differentiation from human ESC- and iPSC-derived cells, we used a fiber-modified Ad vector.¹⁹ Initially, we optimized the time period for Ad-HNF4 α transduction. Human ESC (H9)-derived DE cells (day 6) (Supplementary Figures S2 and S3a), hepatoblasts (day 9) (Supplementary Figures S2 and S3b), or a heterogeneous population consisting of hepatoblasts, hepatocytes, and cholangiocytes (day 12) (Supplementary Figures S2 and S3c) were transduced with Ad-HNF4 α and then the Ad-HNF4 α -transduced cells were cultured until day 20 of differentiation (Figure 1). We ascertained the expression of exogenous HNF4 α in human ESC-derived hepatoblasts (day 9) transduced with Ad-HNF4 α (Supplementary Figure S4). The transduction of Ad-HNF4 α into human ESC-derived hepatoblasts (day 9) led to the highest expression levels of the hepatocyte markers *albumin* (ALB)²⁰ and *α -1-antitrypsin* (Figure 1a). In contrast, the expression levels of the cholangiocyte markers *cytokeratin 7* (CK7)²¹ and *SOX9*²² were

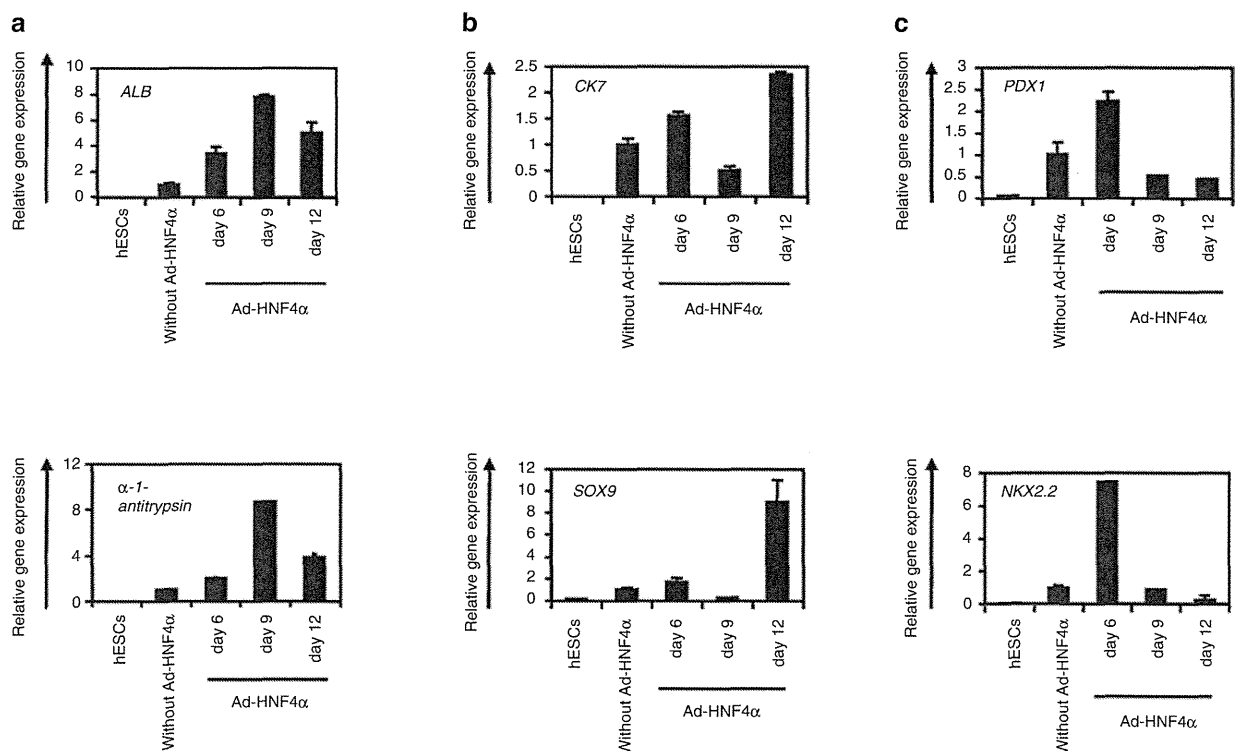


Figure 1 Transduction of HNF4 α into hepatoblasts promotes hepatic differentiation. (a–c) The human ESC (H9)-derived cells, which were cultured for 6, 9, or 12 days according to the protocol described in Figure 2a, were transduced with 3,000 vector particles (VP)/cell of Ad-HNF4 α for 1.5 hours and cultured until day 20. The gene expression levels of (a) hepatocyte markers (ALB and α -1-antitrypsin), (b) cholangiocyte markers (CK7 and SOX9), and (c) pancreas markers (PDX1 and NKX2.2) were examined by real-time RT-PCR on day 0 (human ESCs (hESCs)) or day 20 of differentiation. The horizontal axis represents the days when the cells were transduced with Ad-HNF4 α . On the y-axis, the level of the cells without Ad-HNF4 α transduction on day 20 was taken as 1.0. All data are represented as means \pm SD ($n = 3$). ESC, embryonic stem cell; HNF4 α , hepatocyte nuclear factor 4 α ; RT-PCR, reverse transcription-PCR.

downregulated in the cells transduced on day 9 as compared with nontransduced cells (Figure 1b). This might be because hepatic differentiation was selectively promoted and biliary differentiation was repressed by the transduction of HNF4 α in hepatoblasts. The expression levels of the pancreas markers *PDX1*²³ and *NKX2.2*²⁴ did not make any change in the cells transduced on day 9 as compared with nontransduced cells (Figure 1c). Interestingly, the expression levels of the pancreas markers were upregulated, when Ad-HNF4 α transduction was performed into DE cells (day 6) (Figure 1c). These results suggest that HNF4 α might promote not only hepatic differentiation but also pancreatic differentiation, although the optimal stage of HNF4 transduction for the differentiation of each cell is different. We have confirmed that there was no difference between nontransduced cells and Ad-LacZ-transduced cells in the gene expression levels of all the markers investigated in Figure 1a–c (data not shown). We also confirmed that Ad vector-mediated gene expression in the human ESC-derived hepatoblasts (day 9) continued until day 14 and almost disappeared on day 18 (Supplementary Figure S5). These results indicated that the stage-specific HNF4 α overexpression in human ESC-derived hepatoblasts (day 9) was essential for promoting efficient hepatic differentiation.

Transduction of HNF4 α into human ESC- and iPSC-derived hepatoblasts efficiently promotes hepatic maturation

From the results of Figure 1, we decided to transduce hepatoblasts (day 9) with Ad-HNF4 α . To determine whether hepatic maturation is promoted by Ad-HNF4 α transduction, Ad-HNF4 α -transduced cells were cultured until day 20 of differentiation according to the schematic protocol described in Figure 2a. After the hepatic maturation, the morphology of human ESCs was gradually changed into that of hepatocytes: polygonal with distinct round nuclei (day 20) (Figure 2b). Interestingly, a portion of the hepatocyte-like cells, which were ALB²⁰-, CK18²¹-, CYP2D6-, and CYP3A4²⁵-positive cells, had double nuclei, which was also observed in primary human hepatocytes (Figure 2b,c, and Supplementary Figure S6). We also examined the hepatic gene expression levels on day 20 of differentiation (Figure 3a,b). The gene expression analysis of *CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, and *CYP7A1*²⁵ showed higher expression levels in all of Ad-SOX17-, Ad-HEX-, and Ad-HNF4 α -transduced cells (three factors-transduced cells) as compared with those in both Ad-SOX17- and Ad-HEX-transduced cells (two factors-transduced cells) on day 20 (Figure 3a). The gene expression level of NADPH-CYP reductase

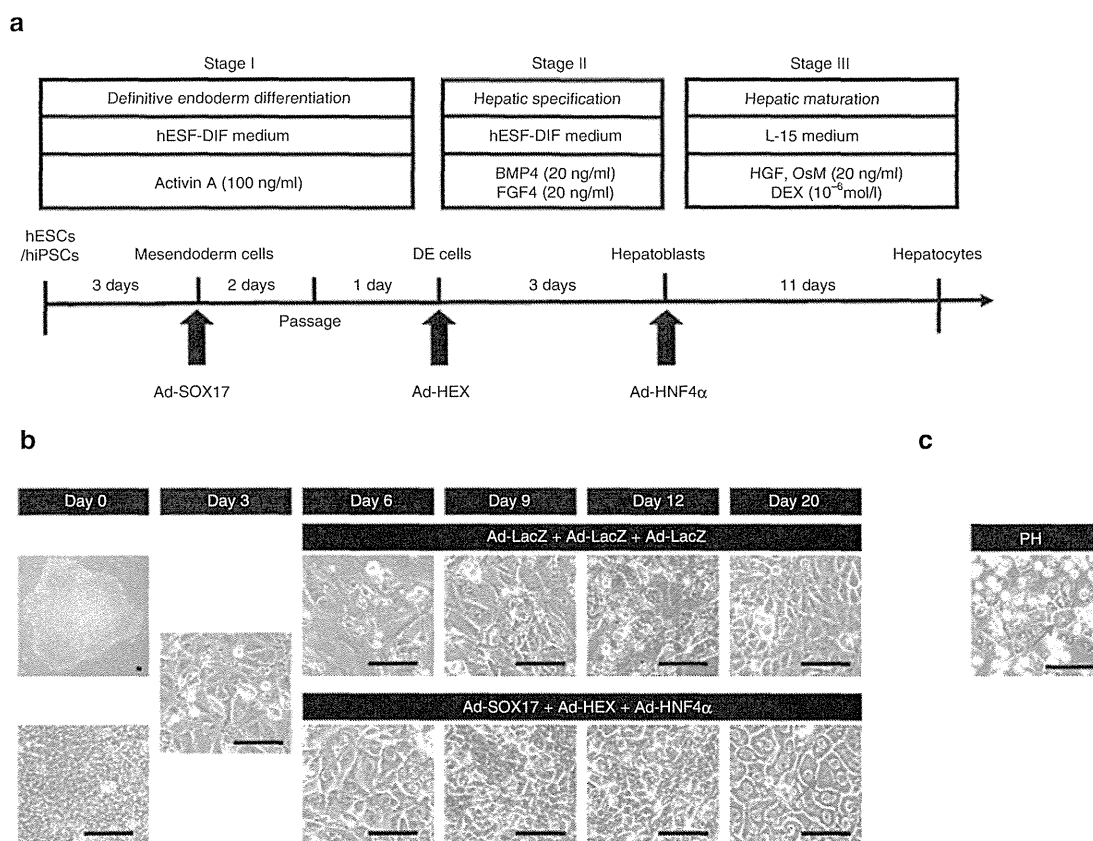


Figure 2 Hepatic differentiation of human ESCs and iPSCs transduced with three factors. **(a)** The procedure for differentiation of human ESCs and iPSCs into hepatocytes via DE cells and hepatoblasts is presented schematically. The hESF-DIF medium was supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, and 0.5 mg/ml fatty-acid-free BSA. The L15 medium was supplemented with 8.3% tryptose phosphate broth, 8.3% FBS, 10 μ mol/l hydrocortisone 21-hemisuccinate, 1 μ mol/l insulin, and 25 mmol/l NaHCO₃. **(b)** Sequential morphological changes (day 0–20) of human ESCs (H9) differentiated into hepatocytes via DE cells and hepatoblasts are shown. Red arrow shows the cells that have double nuclei. **(c)** The morphology of primary human hepatocytes is shown. Bar represents 50 μ m. BSA, bovine serum albumin; DE, definitive endoderm; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell.

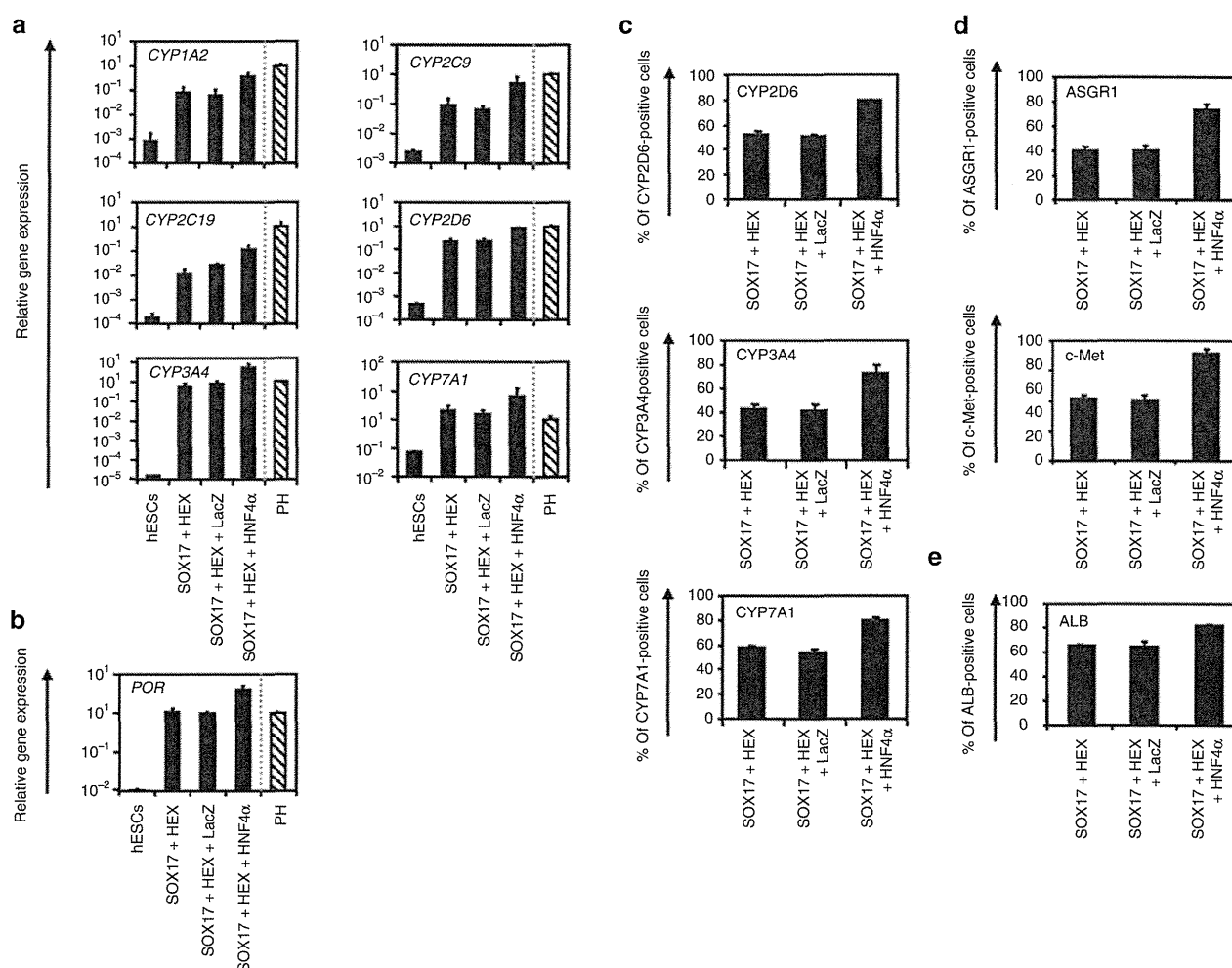


Figure 3 Transduction of HNF4 α promotes hepatic maturation from human ESCs and iPSCs. **(a,b)** The human ESCs were differentiated into hepatocytes according to the protocol described in **Figure 2a**. On day 20 of differentiation, the gene expression levels of **(a)** CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP7A1) and **(b)** POR were examined by real-time RT-PCR in undifferentiated human ESCs (hESCs), the hepatocyte-like cells, and primary human hepatocytes (PH, hatched bar). On the y-axis, the expression level of primary human hepatocytes, which were cultured for 48 hours after the cells were plated, was taken as 1.0. **(c–e)** The hepatocyte-like cells (day 20) were subjected to immunostaining with **(c)** anti-drug-metabolizing enzymes (CYP2D6, CYP3A4, and CYP7A1), **(d)** anti-hepatic surface protein (ASGR1 and c-Met), and **(e)** anti-ALB antibodies, and then the percentage of antigen-positive cells was examined by flow cytometry on day 20 of differentiation. All data are represented as means \pm SD ($n = 3$). ESC, embryonic stem cell; HNF4 α , hepatocyte nuclear factor 4 α ; iPSC, induced pluripotent stem cell.

(POR)²⁶, which is required for the normal function of CYPs, was also higher in the three factors-transduced cells (Figure 3b). The gene expression analysis of ALB, α -1-antitrypsin (α -1-AT), transthyretin, hepatic conjugating enzymes, hepatic transporters, and hepatic transcription factors also showed higher expression levels in the three factors-transduced cells (**Supplementary Figures S7 and S8**). Moreover, the gene expression levels of these hepatic markers of three factor-transduced cells were similar to those of primary human hepatocytes, although the levels depended on the type of gene (Figure 3a,b, and **Supplementary Figures S7 and S8**). To confirm that similar results could be obtained with human iPSCs, we used three human iPS cell lines (201B7, Dotcom, and Tic). The gene expression of hepatic markers in human ESC- and iPSC-derived hepatocytes were analyzed by real-time reverse transcription-PCR on day 20 of differentiation. Three human iPS cell lines as well as human ESCs also effectively differentiated into hepatocytes in response to transduction of the three factors

(**Supplementary Figure S9**). Interestingly, we observed differences in the hepatic maturation efficiency among the three human iPS cell lines. That is, two of the human iPS cell lines (Tic and Dotcom) were more committed to the hepatic lineage than another human iPS cell line (201B7). Because almost homogeneous hepatocyte-like cells would be more useful in basic research, regenerative medicine, and drug discovery, we also examined whether our novel methods for hepatic maturation could generate a homogeneous hepatocyte population by flow cytometry analysis (Figure 3c–e). The percentages of CYP2D6-, CYP3A4-, and CYP7A1-positive cells were ~80% in the three factors-transduced cells, while they were ~50% in the two factors-transduced cells (Figure 3c). The percentages of hepatic surface antigen (asialoglycoprotein receptor 1 [ASGR1] and met proto-oncogene (c-Met))-positive cells (Figure 3d) and ALB-positive cells (Figure 3e) were also ~80% in the three factors-transduced cells. These results indicated that a nearly homogeneous population was obtained by our differentiation protocol

using the transduction of three functional genes (SOX17, HEX, and HNF4 α).

The three factors-transduced cells have characteristics of functional hepatocytes

The hepatic functions of the hepatocyte-like cells, such as the uptake of low-density lipoprotein (LDL) and CYP enzymes activity, of the hepatocyte-like cells were examined on day 20 of differentiation. Approximately 87% of the three factors-transduced cells uptook LDL in the medium, whereas only 44% of the two factors-transduced cells did so (Figure 4a). The activities of CYP enzymes of the hepatocyte-like cells were measured according to the metabolism of the CYP3A4, CYP2C9, or CYP1A2 substrates (Figure 4b). The metabolites were detected in the three factors-transduced cells and their activities were higher than those of the two factors-transduced cells (dimethyl sulfoxide (DMSO) column). We further tested the induction of CYP3A4, CYP2C9, and CYP1A2 by chemical stimulation, since CYP3A4, CYP2C9, and CYP1A2 are the important prevalent CYP isozymes in the liver and are involved in the metabolism of a significant proportion of the currently available commercial drugs (rifampicin or omeprazole column). It is well known that CYP3A4 and CYP2C9 can be induced by rifampicin, whereas CYP1A2 can be induced by omeprazole. The hepatocyte-like cells were treated with either of these. Although undifferentiated human ESCs responded to neither rifampicin nor omeprazole (data not shown), the hepatocyte-like cells produced more metabolites in response to chemical stimulation as well as primary hepatocytes (Figure 4b). The activity levels of the hepatocyte-like cells as compared with those of primary human hepatocytes depended on the types of CYP; the CYP3A4 activity of the hepatocyte-like cells was similar to that of primary human hepatocytes, whereas the CYP2C9 and CYP1A2 activities of the hepatocyte-like cells were slightly lower than those of primary human hepatocytes (Figure 3a). These results indicated that high levels of functional CYP enzymes were detectable in the hepatocyte-like cells.

The metabolism of diverse compounds involving uptake, conjugation, and the subsequent release of the compounds is an important function of hepatocytes. Uptake and release of Indocyanine green (ICG) can often be used to identify hepatocytes in ESC differentiation models.²⁷ To investigate this function in our hepatocyte-like cells, we compared this ability of the three factors-transduced cells with that of the two factors-transduced cells on day 20 of differentiation (Figure 4c). The three factors-transduced cells had more ability to uptake ICG and to excrete ICG by culturing without ICG for 6 hours. We also examined whether the hepatocyte-like cells could store glycogen, a characteristic of functional hepatocytes (Figure 4d). On day 20 of differentiation, the three factors-transduced cells and the two factors-transduced cells were stained for cytoplasmic glycogen using the Periodic Acid-Schiff staining procedure. The three factors-transduced cells exhibited more abundant storage of glycogen than the two-factors-transduced cells. These results showed that abundant hepatic functions, such as uptake and excretion of ICG and storage of glycogen, were obtained by the transduction of three factors.

Many adverse drug reactions are caused by the CYP-dependent activation of drugs into reactive metabolites.²⁸ In order to examine

metabolism-mediated toxicity and to improve the safety of drug candidates, primary human hepatocytes are widely used.²⁸ Because primary human hepatocytes have quite different characteristics among distinct lots and because it is difficult to purchase large amounts of primary human hepatocytes that have the same characteristics, hepatocyte-like cells are expected to be used for this purpose. To examine whether our hepatocyte-like cells could be used to predict metabolism-mediated toxicity, the hepatocyte-like cells were incubated with four substrates (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine), which are known to generate toxic metabolites by CYP enzymes, and then the cell viability was measured (Figure 4e). The cell viability of the two factors plus Ad-LacZ-transduced cells were higher than that of the three factors-transduced cells at each different concentration of four test compounds. These results indicated that the three factors-transduced cells could more efficiently metabolize the test compounds and thereby induce higher toxicity than either the two factors-transduced cells or undifferentiated human ESCs. The cell viability of the three factors-transduced cells was slightly higher than that of primary human hepatocytes.

HNF4 α promotes hepatic maturation by activating mesenchymal-to-epithelial transition

HNF4 α is known as a dominant regulator of the epithelial phenotype because its ectopic expression in fibroblasts (such as NIH 3T3 cells) induces mesenchymal-to-epithelial transition (MET)¹¹, although it is not known whether HNF4 α can promote MET in hepatic differentiation. Therefore, we examined whether HNF4 α transduction promotes hepatic maturation from hepatoblasts by activating MET. To clarify whether MET is activated by HNF4 α transduction, the human ESC-derived hepatoblasts (day 9) were transduced with Ad-LacZ or Ad-HNF4 α , and the resulting phenotype was analyzed on day 12 of differentiation (Figure 5). This time, we confirmed that HNF4 α transduction decreased the population of N-cadherin (hepatoblast marker)-positive cells,²⁹ whereas it increased that of ALB (hepatocyte marker)-positive cells (Figure 5a). The number of CK7 (cholangiocyte marker)-positive population did not change (Figure 5a). To investigate whether these results were attributable to MET, the alteration of the expression of several mesenchymal and epithelial markers was examined (Figure 5b). The human ESC-derived hepatoblasts (day 9) were almost homogeneously N-cadherin³⁰ (mesenchymal marker)-positive and E-cadherin¹¹ (epithelial marker)-negative, demonstrating that human ESC-derived hepatoblasts have mesenchymal characteristics (Figure 5a,b). After HNF4 α transduction, the number of E-cadherin-positive cells was increased and reached ~90% on day 20, whereas that of N-cadherin-positive cells was decreased and was less than 5% on day 20 (Supplementary Figure S10). These results indicated that MET was promoted by HNF4 α transduction in hepatic differentiation from hepatoblasts. Interestingly, the number of growing cells was decreased by HNF4 α transduction (Figure 5c), and the cell growth was delayed by HNF4 α transduction (Supplementary Figure S11). This decrease in the number of growing cells might have been because the differentiation was promoted by HNF4 α transduction. We also confirmed that MET was promoted by HNF4 α transduction in the gene expression levels (Figure 5d).

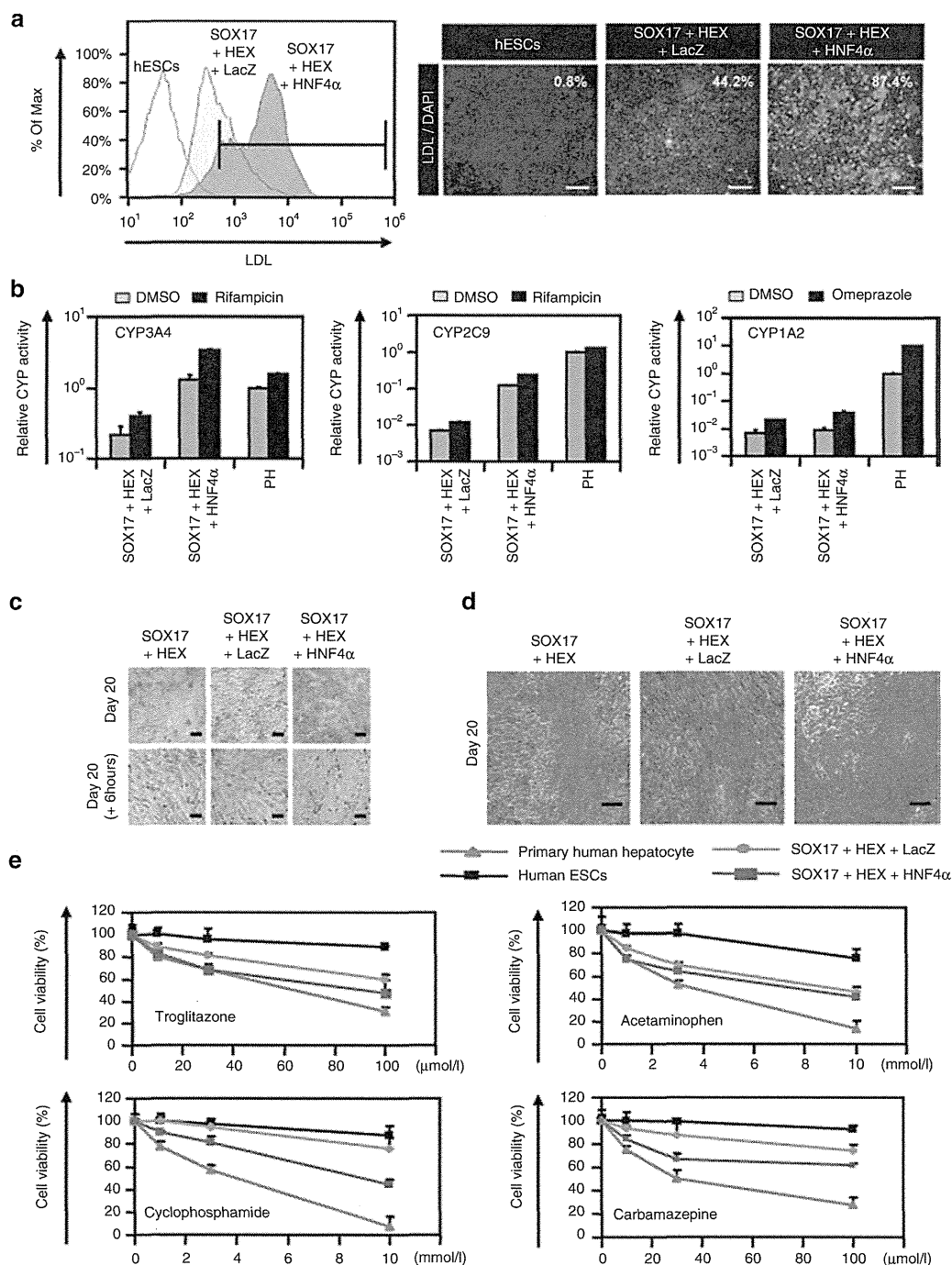


Figure 4 Transduction of the three factors enhances hepatic functions. The human ESCs were differentiated into hepatoblasts and transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hours and cultured until day 20 of differentiation according to the protocol described in **Figure 2a**. The hepatic functions of the two factors plus Ad-LacZ-transduced cells (SOX17+HEX+LacZ) and the three factors-transduced cells (SOX17+HEX+HNF4 α) were compared. **(a)** Undifferentiated human ESCs (hESCs) and the hepatocyte-like cells (day 20) were cultured with medium containing Alexa-Fluor 488-labeled LDL (green) for 1 hour, and immunohistochemistry and flow cytometry analysis were performed. The percentage of LDL-positive cells was measured by flow cytometry. Nuclei were counterstained with DAPI (blue). The bar represents 100 μ m. **(b)** Induction of CYP3A4 (left), CYP2C9 (middle), or CYP1A2 (right) by DMSO (gray bar), rifampicin (black bar), or omeprazole (black bar) in the hepatocyte-like cells (day 20) and primary human hepatocytes (PH), which were cultured for 48 hours after the cells were plated. On the y-axis, the activity of primary human hepatocytes that have been cultured with medium containing DMSO was taken as 1.0. **(c)** The hepatocyte-like cells (day 20) (upper column) were examined for their ability to take up Indocyanin Green (ICG) and release it 6 hours thereafter (lower column). **(d)** Glycogen storage of the hepatocyte-like cells (day 20) was assessed by Periodic Acid-Schiff (PAS) staining. PAS staining was performed on day 20 of differentiation. Glycogen storage is indicated by pink or dark red-purple cytoplasm. The bar represents 100 μ m. **(e)** The cell viability of undifferentiated human ESCs (black), two factors plus Ad-LacZ-transduced cells (green), the three factors-transduced cells (blue), and primary human hepatocytes (red) was assessed by Alamar Blue assay after 48 hours exposure to different concentrations of four test compounds (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine). The cell viability is expressed as a percentage of cells treated with solvent only treat: 0.1% DMSO except for carbamazepine: 0.5% DMSO. All data are represented as means \pm SD ($n = 3$). ESC, embryonic stem cell; DMSO, dimethyl sulfoxide; LDL, low-density lipoprotein.

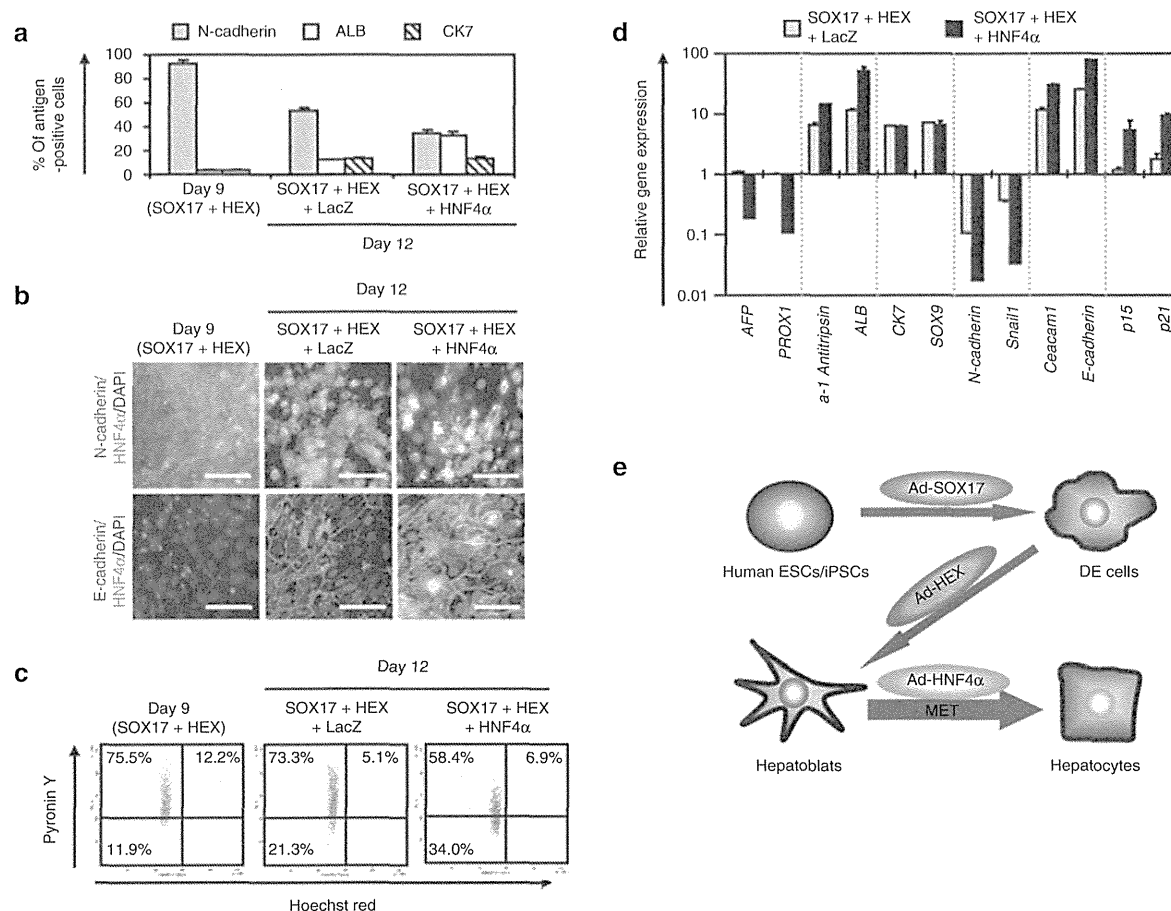


Figure 5 HNF4 α promotes hepatic differentiation by activating MET. Human ESCs were differentiated into hepatoblasts according to the protocol described in **Figure 2a**, and then transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hours, and finally cultured until day 12 of differentiation. **(a)** The hepatoblasts, two factors plus Ad-LacZ-transduced cells (SOX17+HEX+LacZ) (day 12), and the three factors-transduced cells (SOX17+HEX+HNF4 α) (day 12) were subjected to immunostaining with anti-N-cadherin, ALB, or CK7 antibodies. The percentage of antigen-positive cells was measured by flow cytometry. **(b)** The cells were subjected to immunostaining with anti-N-cadherin (green), E-cadherin (green), or HNF4 α (red) antibodies on day 9 or day 12 of differentiation. Nuclei were counterstained with DAPI (blue). The bar represents 50 μ m. Similar results were obtained in two independent experiments. **(c)** The cell cycle was examined on day 9 or day 12 of differentiation. The cells were stained with Pylonin Y (y-axis) and Hoechst 33342 (x-axis) and then analyzed by flow cytometry. The growth fraction of cells is the population of actively dividing cells (G1/S/G2/M). **(d)** The expression levels of *AFP*, *PROX1*, α -1-antitrypsin, *ALB*, *CK7*, *SOX9*, *N-cadherin*, *Snail1*, *Ceacam1*, *E-cadherin*, *p15*, and *p21* were examined by real-time RT-PCR on day 9 or day 12 of differentiation. The expression level of hepatoblasts (day 9) was taken as 1.0. All data are represented as means \pm SD ($n = 3$). **(e)** The model of efficient hepatic differentiation from human ESCs and iPSCs in this study is summarized. The human ESCs and iPSCs differentiate into hepatocytes via definitive endoderm and hepatoblasts. At each stage, the differentiation is promoted by stage-specific transduction of appropriate functional genes. In the last stage of hepatic differentiation, HNF4 α transduction provokes hepatic maturation by activating MET. ESC, embryonic stem cell; HNF4 α , hepatocyte nuclear factor 4 α ; iPSC, induced pluripotent stem cell; MET, mesenchymal-to-epithelial transition; RT-PCR, reverse transcription-PCR; VP, vector particle.

The gene expression levels of hepatocyte markers (α -1-antitrypsin and *ALB*)²⁰ and epithelial markers (*Ceacam1* and *E-cadherin*) were upregulated by HNF4 α transduction. On the other hand, the gene expression levels of hepatoblast markers (*AFP* and *PROX1*)³¹, mesenchymal markers (*N-cadherin* and *Snail*)³², and cyclin dependent kinase inhibitor (*p15* and *p21*)³³ were downregulated by HNF4 α transduction. HNF4 α transduction did not change the expression levels of cholangiocyte markers (*CK7* and *SOX9*). We conclude that HNF4 α promotes hepatic maturation by activating MET.

DISCUSSION

This study has two main purposes: the generation of functional hepatocytes from human ESCs and iPSCs for application to drug toxicity screening in the early phase of pharmaceutical development

and; elucidation of the HNF4 α function in hepatic maturation from human ESCs. We initially confirmed the importance of transcription factor HNF4 α in hepatic differentiation from human ESCs by using a published data set of gene array analysis (**Supplementary Figure S1**).³⁴ We speculated that HNF4 α transduction could enhance hepatic differentiation from human ESCs and iPSCs.

To generate functional hepatocytes from human ESCs and iPSCs and to elucidate the function of HNF4 α in hepatic differentiation from human ESCs, we examined the stage-specific roles of HNF4 α . We found that hepatoblast (day 9) stage-specific HNF4 α transduction promoted hepatic differentiation (Figure 1). Because endogenous HNF4 α is initially expressed in the hepatoblast,^{9,10} our system might adequately reflect early embryogenesis. However, HNF4 α transduction at an inappropriate stage (day 6 or day 12) promoted

bidirectional differentiation; heterogeneous populations, which contain the hepatocytes and pancreas cells or hepatocytes and cholangiocytes, were obtained, respectively (Figure 1), consistent with a previous report that HNF4 α plays an important role not only in the liver but also in the pancreas.¹² Therefore, we concluded that HNF4 α plays a significant stage-specific role in the differentiation of human ESC- and iPSC-derived hepatoblasts to hepatocytes (Figure 5e).

We found that the expression levels of the hepatic functional genes were upregulated by HNF4 α transduction (Figure 3a,b, and **Supplementary Figures S7 and S8**). Although the *c/EBP α* and *GATA4* expression levels of the three factors-transduced cells were higher than those of primary human hepatocytes, the *FOXA1*, *FOXA2*, *FOXA3*, and *HNF1 α* , which are known to be important for hepatic direct reprogramming and hepatic differentiation,^{35,36} expression levels of three factors-transduced cells were slightly lower than those of primary human hepatocytes (**Supplementary Figure S8**). Therefore, additional transduction of *FOXA1*, *FOXA2*, *FOXA3*, and *HNF1 α* might promote further hepatic maturation. Some previous hepatic differentiation protocols that utilized growth factors without gene transfer led to the appearance only of heterogeneous hepatocyte populations.⁴⁻⁶ The HNF4 α transduction led not only to the upregulation of expression levels of several hepatic markers but also to an almost homogeneous hepatocyte population; the differentiation efficacy based on *CYPs*, *ASGR1*, or *ALB* expression was ~80% (Figure 3c-e). The efficient hepatic maturation in this study might be attributable to the activation of many hepatocyte-associated genes by the transduction of HNF4 α , which binds to the promoters of nearly half of the genes expressed in the liver.¹² In the later stage of hepatic maturation, hepatocyte-associated genes would be strongly upregulated by endogenous transcription factors but not exogenous HNF4 α because transgene expression by Ad vectors was almost disappeared on day 18 (**Supplementary Figure S5**). Another reason for the efficient hepatic maturation would be that sequential transduction of *SOX17*, *HEX*, and HNF4 α could mimic hepatic differentiation in early embryogenesis.

Next, we examined whether or not the hepatocyte-like cells had hepatic functions. The activity of many kinds of *CYPs* was upregulated by HNF4 α transduction (Figure 4b). Ad-HNF4 α -transduced cells exhibit many characteristics of hepatocytes: uptake of LDL, uptake and excretion of ICG, and storage of glycogen (Figure 4a,c,d). Many conventional tests of hepatic characteristics have shown that the hepatocyte-like cells have mature hepatocyte functions. Furthermore, the hepatocyte-like cells can catalyze the toxication of several compounds (Figure 4e). Although the activities to catalyze the toxication of test compounds in primary human hepatocytes are slightly higher than those in the hepatocyte-like cells, the handling of primary human hepatocytes is difficult for a number of reasons: since their source is limited, large-scale primary human hepatocytes are difficult to prepare as a homogeneous population. Therefore, the hepatocyte-like cells derived from human ESCs and iPSCs would be a valuable tool for predicting drug toxicity. To utilize the hepatocyte-like cells in a drug toxicity study, further investigation of the drug metabolism capacity and *CYP* induction potency will be needed.

We also investigated the mechanisms underlying efficient hepatic maturation by HNF4 α transduction. Although the

number of cholangiocyte populations did not change by HNF4 α transduction, we found that the number of hepatoblast populations decreased and that of hepatocyte populations increased, indicating that HNF4 α promotes selective hepatic differentiation from hepatoblasts (Figure 5a). As previously reported, HNF4 α regulates the expression of a broad range of genes that code for cell adhesion molecules,¹³ extracellular matrix components, and cytoskeletal proteins, which determine the main morphological characteristics of epithelial cells.^{14,35,37} In this study, we elucidated that *MET* was promoted by HNF4 α transduction (Figure 5b,d). Thus, we conclude that HNF4 α overexpression in hepatoblasts promotes hepatic differentiation by activating *MET* (Figure 5e).

Using human iPSCs as well as human ESCs, we confirmed that the stage-specific overexpression of HNF4 α could promote hepatic maturation (**Supplementary Figure S9**). Interestingly, the differentiation efficacies differed among human iPS cell lines: two of the human iPS cell lines (Dotcom and Tic) were more committed to the hepatic lineage than another human iPS cell line (201B7) (**Supplementary Figure S7**). Therefore, it would be necessary to select a human iPS cell line that is suitable for hepatic maturation in the case of medical applications, such as drug screening and liver transplantation. The difference of hepatic differentiation efficacy among the three iPS lines might be due to the difference of epigenetic memory of original cells or the difference of the inserted position of the foreign genes for the reprogramming.

To control hepatic differentiation mimicking embryogenesis, we employed Ad vectors, which are one of the most efficient transient gene delivery vehicles and have been widely used in both experimental studies and clinical trials.³⁸ We used a fiber-modified Ad vector containing the EF-1 α promoter and a stretch of lysine residue (KKKKKKK, K7) peptides in the C-terminal region of the fiber knob.¹⁹ The K7 peptide targets heparan sulfates on the cellular surface, and the fiber-modified Ad vector containing the K7 peptides was shown to be efficient for transduction into many kinds of cells including human ESCs and human ESC-derived cells.^{7-8,19} Thus, Ad vector-mediated transient gene transfer should be a powerful tool for regulating cellular differentiation.

In summary, the findings described here demonstrate that transcription factor HNF4 α plays a crucial role in the hepatic differentiation from human ESC-derived hepatoblasts by activating *MET* (Figure 5e). In the present study, both human ESCs and iPSCs (three lines) were used and all cell lines showed efficient hepatic maturation, indicating that our protocol would be a universal tool for cell line-independent differentiation into functional hepatocytes. Moreover, the hepatocyte-like cells can catalyze the toxication of several compounds as primary human hepatocytes. Therefore, our technology, by sequential transduction of *SOX17*, *HEX*, and HNF4 α , would be a valuable tool for the efficient generation of functional hepatocytes derived from human ESCs and iPSCs, and the hepatocyte-like cells could be used for the prediction of drug toxicity.

MATERIALS AND METHODS

Human ESC and iPSC culture. A human ES cell line, H9 (WiCell Research Institute, Madison, HI), was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) with Repro Stem (Repro CELL, Tokyo, Japan) supplemented with 5 ng/ml fibroblast

growth factor 2 (FGF2) (Sigma, St Louis, MO). Human ESCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics, Indianapolis, IN) into small clumps and then were subcultured every 4 or 5 days. H9 was 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. Two human iPS cell lines generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Tic, JCRB Number: JCRB1331; and Dotcom, JCRB Number: JCRB1327).^{39,40} These human iPS cell lines were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with iPSellon (Cardio, Kobe, Japan) supplemented with 10 ng/ml FGF2. Another human iPS cell line, 201B7, generated from human dermal fibroblasts was kindly provided by Dr S. Yamanaka (Kyoto University).² The human iPS cell line 201B7 was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with Repro Stem (Repro CELL) supplemented with 5 ng/ml FGF2 (Sigma). Human iPSCs were dissociated with 0.1 mg/ml dispase (Roche Diagnostics) into small clumps and were then subcultured every 5 or 6 days.

In vitro differentiation. Before the initiation of cellular differentiation, the medium of human ESCs and iPSCs was exchanged for a defined serum-free medium, hESF9, and cultured as we previously reported.⁴¹ hESF9 consists of hESF-GRO medium (Cell Science & Technology Institute, Sendai, Japan) supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, oleic acid conjugated with fatty-acid-free bovine albumin (BSA), 10 ng/ml FGF2, and 100 ng/ml heparin (all from Sigma).

The differentiation protocol for the induction of DE cells, hepatoblasts, and hepatocytes was based on our previous report with some modifications.⁷ Briefly, in mesendoderm differentiation, human ESCs and iPSCs were dissociated into single cells and cultured for 3 days on Matrigel (Becton, Dickinson and Company, Tokyo, Japan) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems, Minneapolis, MN). To generate mesendoderm cells and DE cells, human ESC-derived cells were transduced with 3,000 vector particles (VP)/cell of Ad-SOX17 for 1.5 hours on day 3 and cultured until day 6 on Matrigel (BD) in hESF-DIF medium (Cell Science & Technology Institute) supplemented with 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, 0.5 mg/ml BSA, and 100 ng/ml Activin A (R&D Systems). For induction of hepatoblasts, the DE cells were transduced with 3,000 VP/cell of Ad-HEX for 1.5 hours on day 6 and cultured for 3 days on a Matrigel (BD) in hESF-DIF (Cell Science & Technology Institute) medium supplemented with the 10 μ g/ml human recombinant insulin, 5 μ g/ml human apotransferrin, 10 μ mol/l 2-mercaptoethanol, 10 μ mol/l ethanolamine, 10 μ mol/l sodium selenite, 0.5 mg/ml BSA, 20 ng/ml bone morphogenetic protein 4 (R&D Systems), and 20 ng/ml FGF4 (R&D Systems). In hepatic differentiation, hepatoblasts were transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hr on day 9 and were cultured for 11 days on Matrigel (BD) in L15 medium (Invitrogen, Carlsbad, CA) supplemented with 8.3% tryptose phosphate broth (BD), 8.3% fetal bovine serum (Vita, Chiba, Japan), 10 μ mol/l hydrocortisone 21-hemisuccinate (Sigma), 1 μ mol/l insulin, 25 mmol/l NaHCO₃ (Wako, Osaka, Japan), 20 ng/ml hepatocyte growth factor (R&D Systems), 20 ng/ml Oncostatin M (R&D Systems), and 10⁻⁶ mol/l Dexamethasone (Sigma).

Ad vectors. Ad vectors were constructed by an improved *in vitro* ligation method.^{42,43} The human HNF4 α gene (accession number NM_000457) was amplified by PCR using primers designed to incorporate the 5' Not I and 3' Xba I restriction enzyme sites: Fwd 5'-ggcctctagatggaggcaggagaatg-3' and Rev 5'-ccccggcgccgacggcttgctagataac-3'. The human HNF4 α gene was inserted into pBSKII (Invitrogen), resulting in pBSKII-HNF4 α , and

then the human HNF4 α gene was inserted into pHMEF5,⁴⁴ which contains the human elongation factor-1 α (EF-1 α) promoter, resulting in pHMEF-HNF4 α . The pHMEF-HNF4 α was digested with I-CeuI/PI-SceI and ligated into I-CeuI/PI-SceI-digested pAdHM41-K7,¹⁹ resulting in pAd-HNF4 α . The human EF-1 α promoter-driven LacZ-, SOX17-, or HEX-expressing Ad vectors, Ad-LacZ, Ad-SOX17, or Ad-HEX, were constructed previously.^{7,8,45} Ad-LacZ, Ad-SOX17, Ad-HEX, and Ad-HNF4 α , each of which contains a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of human ESCs, iPSCs, and DE cells, were generated and purified as described previously.⁷ The VP titer was determined by using a spectrophotometric method.⁴⁶

LacZ assay. Human ESC- and iPSC-derived cells were transduced with Ad-LacZ at 3,000 VP/cell for 1.5 hours. After culturing for the indicated number of days, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) staining was performed as described previously.⁴⁴

Flow cytometry. Single-cell suspensions of human ESCs, iPSCs, and their derivatives were fixed with methanol at 4°C for 20 minutes and then incubated with the primary antibody, followed by the secondary antibody. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD).

RNA isolation and reverse transcription-PCR. Total RNA was isolated from human ESCs, iPSCs, and their derivatives using ISOGENE (Nippon Gene) according to the manufacturer's instructions. Primary human hepatocytes were purchased from CellzDirect, Durham, NC. complementary DNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time reverse transcription-PCR was performed with Taqman gene expression assays (Applied Biosystems, Foster City, CA) or SYBR Premix Ex Taq (TaKaRa) using an ABI PRISM 7000 Sequence Detector (Applied Biosystems). 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 **Supplementary Table S1**.

Immunohistochemistry. The cells were fixed with methanol or 4% paraformaldehyde (Wako). After blocking with phosphate-buffered saline containing 2% BSA (Sigma) and 0.2% Triton X-100 (Sigma), the cells were incubated with primary antibody at 4°C for 16 hours, followed by incubation with a secondary antibody that was labeled with Alexa Fluor 488 (Invitrogen) or Alexa Fluor 594 (Invitrogen) at room temperature for 1 hour. All the antibodies are listed in **Supplementary Table S2**.

Assay for CYP activity. To measure cytochrome P450 3A4, 2C9, and 1A2 activity, we performed Lytic assays by using a P450-GloTM CYP3A4 Assay Kit (Promega, Madison, WI). For the CYP3A4 and 2C9 activity assay, undifferentiated human ESCs, the hepatocyte-like cells, and primary human hepatocytes were treated with rifampicin (Sigma), which is the substrate for CYP3A4 and CYP2C9, at a final concentration of 25 μ mol/l or DMSO (0.1%) for 48 hours. For the CYP1A2 activity assay, undifferentiated human ESCs, the hepatocyte-like cells, and primary human hepatocytes were treated with omeprazole (Sigma), which is the substrate for CYP1A2, at a final concentration of 10 μ M or DMSO (0.1%) for 48 hours. We measured the fluorescence activity with a luminometer (Lumat LB 9507; Berthold, Oak Ridge, TN) according to the manufacturer's instructions.

Pyronin Y/Hoechst Staining. Human ESC-derived cells were stained with Hoechst33342 (Sigma) and Pyronin Y (PY) (Sigma) in Dulbecco's modified Eagle medium (Wako) supplemented with 0.2 mmol/l HEPES and 5% FCS (Invitrogen). Samples were then placed on ice for 15 minutes, and 7-AAD was added to a final concentration of 0.5 mg/ml for exclusion of dead cells. Fluorescence-activated cell-sorting analysis of these cells was

performed on a FACS LSR Fortessa flow cytometer (Becton Dickinson) equipped with a UV-laser.

Cellular uptake and excretion of ICG. ICG (Sigma) was dissolved in DMSO at 100 mg/ml, then added to a culture medium of the hepatocyte-like cells to a final concentration of 1 mg/ml on day 20 of differentiation. After incubation at 37°C for 60 minutes, the medium with ICG was discarded and the cells were washed with phosphate-buffered saline. The cellular uptake of ICG was then examined by microscopy. Phosphate-buffered saline was then replaced by the culture medium and the cells were incubated at 37°C for 6 hours. The excretion of ICG was examined by microscopy.

Periodic Acid-Schiff assay for glycogen. The hepatocyte-like cells were fixed with 4% paraformaldehyde and stained using a Periodic Acid-Schiff staining system (Sigma) on day 20 of differentiation according to the manufacturer's instructions.

Cell viability tests. Cell viability was assessed by Alamar Blue assay kit (Invitrogen). After treatment with test compounds^{47–50} (troglitazone, acetaminophen, cyclophosphamide, and carbamazepine) (all from Wako) for 2 days, the culture medium was replaced with 0.5 mg/ml solution of Alamar Blue in culturing medium and cells were incubated for 3 hours at 37°C. The supernatants of the cells were measured at a wavelength of 570 nm with background subtraction at 600 nm in a plate reader. Control refers to incubations in the absence of test compounds and was considered as 100% viability value.

Uptake of LDL. The hepatocyte-like cells were cultured with medium containing Alexa-488-labeled LDL (Invitrogen) for 1 hour, and then the cells that could uptake LDL were assessed by immunohistochemistry and flow cytometry.

Primary human hepatocytes. Cryopreserved human hepatocytes were purchased from CellzDirect (lot Hu8072). 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 (CellzDirect) and the suspension was centrifuged at 100g for 10 minutes at room temperature. The hepatocytes were seeded at 1.25×10^5 cells/cm² in hepatocyte culture medium (Lonza, Walkersville, MD) containing 10% FCS (GIBCO-BRL) onto type I collagen-coated 12-well plates. The medium was replaced with hepatocyte culture medium containing 10% FCS (GIBCO-BRL) 6 hours after seeding. The hepatocytes, which were cultured 48 hours after plating the cells, were used in the experiments.

SUPPLEMENTARY MATERIAL

Figure S1. Genome-wide screening of transcription factors involved in hepatic differentiation emphasizes the importance of the transcription factor HNF4 α .

Figure S2. Summary of specific markers for DE cells, hepatoblasts, hepatocytes, cholangiocytes, and pancreas cells.

Figure S3. The formation of DE cells, hepatoblasts, hepatocytes, and cholangiocytes from human ESCs.

Figure S4. Overexpression of HNF4 α mRNA in hepatoblasts by Ad-HNF4 α transduction.

Figure S5. Time course of LacZ expression in hepatoblasts transduced with Ad-LacZ.

Figure S6. The morphology of the hepatocyte-like cells.

Figure S7. Upregulation of the expression levels of conjugating enzymes and hepatic transporters by HNF4 α transduction.

Figure S8. Upregulation of the expression levels of hepatic transcription factors by HNF4 α transduction.

Figure S9. Generation of hepatocytes from various human ES or iPS cell lines.

Figure S10. Promotion of MET by HNF4 α transduction.

Figure S11. Arrest of cell growth by HNF4 α transduction.

Table S1. List of Taqman probes and primers used in this study.

Table S2. List of antibodies used in this study.

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RESEARCH

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L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in neuroinflammation: the 'collusion' hypothesis for increased extracellular L-glutamate concentration in neuroinflammation

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Abstract

Background: In the central nervous system, astrocytic L-glutamate (L-Glu) transporters maintain extracellular L-Glu below neurotoxic levels, but their function is impaired with neuroinflammation. Microglia become activated with inflammation; however, the correlation between activated microglia and the impairment of L-Glu transporters is unknown.

Methods: We used a mixed culture composed of astrocytes, microglia, and neurons. To quantify L-Glu transporter function, we measured the extracellular L-Glu that remained 30 min after an application of L-Glu to the medium (the starting concentration was 100 μ M). We determined the optimal conditions of lipopolysaccharide (LPS) treatment to establish an inflammation model without cell death. We examined the predominant subtypes of L-Glu transporters and the changes in the expression levels of these transporters in this inflammation model. We then investigated the role of activated microglia in the changes in L-Glu transporter expression and the underlying mechanisms in this inflammation model.

Results: Because LPS (10 ng/mL, 72 h) caused a significant increase in the levels of L-Glu remaining but did not affect cell viability, we adopted this condition for our inflammation model without cell death. GLAST was the predominant L-Glu transporter subtype, and its expression decreased in this inflammation model. As a result of their release of L-Glu, activated microglia were shown to be essential for the significant decrease in L-Glu uptake. The serial application of L-Glu caused a significant decrease in L-Glu uptake and GLAST expression in the astrocyte culture. The hemichannel inhibitor carbenoxolone (CBX) inhibited L-Glu release from activated microglia and ameliorated the decrease in GLAST expression in the inflammation model. In addition, the elevation of the astrocytic intracellular L-Glu itself caused the downregulation of GLAST.

Conclusions: Our findings suggest that activated microglia trigger the elevation of extracellular L-Glu through their own release of L-Glu, and astrocyte L-Glu transporters are downregulated as a result of the elevation of astrocytic intracellular L-Glu levels, causing a further increase of extracellular L-Glu. Our data suggest the new hypothesis that activated microglia collude with astrocytes to cause the elevation of extracellular L-Glu in the early stages of neuroinflammation.

Keywords: L-glutamate, Microglia, Transporter, Astrocytes, Inflammation, Hemichannel

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Background

L-glutamate (L-Glu) is one of the most important excitatory neurotransmitters in the mammalian central nervous system (CNS). However, high concentrations of L-Glu cause excessive stimulation of L-Glu receptors and lead to neurotoxicity [1,2]. In astrocytes, GLAST (EAAT1 in humans) and GLT-1 (EAAT2) are the major functional L-Glu transporters in the CNS, and they play an important role in maintaining extracellular L-Glu concentrations below neurotoxic levels [3]. Impairments of L-Glu transporter function have been reported in numerous neurological diseases associated with inflammation, for example, Alzheimer's disease [4], amyotrophic lateral sclerosis [5], major depressive disorder [6,7], and epilepsy [8-10]. Furthermore, elevated extracellular L-Glu content has been reported in *in vivo* and *in vitro* inflammation models [11,12]. Accordingly, the impairment of L-Glu transporters has been suggested to contribute to elevated extracellular L-Glu concentrations in inflammation; however, the specific role of such transporters remains unknown, as some inflammation models also cause cell death.

The CNS is composed of neurons and the following three types of glial cells: astrocytes, microglia, and oligodendrocytes [13]. Microglia are the primary cells that are activated in response to inflammatory stimulation [14,15] and are the resident innate immune cells in the CNS. Once activated, microglia exhibit a phenotypic switch from a resting ramified type to a motile amoeboid type [16,17] and release various soluble factors, including pro-inflammatory cytokines [18,19], reactive oxygen species [20], nitric oxide (NO) [16], L-Glu [21,22], and ATP [23,24]. Although the direct application of some of these factors has been reported to inhibit L-Glu transporters [25-28], few studies have examined the interaction between activated microglia and astrocyte L-Glu transporters in inflammation.

In this study, we aimed to clarify the interaction between activated microglia and astrocyte L-Glu transporters in inflammation. To quantify L-Glu transporter function, we measured the extracellular concentrations of L-Glu (that is, the concentration of L-Glu remaining) after a single exogenous application of L-Glu to the medium. To ensure that we measured the effects on live cells (and not L-Glu released from dying cells), we identified a condition of lipopolysaccharide (LPS) application that was suitable to induce inflammation without cell death. In this model, we found that activated microglia released L-Glu, the resultant elevation in extracellular L-Glu led to the elevation of intracellular L-Glu content in astrocytes through L-Glu transporters, and the increased level of intracellular L-Glu in astrocytes decreased GLAST expression. These reactions caused a further elevation of the extracellular concentration of

L-Glu. Our data suggest a new hypothesis in which activated microglia collude with astrocytes to cause the elevation of extracellular L-Glu in the early stages of neuroinflammation.

Methods

All procedures using live animals in this study were conducted in accordance with the guidelines of the National Institute of Health Sciences (NIHS), Japan, as developed under the Guide for the Care and Use of Laboratory Animals by the National Research Council. Also all experiments were approved by the ethics committee of the NIHS.

Materials

L-Glu, LPS, CBX, anti-rabbit Iba-1 polyclonal antibody (019-19741), and paraformaldehyde (PFA) were purchased from Wako (Osaka, Japan). Dihydrokainic acid (DHK), adenosine 5'-triphosphate disodium salt hydrate (ATP), 2' (3')-O-(4-benzoylbenzoyl)ATP triethylammonium salt (BzATP), 2',3'-O-(2,4,6-trinitrophenyl)ATP salt hydrate (TNP-ATP), adenosine 5'-triphosphate, periodate oxidized sodium salt (OxATP), poly-L-lysine hydrobromide, polyethylenimine, β -nicotinamide adenine dinucleotide (β -NAD), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1-methoxy-5-methyl-phenazinium methyl sulfate (MPMS), Triton-X100, lactate lithium salt, anti-mouse β -actin monoclonal antibody (A5316), sodium deoxycholate, 2-mercaptoethanol, bromophenol blue sodium salt (BPB), and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). DL-threo- β -benzyloxyaspartic acid (TBOA) was purchased from TOCRIS (Ellisville, MO, USA). An MTT Cell proliferation assay kit was purchased from Life Technologies (Grand Island, NY, USA). Rat glutamate transporter (GLAST/EAAT1) control peptide (GLAST11-P) and rat glutamate transporter (GLT1/EAAT2) control peptide (GLT11-P) were purchased from Alpha Diagnostic (San Antonio, TX, USA). Clodronate disodium salt and polyoxyethylene (9) octylphenyl ether (NP-40) were purchased from Calbiochem (Darmstadt, Germany). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and horse serum (HS) were purchased from GIBCO (Grand Island, NY, USA). Bovine liver glutamate dehydrogenase (GLDH) was purchased from Roche (Mannheim, Germany). RNeasy Mini Kits and an RNase-Free DNase set were purchased from Qiagen (Hilden, Germany). TaqMan one-step RT-PCR master mix reagents and TaqMan ribosomal RNA control reagents (VIC Probe) were purchased from Applied Biosystems (Foster City, CA, USA). 2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4H-chromene-3-carbonitrile (UCPH-101), rabbit anti-GLAST polyclonal antibody (ab65978), and anti-chicken glial fibrillary acidic protein (GFAP) polyclonal antibody (ab4674)

were purchased from Abcam (Cambridge, UK). Goat anti-EAAT2 (GLT1) antibody (sc-7760) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse Tuj1 (β 3 tubulin) antibody (MAB5564) was purchased from Chemicon (Temecula, CA, USA). Donkey anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Amersham Biosciences (Washington, DC, USA). The SuperSignal West Femto Trial Kit was purchased from Thermo Scientific (Rockford, IL, USA). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl) was purchased from Bio-Rad (Hercules, CA, USA). Ethylenediaminetetraacetate (EDTA) and ethyleneglycoldiaminetetraacetate (EGTA) were purchased from Dojindo (Kumamoto, Japan). Goat serum, anti-mouse IgG-conjugated Alexa Fluor 488, anti-chicken IgG-conjugated Alexa Fluor 594, and anti-rabbit IgG-conjugated Alexa Fluor 647 were purchased from Vector Laboratories (Burlingame, CA, USA).

Cell culture

Astrocyte-microglia-neuron mixed culture

The brains of 2-day-old Sprague-Dawley (SD) rats were aseptically removed, and the cerebral cortices were dissected. The tissues were dissociated by trituration and trypsinization. After centrifugation at 1,500 rpm for 5 min, the cells were suspended in DMEM supplemented with 10% FBS and 1% antibiotic-antimitotic agent, and the residual tissue aggregates were removed by filtration through a cell strainer with a pore size of 40 to 45 μ m. The cells were seeded onto appropriately sized poly-L-lysine-coated plastic dishes or polyethyleneimine-coated cover glass, and grown for 8 days at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every 2 days.

Astrocyte culture

The primary culture of rat astrocytes was prepared according to a method previously described [29,30]. The cortical cells were obtained by the methods described above and were seeded in uncoated 75-cm² flasks at a density of 5×10^4 cells/cm². The medium was changed 24 h after plating and then every 3 to 4 days. When the cells became confluent (10 to 14 days *in vitro* (DIV)), the non-astrocyte cells were detached from the flasks by shaking and removed by changing the medium. The remaining cells were dissociated by trypsinization and seeded onto appropriately sized poly-L-lysine-coated plastic dishes or polyethyleneimine-coated cover glass at a density of 3×10^5 cells/cm². The cells became confluent again 2 to 3 days after plating.

Microglia culture

The primary culture of the rat microglia was prepared according to a method previously described [31]. In

brief, the cells were obtained from the cerebral cortices of 1-day-old SD rats and seeded in poly-L-lysine-coated 75-cm² flasks at a density of 2.5×10^5 cells/cm². The cells were then grown in DMEM containing 10% FBS, 10 U/mL penicillin, and 10 mg/mL streptomycin for 10 to 14 days at 37°C. The medium was changed every 2 to 3 days. The microglia were removed from the flask bottoms by gentle shaking (60 rpm, 2 min) and collected by centrifugation at $1,000 \times g$ for 5 min. The microglia were resuspended and seeded at a density of 6×10^4 cells/cm² onto appropriately sized poly-L-lysine-coated plastic dishes or polyethyleneimine-coated cover glass.

Astrocyte-microglia co-culture

The microglia prepared by the methods described above were seeded at a density of 6×10^4 cells/cm² onto confluent astrocytes and cultured for 1 to 2 days.

Astrocyte-neuron co-culture

The astrocyte-microglia-neuron co-culture was treated with clodronate [32] at a concentration of 10 μ g/mL for 4 days from 5 DIV to reduce the density of microglia to below 1.2×10^3 cells/cm².

Drug treatment

Stock solutions of 100 mM L-Glu, 10 μ g/mL LPS, 100 μ g/mL clodronate, 10 mM TBOA (non-selective L-Glu transporter inhibitor, IC₅₀: 48 μ M for GLAST, 7 μ M for GLT-1), 10 mM UCPH-101 (a GLAST specific inhibitor, IC₅₀: 0.66 μ M for GLAST, >400-fold selectivity over EAAT2 and EAAT3), 10 mM DHK (a GLT-1-specific inhibitor, IC₅₀: >3,000 μ M for GLAST, 23 μ M for GLT-1), 100 mM ATP, 10 mM BzATP, 10 mM, and 100 mM CBX in phosphate-buffered saline (PBS) were dissolved into the culture medium at the time of application. At 8 DIV, the astrocyte-microglia-neuron culture was treated with LPS at concentrations of 1 to 100 ng/mL for 6 to 72 h. TBOA, UCPH-101, or DHK was applied to the astrocyte-microglia-neuron co-culture for 24 h at 10 DIV. ATP or BzATP was applied to the astrocyte culture at concentrations of 100 to 3,000 μ M or 10 to 300 μ M, respectively, for 72 h. At 8 DIV, CBX was applied to the astrocyte-microglia-neuron culture at concentrations of 10 to 100 μ M from 1 h before to the end of the LPS treatment.

The measurement of the extracellular L-Glu concentration in the medium

The measurement of L-Glu concentration in the medium was performed according to a previously described method [33]. The culture medium in the 96-well plates was replaced with fresh medium containing 100 μ M L-Glu. After 30 min, 50 μ L of the culture medium in each well was collected. The L-Glu concentration was