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Hepatic maturation of human iPS cell-derived hepatocyte-like cells by ATF5, c/EBPα, and PROX1 transduction

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ABSTRACT

Hepatocyte-like cells differentiated from human iPS cells (human iPS-HLCs) are expected to be utilized in drug development and research. However, recent hepatic characterization of human iPS-HLCs showed that these cells resemble fetal hepatocytes rather than adult hepatocytes. Therefore, in this study, we aimed to develop a method to enhance the hepatic function of human iPS-HLCs. Because the gene expression levels of the hepatic transcription factors (activating transcription factor 5 (ATF5), CCAAT/enhancer-binding protein alpha (c/EBPα), and prospero homeobox protein 1 (PROX1)) in adult liver were significantly higher than those in human iPS-HLCs and fetal liver, we expected that the hepatic functions of human iPS-HLCs could be enhanced by adenovirus (Ad) vector-mediated ATF5, c/EBPα, and PROX1 transduction. The gene expression levels of *cytochrome P450 (CYP) 2C9*, *2E1*, *alpha-1 antitrypsin*, *transthyretin*, *Na+/taurocholate cotransporting polypeptide*, and *uridine diphosphate glucuronosyl transferase 1A1* and protein expression levels of CYP2C9 and CYP2E1 were upregulated by ATF5, c/EBPα, and PROX1 transduction. These results suggest that the hepatic functions of the human iPS-HLCs could be enhanced by ATF5, c/EBPα, and PROX1 transduction. Our findings would be useful for the hepatic maturation of human iPS-HLCs.

1. Introduction

The liver is an important organ in term of the absorption, distribution, metabolism, and excretion of drugs. Therefore, to develop safe and effective drugs, primary human hepatocytes (PHHs) are widely used in the early stage of drug development. However, alternative hepatocyte resources for drug screening are required because PHHs are costly and not abundantly available. Hepatocyte-like cells (HLCs) that are generated from human induced pluripotent stem (iPS) cells are expected to be used in drug screening in place of PHHs. We previously reported that the stage-specific transient transduction of hepatic transcription factors mediated by adenovirus (Ad) vector was useful for promoting hepatic differentiation [1–4]. However, the drug metabolism capacity of human iPS-HLCs is still lower than that of PHHs [4]. In addition, Baxter

Abbreviations: Human iPS-HLCs, Human induced pluripotent stem cells-derived hepatocyte-like cells; HLCs, Hepatocyte-like cells; ATF5, Activating transcription factor 5; c/EBPα, CCAAT/enhancer-binding protein alpha; PROX1, Prospero homeobox protein 1; 3 TFs, The three transcription factors (ATF5, c/EBPα, and PROX1); Ad, Adenovirus; CYP, cytochrome P450; αAT, alpha-1 antitrypsin; TTR, transthyretin; NTCP, Na+/taurocholate cotransporting polypeptide; UGT1A1, Uridine diphosphate glucuronosyltransferase 1A1; HNF, Hepatocyte nuclear factor; FGF, Fibroblast growth factor; FBS, Fetal bovine serum; BMP, Bone morphogenetic protein; HCM, Hepatocyte culture medium; EGF, Epidermal growth factor; HGF, Hepatocyte growth factor; OsM, Oncostatin M; BSA, Bovine serum albumin; FOXA2, Forkhead box protein A2; AhR, Aryl hydrocarbon receptor; CAR, Constitutive androstane receptor; PPARα, Peroxisome proliferator-activated receptor alpha; LXR, Liver X receptor.

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et al. have shown by means of phonotypic and functional analyses that the human iPS-HLCs better mimic fetal hepatocytes rather than adult hepatocytes [5]. Therefore, it is necessary to promote the hepatic maturation of human iPS-HLCs.

Recently, Du et al. showed that metabolically functioning human induced hepatocytes (hiHeps) can be converted from fibroblasts by overexpressing the hepatic fate conversion factors hepatocyte nuclear factor 1 alpha, 4 alpha, and 6 (HNF1α, HNF4α, HNF6, respectively) [6]. In addition, they demonstrated that hiHeps could be further maturated by overexpressing activating transcription factor 5 (ATF5), CCAAT/enhancer-binding protein alpha (c/EBPα), and prospero homeobox protein 1 (PROX1). Consistently, all the hepatic transcription factors (ATF5, c/EBPα, and PROX1) are known to play an important role in the maturation of hepatocytes and maintenance of hepatic function. Specifically, it is known that the level of cytochrome P450 (CYP) 2B6 expression in hepatocytes is regulated by ATF5, and also cooperatively regulated with the hepatic nuclear receptor, constitutive androstane receptor (CAR) [7]. The hepatic functions, such as albumin secretion and urea production, are positively regulated by c/EBPα [8]. It has also been reported that PROX1 plays a key role in the metabolic maturation of hepatocytes, as well as FOXA2 [9]. Considering these results together, we expected that the hepatic functions of human iPS-HLCs could be improved by the transduction of three transcription factors, ATF5, c/EBPα, and PROX1.

In this study, we attempt to produce matured human iPS-HLCs by Ad vector-mediated ATF5, c/EBPα, and PROX1 (3TFs) transduction. To investigate whether the hepatic functions were enhanced, we analyzed the expression of hepatocyte markers in the Ad-3TFs-transduced human iPS-HLCs. Moreover, the protein expression levels of CYP2C9, CYP2E1, and α-1 antitrypsin (αAT) were examined by western blotting and immunofluorescent staining.

2. Materials and methods

2.1. Human iPS cells

Human iPS cells generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Dotcom, JCRB Number: JCRB1327). Human iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with ReproStem (ReproCELL) supplemented with 10 ng/ml fibroblast growth factor (FGF) 2 (KATAYAMA Kogyo Kagaku).

2.2. Hepatocyte differentiation

Before the initiation of hepatocyte differentiation, human iPS cells were dissociated into clumps by using dispase (Roche Diagnostics) and plated onto BD Matrigel Basement Membrane Matrix Growth Factor Reduced (Becton, Dickinson and Company). These cells were cultured in the mouse embryo fibroblasts-conditioned medium for 3–4 days. The differentiation protocol for the induction of definitive endoderm cells, hepatoblast-like cells, and human iPS-HLCs was based on our previous reports with some modifications [10]. Briefly, in the definitive endoderm differentiation, human iPS cells were cultured with the L-Wnt3A-expressing cell (CRL2647; ATCC)-conditioned RPMI1640 medium (Sigma) containing 100 ng/mL Activin A (R&D Systems), 1% GlutaMAX (Invitrogen), 0.2% fetal bovine serum (FBS), and 1 × B27 Supplement Minus Vitamin A (Invitrogen) for 4 days. For the induction of hepatoblasts, the definitive endoderm cells were cultured with RPMI1640 medium containing 30 ng/mL bone morphogenetic protein 4 (BMP4) (R&D Systems) and 20 ng/mL FGF4 (R&D Systems), 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A for 5 days. To perform the hepatocyte differentiation, the hepatoblast-like cells were cultured with RPMI1640 medium containing 20 ng/mL hepatocyte growth factor (HGF) (R&D Systems), 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A for 5 days. Finally, the cells were cultured with hepatic maturation medium (hepatic maturation medium consists of Hepatocyte Culture Medium (HCM; Lonza, without epidermal growth factor (EGF)) containing 20 ng/mL oncostatin M (OsM) and 3% GlutaMAX) for 11 days. To promote hepatic maturation, human iPS-HLCs were transduced with 1000 VP/cell of Ad-ATF5, Ad-c/EBPα, and Ad-PROX1 (total 3000 VP/cell) for 1.5 h on day 25 and were cultured with hepatic maturation medium for 5 days.

2.3. Ad vector

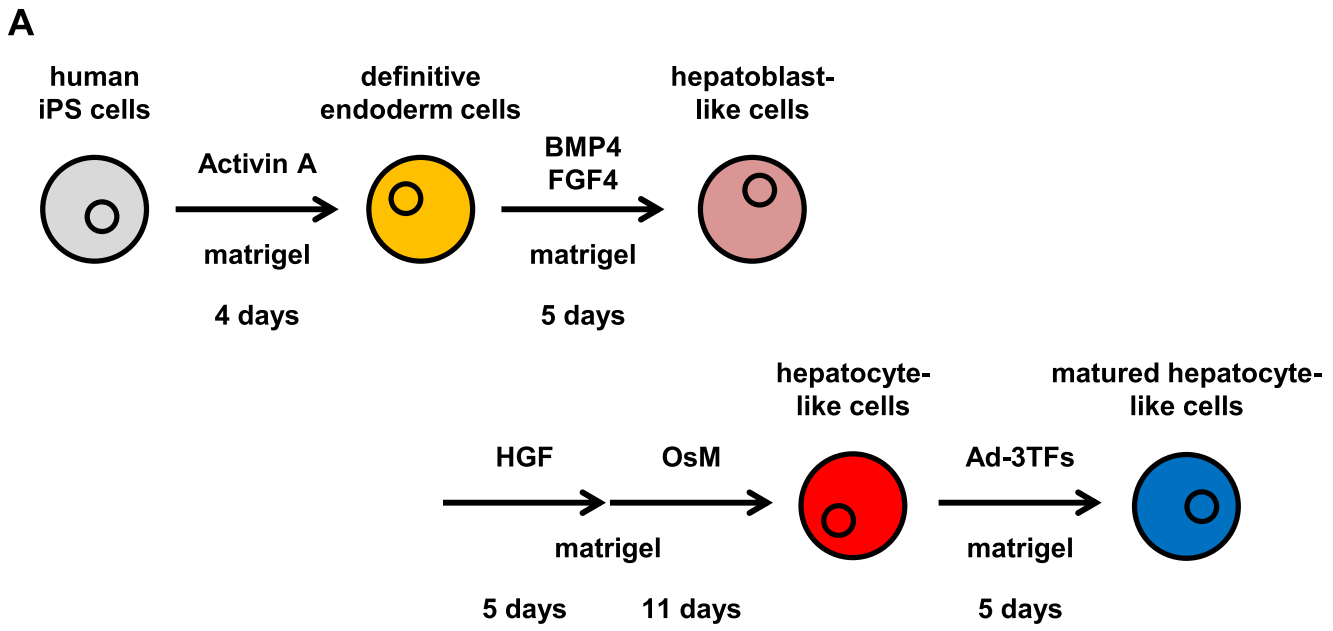
Ad vectors were constructed by an improved *in vitro* ligation method [11,12]. The human ATF5 and PROX1 gene (accession number NM_ 001193646 and NM_ 001270616, respectively) were amplified by PCR using primers: ATF5 Fwd 5′- TGtctagaCCACCATGTCACTCCTGGCGACCCTG -3′ and ATF5 Rev 5′-AAgcggccgcCTAGCAGCTACGGGTCCTCTG -3’; PROX1 Fwd 5′- AGtctagaCCACCATGCCTGACCATGACAGCAC -3′ and PROX1 Rev 5′-TAgcggccgcCTACTCATGAAGCAGCTCTTG -3’. The human ATF5 and PROX1 gene was inserted into pHMEF5 [13], which contains the human elongation factor-1α (EF-1α) promoter, resulting in pHMEF-ATF5 and -PROX1, respectively. The pHMEF-ATF5 and -PROX1 was digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested pAdHM41-K7 [14], resulting in pAd-ATF5 and pAd-PROX1, respectively. The human EF-1α promoter-driven LacZ- and c/EBPα-expressing Ad vectors (Ad-LacZ and Ad-c/EBPα, respectively) were constructed previously [15,16]. Ad-LacZ, Ad-ATF5, Ad-c/EBPα, and Ad-PROX1, 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 iPS cells, definitive endoderm cells, and hepatoblast-like cells were used in this study. The vector particle (VP) titer was determined by using a spectrophotometric method [17].

2.4. Real-time RT-PCR

Total RNA was isolated from human iPS cells and their derivatives using ISOGENE (NIPPON GENE). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus real-time PCR system (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* (*GAPDH*). The primer sequences used in this study are described in Table S1. Fetal liver and Adult liver total RNA were purchased from Clontech and BioChain, respectively.

2.5. Western blotting

The human iPS cell-derivatives were homogenized with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing a protease inhibitor mixture (Sigma). After being frozen and thawed, the homogenates were centrifuged at 15,000 *g* at 4 °C for 10 min, and the supernatants were collected. The lysates were subjected to SDS-PAGE on 7.5% polyacrylamide gel, and then transferred onto polyvinylidene fluoride membranes (Millipore). After the reaction was blocked with 1% skim milk in TBS containing 0.1% Tween 20 (Sigma) at room temperature for 1 h, the membranes were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HGF
OsM
matrigel
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11 days
Ad-3TFs
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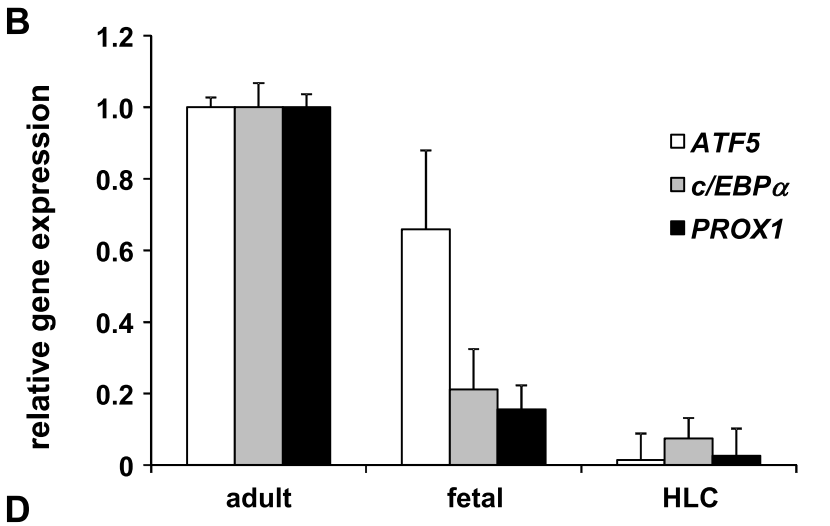
B
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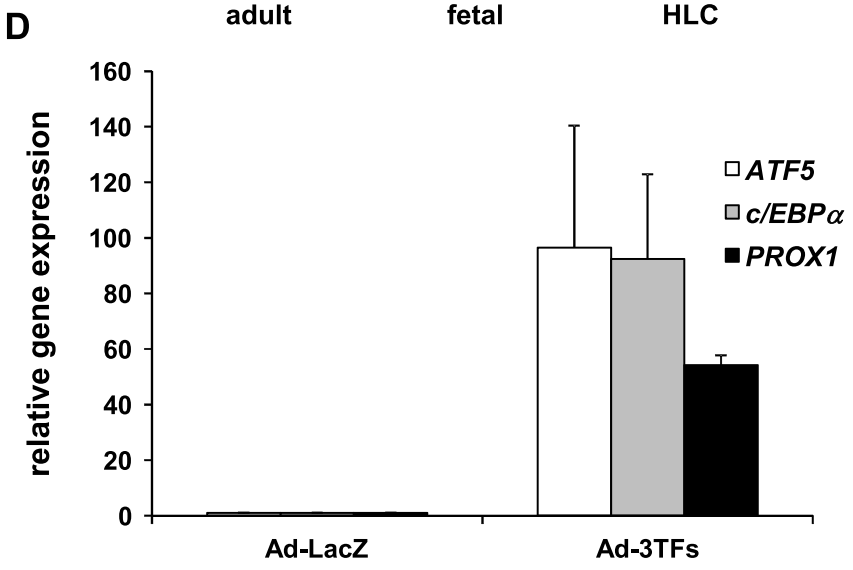
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ATF5
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PROX1
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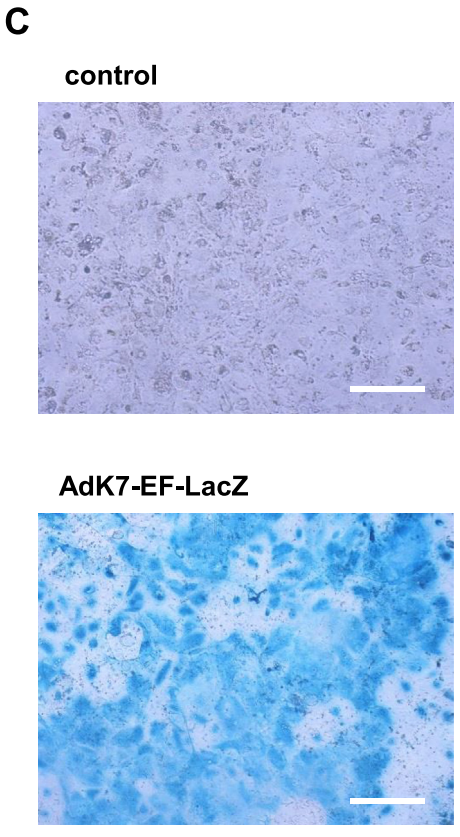
Ad-3TFs


C
control
AdK7-EF-LacZ

incubated with anti-human CYP2C9, 2E1, or β-actin antibodies at 4 °C overnight, followed by reaction with horseradish peroxidase-conjugated anti-mouse or anti-goat IgG antibodies at room temperature for 1 h. All the antibodies are listed in Table S2. The band was visualized by ECL Plus Western blotting detection reagents (GE Healthcare) and the signals were read using an LAS-4000 imaging system (Fuji Film).

2.6. Immunohistochemistry

To perform the immunohistochemistry, the cells were fixed with 4% paraformaldehyde (PFA, Wako) in PBS for 20 min. After incubation with 0.1% Triton X-100 (Sigma) in PBS for 10 min, the cells were blocked with PBS containing 2% FBS and 2% bovine serum albumin (BSA) for 50 min, the cells were incubated with a primary antibody at 4 °C overnight, and finally, incubated with a secondary antibody at room temperature for 1 h. All the antibodies are listed in Table S3.

2.7. X-gal staining

Human iPS-HLCs were transduced with Ad-LacZ at 3000 VP/cell for 1.5 h. After culturing for 2 days, *5-bromo-4-chloro-3-indolyl* β-D-*galactopyranoside* (X-Gal) (NIPPON GENE) staining was performed as described previously [13].

3. Results

3.1. Expression analysis of ATF5, c/EBPα, and PROX1 among human iPS-HLCs, fetal liver, and adult liver

To generate the human iPS-HLCs, hepatic differentiation was performed according to the protocol shown in Fig. 1A. Because the expression levels of ATF5, c/EBPα and PROX1 (three transcription factors: 3TFs) in the human iPS-HLCs and fetal liver were lower than those in adult liver (Fig. 1B), we expected that the hepatic functions of the human iPS-HLCs would be enhanced by 3TFs overexpression. To overexpress 3TFs in the human iPS-HLCs, we produced an ATF5-expressing Ad vector (Ad-ATF5), a c/EBPα-expressing Ad vector (Ad-c/EBPα), and a PROX1-expressing Ad vector (Ad-PROX1). First, we prepared LacZ-expressing Ad vector (Ad-LacZ) to examine the transduction efficiency in the human iPS-HLCs. The human iPS-HLCs were transduced with Ad-LacZ at 3000 VP/cell, and LacZ expression in the cells was examined by X-Gal staining. The Ad-LacZ-transduced human iPS-HLCs homogeneously expressed LacZ (Fig. 1C). This result indicated that the transgene could be efficiently overexpressed in the human iPS-HLCs by using our Ad vectors. Next, the human iPS-HLCs were transduced with Ad-ATF5, Ad-c/EBPα, and Ad-PROX1 at 1000 VP/cell per each Ad vector. We confirmed that 3TFs overexpression was successfully performed by using Ad-3TFs (Fig. 1D).

3.2. Hepatic maturation of the human iPS-HLCs by transduction of ATF5, c/EBPα, and PROX1

To examine whether the human iPS-HLCs could be maturated by ATF5, c/EBPα, and PROX1 transduction, the human iPS-HLCs were transduced with Ad-ATF5, Ad-c/EBPα, and Ad-PROX1 (Ad-3TFs) on day 25 of differentiation. At 5 days after the transduction, the gene expression levels of hepatocyte-related markers, including major drug metabolizing enzymes, in the human iPS-HLCs were examined by real-time RT-PCR. The gene expression levels of *CYP2C9*, *CYP2E1*, *αAT*, *transthyretin* (*TTR*), *Na+/taurocholate cotransporting polypeptide* (*NTCP*), and *uridine diphosphate glucuronosyl transferase 1A1* (*UGT1A1*) in the Ad-3TFs-transduced human iPS-HLCs were significantly higher than those in the Ad-LacZ-transduced human iPS-HLCs (Fig. 2A). The protein expression levels of CYP2C9 and CYP2E1 were also upregulated by Ad-3TFs transduction (Fig. 2B). Moreover, immunofluorescent staining of αAT showed that its expression level in the human iPS-HLCs was enhanced by 3TFs transduction (Fig. 2C). Interestingly, the gene expression levels of *forkhead box protein A2* (*FOXA2*), *HNF1α*, *HNF4α*, *aryl hydrocarbon receptor* (*AhR*), *constitutive androstane receptor* (*CAR*), *peroxisome proliferator-activated receptor alpha* (*PPARα* and *liver X receptor* (*LXR*) were also increased (Fig. 3). These results suggest that the hepatic functions of the human iPS-HLCs were enhanced by 3TFs transduction.

4. Discussion

In this study, the human iPS-HLCs were transduced with Ad-3TFs to enhance the hepatic functions of these cells. As shown in Fig. 2, the gene expression levels of hepatic markers (*CYP2C9*, *2E1*, *αAT*, *TTR*, *NTCP*, and *UGT1A1*), the protein expression levels of hepatic markers (CYP2C9, 2E1, and αAT), and the gene expression levels of hepatic transcription factors and nuclear receptors (*FOXA2*, *HNF1α*, *HNF4α*, *GATA4*, *AhR*, *CAR*, *PPARα*, and *LXR*) were upregulated by Ad-3TFs transduction.

We consider that the hepatic functions of human iPS-HLCs could be successfully enhanced by 3TFs, because the expression of many hepatocyte transcription factors and nuclear receptors that play crucial roles in hepatocyte differentiation and maturation were significantly upregulated (Fig. 3). Specifically, the gene expression levels of *HNF4α*, *FOXA2*, *HNF1α*, and *LXR* were increased by Ad-3TFs transduction. It has been shown that the maturation of hepatocytes from hepatoblasts [3], direct reprogramming of fibroblasts into hepatocytes [6,18], and transdifferentiation into hepatocytes [19] could be promote by HNF4α overexpression. It is also known that the combination of two transcription factors, FOXA2 and HNF1α, promoted efficient hepatic differentiation from human iPS cells [4]. Finally, the transcription factor LXR was shown to contribute to the differentiation of functional hepatocytes from HepaRG cells [20]. Taken together, these results suggest that the Ad-3TFs transduction could enhance the hepatic functions of human iPS-HLCs through upregulation of *HNF4α*, *FOXA2*, *HNF1α*, and *LXR* expression levels.

We have previously reported that the hepatocyte differentiation of human iPS cells could be promoted by overexpression of hepatic

Fig. 1. Efficient Ad vector-mediated hepatic gene transfer into the human iPS cell-derived hepatocyte-like cells. (**A**) The procedure for differentiation of human iPS cells (Dotcom) into hepatocyte-like cells (human iPS-HLCs) via definitive endoderm cells and hepatoblast-like cells is presented schematically. The human iPS-HLCs (day 25) were transduced with 1000 VP/cell of three transcription factors (3TFs; ATF5, c/EBPα, and PROX1)-expressing Ad vector (Ad-3TFs; total 3000 VP/cell) for 1.5 h, and cultured until day 30. Details of the hepatic differentiation procedure are described in the Materials and Methods section. (**B**) The gene expression levels of *ATF5*, *c/EBPα*, and *PROX1* in human iPS-HLCs, fetal liver, and adult liver were examined by real-time RT-PCR. On the y axis, the gene expression level in adult liver was taken as 1.0. (**C**) Human iPS cells were differentiated into human iPS-HLCs according to the protocol described in Fig. 1A, and then transduced with 3000 VP/cell of Ad-LacZ for 1.5 h. On day 27, X-gal staining was performed. The scale bars represent 50 μm. (**D**) The gene expression levels of *ATF5*, *c/EBPα*, and *PROX1* in Ad-LacZ- or Ad-3TFs-transduced cells were examined by real-time RT-PCR. On the y axis, the gene expression levels in Ad-LacZ-transduced cells were taken as 1.0. All data are represented as means ± SD ($n = 3$).

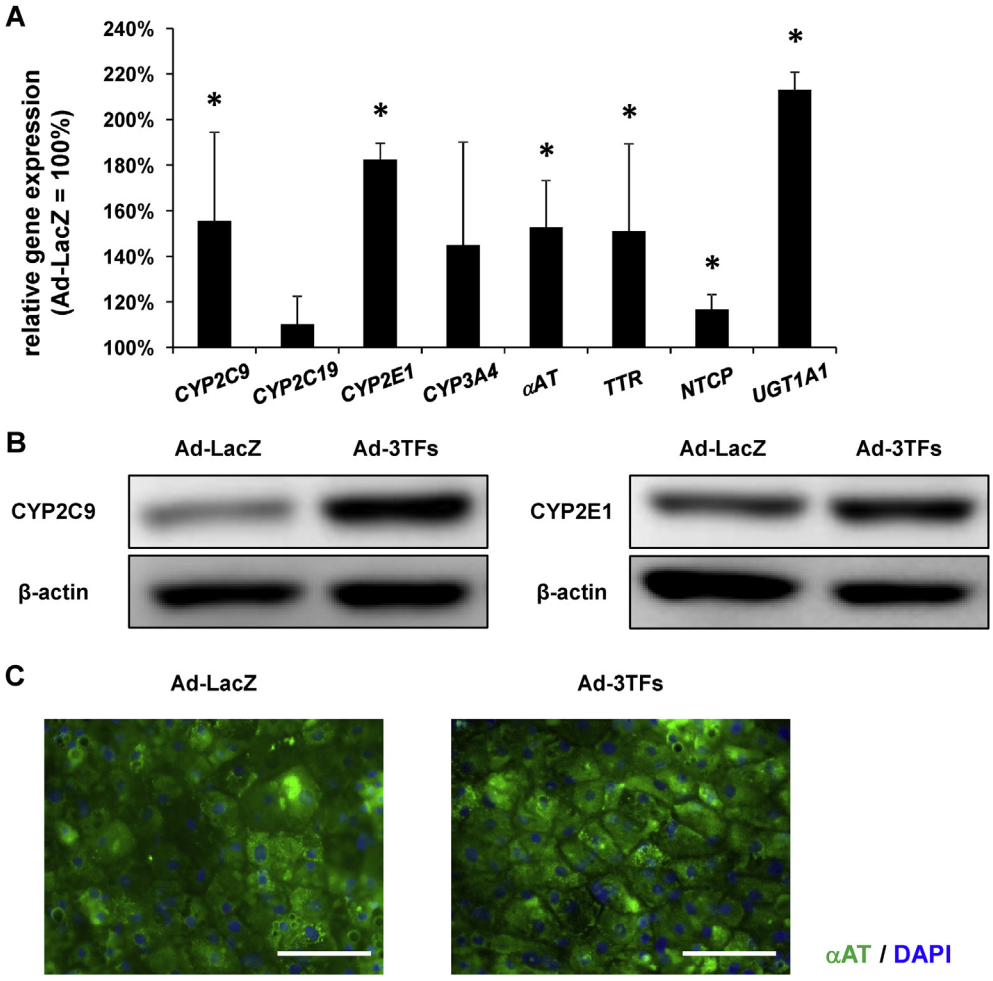


Fig. 2. Hepatic maturation of human iPS cell-derived hepatocyte-like cells by ATF5, c/EBPα, and PROX1 transduction. Human iPS cells (Dotcom) were differentiated into human iPS-HLCs as described in Fig. 1A. (**A**) The gene expression levels of *CYP2C9*, *2C19*, *2E1*, *3A4*, *αAT*, *TTR*, *NTCP*, and *UGT1A1* in Ad-LacZ- or Ad-TFs-transduced cells were examined by real-time RT-PCR. On the y axis, the expression levels in Ad-LacZ-transduced cells were taken as 100. (**B**) The protein expression levels of CYP2C9 and CYP2E1 in Ad-LacZ- or Ad-TFs-transduced cells were examined by western blotting. (**C**) Ad-LacZ- or Ad-TFs-transduced cells were subjected to immunostaining with anti-αAT (green) antibodies. Nuclei were counterstained with DAPI (blue). The scale bars represent 50 μm. All data are represented as means ± SD ($n = 3$).

transcription factors [1–4]. For these studies, we used hepatic transcription factors that are known to be involved in fetal liver development. In the present study, our focus was on hepatic transcription factors that play an important role in postnatal liver development, and these genes were transduced into the human iPS-HLCs (Fig. 1B). Because it is known that hepatic functions are still rapidly enhanced during the postnatal period [21], further maturated human iPS-HLCs might be possible to generate by establishing a differentiation protocol which mimic the environment of postnatal liver.

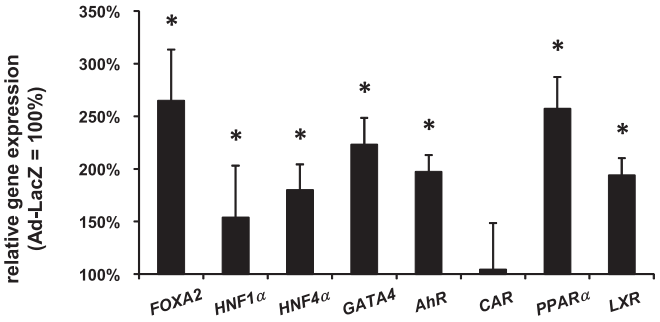


Fig. 3. Gene expression analysis of hepatic transcription factors and nuclear receptors in the human iPS cell-derived hepatocyte-like cells. Human iPS cells (Dotcom) were differentiated into human iPS-HLCs as described in Fig. 1A. The gene expression levels of *FOXA2*, *HNF1α*, *HNF4α*, *GATA4*, *AhR*, *CAR*, *PPARα* and *LXR* in Ad-LacZ- or Ad-TFs-transduced cells were examined by real-time RT-PCR. On the y axis, the expression levels in Ad-LacZ-transduced cells were taken as 100. All data are represented as means ± SD ($n = 3$).

In summary, we succeeded in generating more matured human iPS-HLCs by overexpressing 3TFs. The expression levels of hepatic markers, including drug metabolism enzymes and hepatic transporters, were upregulated by Ad-3TFs transduction. We believe that these more matured human iPS-HLCs would be useful tools for drug screening and evaluation of drug response.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.12.007.

Conflict of interest statement

The authors declare no competing financial interests.

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