

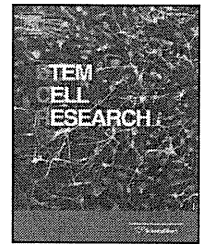
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Appendix A. Supplementary data

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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SHORT REPORT

Promotion of hematopoietic differentiation from mouse induced pluripotent stem cells by transient HoxB4 transduction

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Abstract Ectopic expression of HoxB4 in embryonic stem (ES) cells leads to an efficient production of hematopoietic cells, including hematopoietic stem/progenitor cells. Previous studies have utilized a constitutive HoxB4 expression system or tetracycline-regulated HoxB4 expression system to induce hematopoietic cells from ES cells. However, these methods cannot be applied therapeutically due to the risk of transgenes being integrated into the host genome. Here, we report the promotion of hematopoietic differentiation from mouse ES cells and induced pluripotent stem (iPS) cells by transient HoxB4 expression using an adenovirus (Ad) vector. Ad vector could mediate efficient HoxB4 expression in ES cell-derived embryoid bodies (ES-EBs) and iPS-EBs, and its expression was decreased during cultivation, showing that Ad vector transduction was transient. A colony-forming assay revealed that the number of hematopoietic progenitor cells with colony-forming potential in HoxB4-transduced cells was significantly increased in comparison with that in non-transduced cells or LacZ-transduced cells. HoxB4-transduced cells also showed more efficient generation of CD41⁺, CD45⁺, or Sca-1⁺ cells than control cells. These results indicate that transient, but not constitutive, HoxB4 expression is sufficient to augment the hematopoietic differentiation of ES and iPS cells, and that our method would be useful for clinical applications, such as cell transplantation therapy.

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Introduction

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, each of which is derived from the inner cell mass of blastocysts and somatic cells by transducing three or four

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transcription factors, respectively, can differentiate into various types of cells *in vitro*. They are thus considered as a valuable model to understand the processes involved in the differentiation of lineage-committed cells as well as an unlimited source of cells for therapeutic applications such as hematopoietic stem/progenitor cell (HSPC) transplantation (Evans and Kaufman, 1981; Thomson et al., 1998; Keller, 2005; Takahashi and Yamanaka, 2006; Takahashi et al., 2007).

Differentiation of ES and iPS cells into mature hematopoietic cells, including erythrocytes, myeloid cells, and lymphoid cells, has been performed by embryoid body (EB) formation or coculture with stromal cells (Nakano et al., 1994; Chadwick et al., 2003; Schmitt et al., 2004; Vodyanik et al., 2005). However, the development of an efficient differentiation method for immature hematopoietic cells, including HSPCs, from ES and iPS cells has been challenging. Previously, Daley and his colleagues have shown that enforced expression of HoxB4 in mouse ES cells by a retrovirus vector robustly enhanced the differentiation of ES cells into HSPCs *in vitro*, and these ES cell-derived HSPCs had a long-term reconstitution potential *in vivo* (Kyba et al., 2002; Wang et al., 2005). In addition, constitutive expression of HoxB4 was shown to induce the hematopoietic differentiation from human ES cells (Bowles et al., 2006). These findings indicated that manipulation of HoxB4 expression would be effective for production of HSPCs from ES and iPS cells. However, it is known that long-term constitutive HoxB4 expression in HSPCs has an inhibitory effect on the differentiation of certain hematopoietic lineages, such as lymphoid cells and erythroid cells (Kyba et al., 2002; Pilat et al., 2005), and can lead to a significant risk of leukemogenesis in large animals (Zhang et al., 2008). Although a tetracycline-inducible HoxB4 expression system has been utilized to overcome these unwanted effects, this gene expression system is complex, and cannot be directly applied to therapeutic use. Foreign genes can be integrated into the host chromosome in a stable gene expression system that includes a tetracycline-regulated system, and this could cause an increased risk of cellular transformation (Li et al., 2002; Hacein-Bey-Abina et al., 2003; Williams and Baum, 2004). Therefore, to apply ES cell- and iPS cell-derived HSPCs to clinical medicine, development of a simple and transient HoxB4 transduction method in ES and iPS cells is required.

We have utilized an adenovirus (Ad) vector as a tool for transduction of functional genes into stem cells, because Ad vectors are relatively easy to construct, can be produced at high titers, and mediate efficient and transient gene expression in both dividing and nondividing cells. We have demonstrated that Ad vectors could efficiently transduce a foreign gene in stem cells, including ES and iPS cells (Kawabata et al., 2005; Tashiro et al., 2009, 2010). We also succeeded in promoting the differentiation of osteoblasts, adipocytes, or hepatoblasts from ES and iPS cells by Ad vector-mediated transient transduction of Runx2, PPAR γ , or Hex, respectively (Tashiro et al., 2009, 2008; Inamura et al., 2011).

Our data led us to examine whether HSPCs could also be efficiently differentiated from ES and iPS cells by Ad vector-mediated transduction of a HoxB4. In the present study, we investigated whether or not differentiation of HSPCs from mouse ES and iPS cells could be promoted by

transient HoxB4 expression. Our results showed that Ad vector-mediated transient HoxB4 expression in mouse ES and iPS cells are sufficient to augment the differentiation of hematopoietic cells, including HSPCs, from mouse ES and iPS cells. This result indicates that an Ad vector-mediated transient gene expression system would be a powerful and safe tool to induce hematopoietic differentiation from mouse ES and iPS cells.

Results

Transduction with Ad vectors in ES-EBs or iPS-EBs

A previous study using a tetracycline-inducible HoxB4 expression system showed that hematopoietic stem/progenitor cells (HSPCs) were generated by induction of HoxB4 expression in ES cell-derived embryoid bodies (ES-EBs) from day 4 to day 6 of differentiation (Kyba et al., 2002), suggesting that HoxB4 expression within this time range would be effective for induction of hematopoietic cells. In addition, CD41⁺c-kit⁺ cells in EBs are reported to be early hematopoietic progenitor cells (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003). Thus, we planned to transduce HoxB4 in total cells derived from ES- or iPS-EBs on day 5 of differentiation or in CD41⁺c-kit⁺ cells derived from ES- or iPS-EBs on day 6. We initially investigated the expression of coxsackievirus and adenovirus receptor (CAR) in ES-EB- or iPS-EB-derived cells, because CAR was indispensable for transduction of an exogenous gene using Ad vector (Bergelson et al., 1997; Tomko et al., 1997). Flow cytometric analysis showed the expression of CAR in ES-EB- and iPS-EB-derived total cells and CD41⁺c-kit⁺ cells, although the expression levels of CAR in CD41⁺c-kit⁺ cells were decreased in comparison with that in total cells (Figs. 1a and b). These results indicate that ES-EB- and iPS-EB-derived total cells and CD41⁺c-kit⁺ cells could be transduced with Ad vectors. We also observed the expression of green fluorescent protein (GFP) in iPS-EB-derived total cells. Because the mouse iPS cells used in this study express GFP under the control of Nanog promoter (Okita et al., 2007), the existence of GFP-positive cells showed that undifferentiated iPS cells would still be present in iPS-EB-derived total cells.

We next examined the transduction efficiency in EB-derived total cells or EB-derived CD41⁺c-kit⁺ cells using DsRed- or GFP-expressing Ad vectors, respectively. After transduction with Ad-DsRed or Ad-GFP at 3000 vector particles (VPs)/cell, the cells were cultured with the hematopoietic cytokines for 2 days. The results showed that, at 3000 VPs/cell, approximately 60% or 40% of the EB-derived total cells or EB-derived CD41⁺c-kit⁺ cells, respectively, expressed transgenes (Figs. 1c and d). Although the number of transgene-expressing cells was increased in the case of transduction with Ad vectors at 10,000 VPs/cell, the number of viable cells was markedly reduced (data not shown). Therefore, we decided to use Ad vectors at 3000 VPs/cell for transducing human HoxB4 (hHoxB4) into ES-EBs and iPS-EBs. RT-PCR analysis on day 3 after transduction with Ad-hHoxB4 into EB-derived total cells showed an elevation of hHoxB4 mRNA expression in hHoxB4-transduced cells, while neither non-transduced cells nor LacZ-transduced cells showed hHoxB4 expression (Fig. 1e). Importantly, the expression level of hHoxB4 in the cells was markedly decreased on day 6 after Ad

transduction. This result showed that the ES-EB- or iPS-EB-derived cells could express transgenes by Ad vectors, and that Ad vector mediated the transient transgene expression in these cells.

Transient HoxB4 expression augments the generation of hematopoietic cells from mouse ES and iPS cells

To induce and expand the hematopoietic cells from the iPS cell line 38C2, EB-derived total cells were plated and cultured on OP9 stromal cells with the hematopoietic cytokines. On day 10 after plating on OP9 cells, the number of 38C2-derived hematopoietic cells in LacZ-transduced cells was similar to that in non-transduced cells. On the other hand, transient transduction of HoxB4 with Ad-hHoxB4 resulted in a significant increase in the number of hematopoietic cells compared with non-transduced cells or LacZ-transduced cells (Fig. 2a, middle). Likewise, an increase in the hematopoietic cell number by Ad vector-mediated hHoxB4 transduction was also observed in ES cell derived-hematopoietic cells or the other iPS line 20D17-derived hematopoietic cells (Fig. 2a, left and right). Additionally, ES-EB- or iPS-EB-derived CD41⁺c-kit⁺ cells, which were transiently transduced with hHoxB4, could proliferate on OP9 stromal cells for over 20 days (Fig. 2b). This result is mostly in agreement with the previous report that ES cell-derived hematopoietic cells stably expressing HoxB4 had a growth advantage in the presence of hematopoietic cytokines (Pilat et al., 2005). Transient, but not stable, HoxB4 expression in ES-EB- or iPS-EB-derived cells would be sufficient to augment the generation of hematopoietic cells from ES and iPS cells.

We next investigated the surface antigen expression in non-transduced cells, LacZ-transduced cells, or hHoxB4-transduced cells after expansion on OP9 stromal cells. Flow cytometric analysis revealed an increase of CD45 and CD41 expressions in HoxB4-transduced cells, compared with non-transduced cells and LacZ-transduced cells (Figs. 3a and b). CD45 is known as a marker of hematopoietic cells. In both *in vitro* ES cell differentiation and a developing mouse embryo, the expression of CD45 was developmentally controlled, and CD45 expression was observed on hematopoietic cells after expression of CD41 (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003). Thus, a higher percentage of CD45⁺ cells in HoxB4-transduced cells would be due, at least in part, to an increase of CD41 expression in HoxB4-transduced cells relative to non-transduced cells and LacZ-transduced cells. We also

found a significant elevation of Sca-1 in hHoxB4-transduced cells (Figs. 3a and b). Sca-1 is expressed in fetal and adult HSPCs (Arai et al., 2004; McKinney-Freeman et al., 2009), although Sca-1 expression was observed in other types of cells. Therefore, our data suggest that immature hematopoietic cells would be generated in hHoxB4-transduced cells more efficiently than in non-transduced cells or LacZ-transduced cells.

In parallel with the flow cytometric analysis, we also analyzed the expression levels of hematopoietic marker genes in iPS cell-derived hematopoietic cells by RT-PCR (Fig. 3c). The expression levels of marker genes in LacZ-transduced cells were mostly equal to those in non-transduced cells. In contrast, among the genes we assayed, the expression levels of *Gata-1*, *c-myb*, and *Cxcr4* mRNA were slightly but significantly up-regulated in hHoxB4-transduced cells. GATA-1 reflects early hematopoietic development, whereas c-Myb is a marker of definitive hematopoiesis (Godin and Cumano, 2002). Increased expression of these genes in HoxB4-transduced cells suggests that transient hHoxB4 expression promotes the production of both primitive and definitive hematopoietic progenitor cells from mouse ES and iPS cells. We could not detect the hHoxB4 mRNA expression in Ad-hHoxB4-transduced cells, confirming the transient hHoxB4 expression by Ad vectors (Fig. 3c).

HoxB4 expression enhances development of hematopoietic progenitor cells from mouse ES and iPS cells

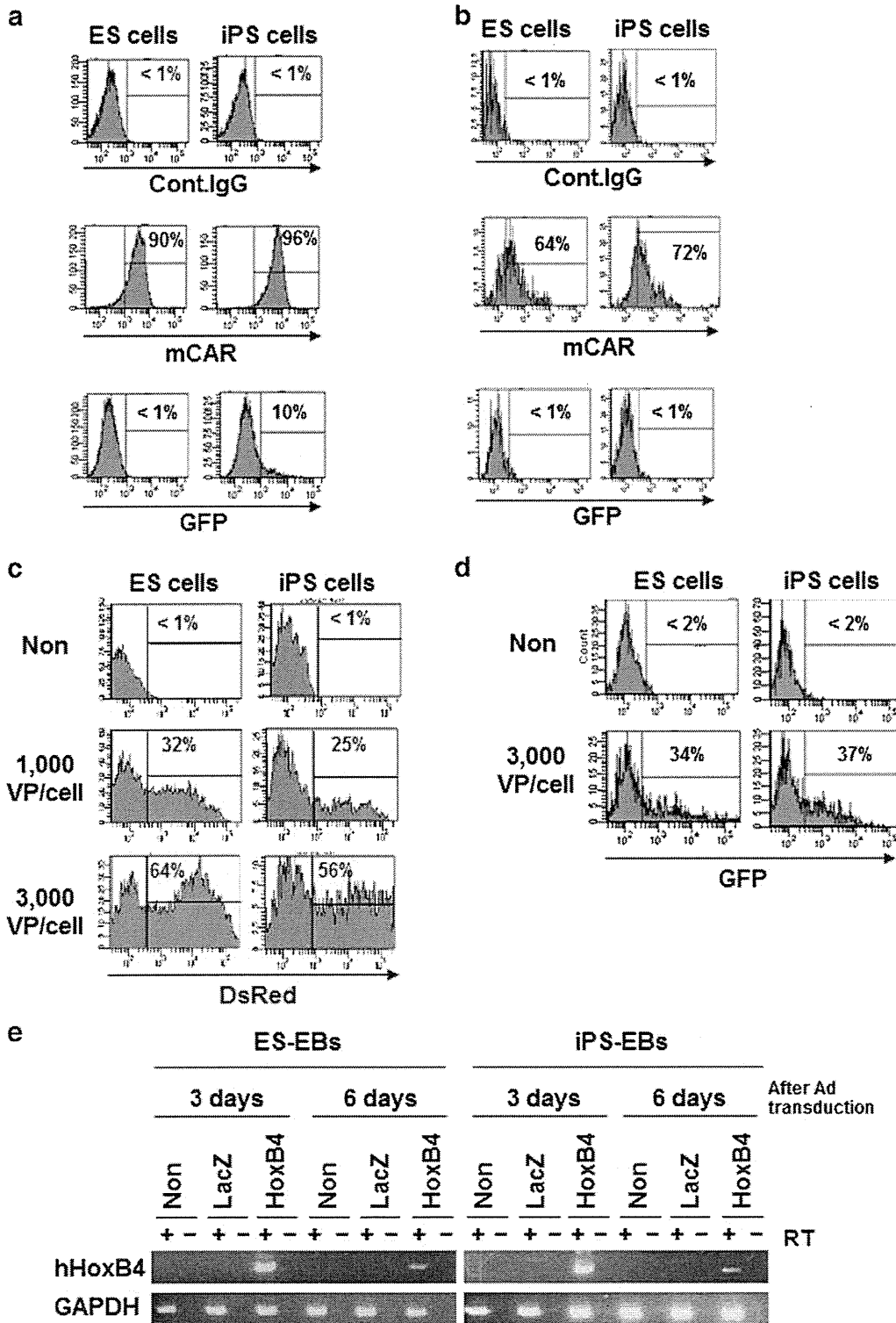
To examine whether hematopoietic immature cells with hematopoietic colony-forming potential could be generated from ES and iPS cells, ES cell-derived hematopoietic cells and iPS cell-derived hematopoietic cells, both of which were cultured on OP9 stromal cells for 10 days, were plated and cultured in methylcellulose-containing media with hematopoietic cytokines. Without Ad transduction, the number of total hematopoietic colonies in the iPS cell line 38C2 was five times as high as that in ES cells, whereas another iPS cell line, 20D17, had nearly the same hematopoietic differentiation potential as ES cells (Fig. 4a). These results indicate that there is a difference in hematopoietic differentiation potential among iPS cell lines.

We next examined the hematopoietic colony potential in LacZ-transduced cells or HoxB4-transduced cells. The colony assay revealed a significant increase in the number of total hematopoietic colonies in hHoxB4-transduced cells compared with control cells, whereas there was no significant difference in the number of hematopoietic colonies between

Figure 1 Transduction with Ad vectors in ES-EB- or iPS-EB-derived cells. (a, b) The expression levels of CAR, a primary receptor for Ad, in ES-EB- or iPS-EB-derived total cells (a) or CD41⁺c-kit⁺ cells (b) were detected with anti-mouse CAR monoclonal antibody by flow cytometric analysis. As a negative control, the cells were incubated with an irrelevant antibody. Data shown are from one representative experiment of three performed. (c, d) EB-derived total cells (c) or CD41⁺c-kit⁺ cells (d), purified by FACS (Supplemental Fig. 1), were transduced with Ad-DsRed or Ad-GFP for 1.5 h, and transgene-expressing cells were then analyzed by flow cytometry. Because CD41⁺c-kit⁺ cells do not express GFP (Fig. 1b), Ad-GFP was used for transduction into CD41⁺c-kit⁺ cells. Similar results were obtained in three independent experiments. (e) The expression level of human HoxB4 mRNA in the cells was examined by conventional RT-PCR on days 3 and 6 after transduction with Ad-hHoxB4 at 3000 VPs/cell into EB-derived total cells. Abbreviations: ES, embryonic stem; iPS, induced pluripotent stem; mCAR, mouse coxsackievirus and adenovirus receptor; GFP, green fluorescent protein; Cont., control.; VP, vector particle; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Transduction with Ad vectors

non-transduced cells and LacZ-transduced cells (Fig. 4a). Note that the number of the most immature multipotent progenitor cells, CFU-GEMM/CFU-Mix, in hHoxB4-transduced cells was approximately seven times as great as that in non-transduced cells or LacZ-transduced cells, and that large CFU-Mix colonies were more frequently observed in hHoxB4-trans-

duced cells than control cells (Fig. 4b and data not shown). A colony assay after culturing on OP9 stromal cells for 20 days also revealed that much number of myeloid (CFU-G, M, and GM) colonies and CFU-Mix colonies were observed by transient hHoxB4 transduction (Figs. 4c and d). Thus, our data clearly showed that Ad vector-mediated transient hHoxB4 expression



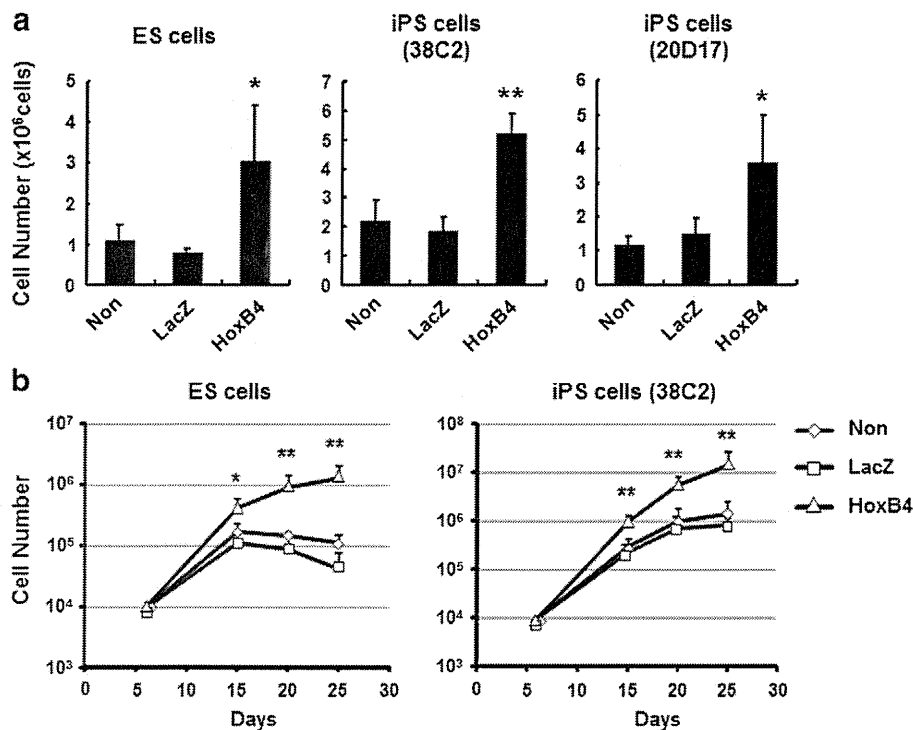


Figure 2 The number of ES cell- or iPS cell-derived hematopoietic cells was significantly increased in Ad-hHoxB4-transduced cells. (a, b) ES-EB- or iPS-EB-derived total cells (a) or CD41⁺c-kit⁺ cells (b) were transduced with Ad-LacZ or Ad-hHoxB4 at 3000 VPs/cell for 1.5 h, and the cells were then plated on OP9 feeder cells. As a control, non-transduced cells were also plated on OP9 cells. After culturing on OP9 feeders with the hematopoietic cytokines for 10 days (a) or 20 days (b), the number of hematopoietic cells per 2 wells of a 6-well plate was counted. (a) Left, ES cells; middle, iPS cell line 38C2; right, iPS cell line 20D17. Results shown were the mean of four independent experiments with indicated standard deviations. * $p < 0.05$, ** $p < 0.01$ as compared with non-transduced or Ad-LacZ-transduced cells.

enhances the differentiation of hematopoietic immature cells, including HSPCs, from mouse ES and iPS cells.

Discussion

Previous studies have shown that enforced expression of HoxB4 is an effective strategy for hematopoietic differentiation from both mouse and human ES cells (Kyba et al., 2002; Bowles et al., 2006; Pilat et al., 2005; Schiedlmeier et al., 2007). These studies usually used recombinant ES cells, such as ES cells constitutively expressing HoxB4 (Pilat et al., 2005) or ES cells containing a tetracycline (Tet)-inducible HoxB4 expression system (Kyba et al., 2002), to induce hematopoietic cells. However, this expression system might raise clinical concerns, including the risk of oncogenesis due to integration of transgenes into host genomes. In the present study, we showed that Ad vector-mediated transient hHoxB4 expression in mouse ES-EB- or iPS-EB-derived cells could result in an efficient production of hematopoietic cells, including HSPCs with a hematopoietic colony-forming ability, from mouse ES and iPS cells (Figs. 2, 3, and 4). Our data obtained in this report are largely consistent with previous reports (Kyba et al., 2002) in which HSPCs were generated by using ES cells containing the Tet-regulated HoxB4 expression cassette. Therefore, a transient HoxB4 expression system using an Ad vector, instead of a Tet-inducible HoxB4 expression

system, would contribute to safer clinical applications of ES or iPS cell-derived hematopoietic cells.

Conventional Ad vector is known to infect the cells through an entry receptor, CAR, on the cellular surface (Bergelson et al., 1997; Tomko et al., 1997). Previously, we showed that undifferentiated ES and iPS cells expressed CAR, and conventional Ad vector could easily transduce a foreign gene in more than 90% of the undifferentiated ES and iPS cells at 3000 VPs/cell (Kawabata et al., 2005; Tashiro et al., 2009). Like undifferentiated ES and iPS cells, we could detect the CAR expression in more than 90% or 70% of EB-derived total cells or EB-derived CD41⁺c-kit⁺ cells, respectively (Figs. 1a and b). However, the transduction efficiency in EB-derived total cells or CD41⁺c-kit⁺ cells was only 60% or 40%, respectively, of the cells at most (Figs. 1c and d). Although we are not certain why transgene expression was not observed in all of CAR⁺ EB-derived cells, it is possible that the promoter might not have worked in all of the cells because the EB-derived total cells and CD41⁺c-kit⁺ cells were heterogeneous, unlike in the case of undifferentiated ES and iPS cells. It is also possible that the Ad binding site of CAR might be disrupted by trypsin treatment during the preparation of the EB-derived cells (Carson, 2000). Because the development of efficient transduction methods in EB-derived cells is considered to be a powerful tool to promote the hematopoietic differentiation from ES and iPS cells, further improvement of the transduction conditions will be needed.

We found a difference in the hematopoietic differentiation potential among mouse iPS cell lines (Fig. 4). Consistent with our data, Kulkeaw et al. showed a difference in the

hematopoietic differentiation capacity among six iPS cell lines (Kulkeaw et al., 2010). In addition, recent studies have reported that iPS cells leave an epigenetic memory of

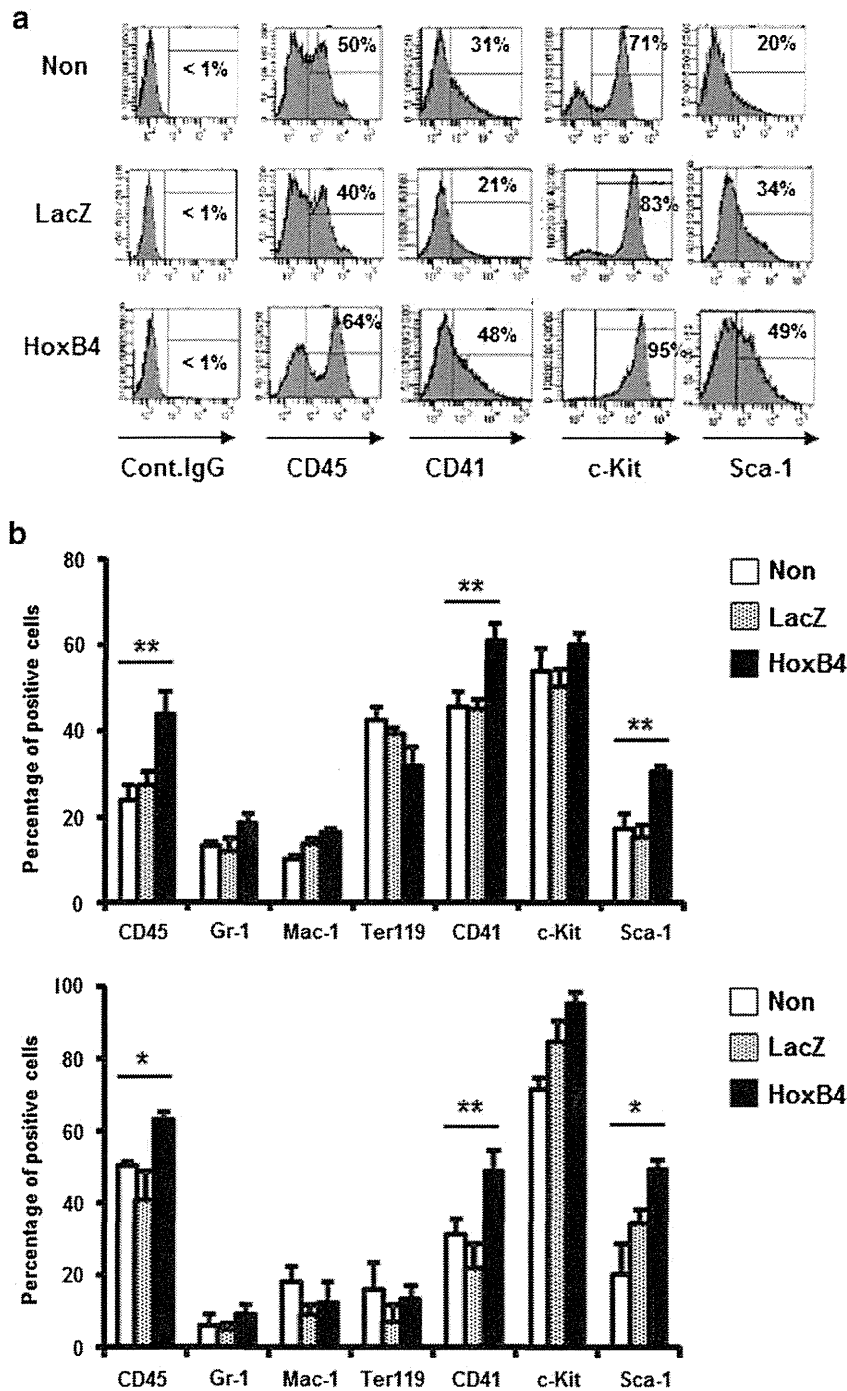


Figure 3 Expression of surface antigen and hematopoietic marker genes in mouse ES cell- or iPS cell-derived cells. (a, b) ES cell- or iPS cell line 38C2-derived cells were reacted with each antibody, and were then subjected to flow cytometric analysis. (a) Representative data from iPS cell line 38C2 are shown. (b) Percentage of each antigen positive cells in ES cell-derived cells (upper) or iPS cell-derived cells (lower) is shown. The data expressed the mean of three independent experiments with indicated standard deviations. * $p < 0.05$, ** $p < 0.01$ as compared with non-transduced or Ad-LacZ-transduced cells. (c) Total RNA was extracted from undifferentiated iPS cells (Day 0), iPS-EB (Day 5), iPS cells-derived hematopoietic cells (day 15), OP9 stromal cells, and MEF feeder, and semi-quantitative PCR (left) or quantitative real-time PCR (right) was then carried out as described in the Materials and methods. The data expressed the mean of three independent experiments with indicated standard deviations. * $p < 0.05$, ** $p < 0.01$ as compared with non-transduced or Ad-LacZ-transduced cells. Abbreviation: EBs, embryoid bodies; MEF, mouse embryonic fibroblast; GATA, GATA-binding protein.

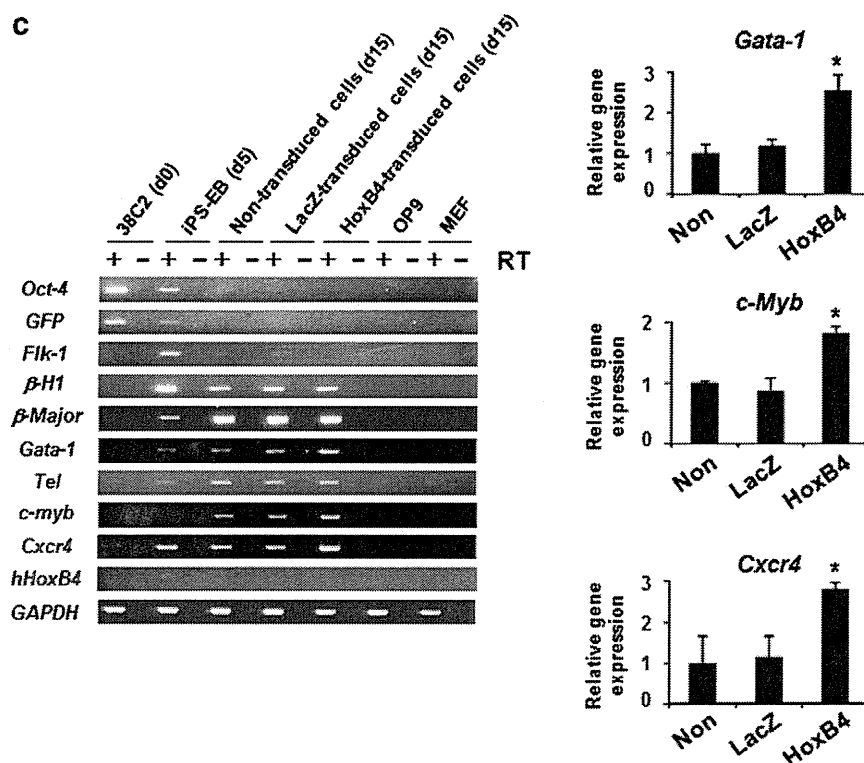


Figure 3 (continued).

their cellular origin, and this memory influences their functional properties, including *in vitro* differentiation (Kim et al., 2010; Polo et al., 2010). Thus, these reports indicate that, in order to obtain a large number of HSPCs from iPS cells, it is necessary to choose an appropriate iPS cell line, such as HSPC-derived iPS cells (Okabe et al., 2009). Importantly, using mouse embryonic fibroblast-derived iPS cells (38C2 and 20D17), we showed that the use of transient hHoxB4 transduction in iPS-EB-derived cells achieved more effective differentiation into HSPCs than the use of non-transduced cells (Fig. 4). Our method therefore should be efficient for the production of HSPCs from any iPS cell line.

An important but unsolved question in this study is whether ES cell-derived hematopoietic cells and iPS cell-derived hematopoietic cells transduced with Ad-hHoxB4 have long-term hematopoietic reconstitution potential *in vivo*. Recent studies have demonstrated that some surface antigen expressions were different between bone marrow-derived HSPCs and ES cell-derived HSPCs, and that CD41⁺ cells had long-term repopulation ability in ES cell-derived HSPCs (McKinney-Freeman et al., 2009; Matsumoto et al., 2009). Our flow cytometric analysis revealed an increase of CD41⁺ cells in hHoxB4-transduced cells compared with non-transduced cells and LacZ-transduced cells (Fig. 3b). We also showed that Ad-hHoxB4-transduced cells could proliferate on OP9 stromal cells more efficiently than control cells (Fig. 2). Thus, these results suggest that immature hematopoietic cells were generated by transient hHoxB4 transduction, and that hHoxB4-transduced cells might have reconstitution potential *in vivo*. This *in vivo* transplantation analysis is now on-going in our laboratory.

In the present study, we succeeded in the promotion of hematopoietic differentiation from mouse ES and iPS cells by Ad vector-mediated hHoxB4 transduction. Ad vector transduction can avoid the integration of transgene into host genomes, and multiple genes can be transduced by Ad vectors in an appropriate differentiation period. Thus, an even more efficient protocol for hematopoietic differentiation from ES and iPS cells could likely be established by co-transduction of HoxB4 and other genes involved in the hematopoiesis, such as Cdx4 (Wang et al., 2005) and Scl/Tal1 (Kurita et al., 2006), using Ad vectors. Taken together, our results show that Ad vector-mediated transient gene expression is valuable tool to induce hematopoietic cell from ES and iPS cells, and this strategy would be applicable to safe therapeutic applications, such as HSPC transplantation.

Materials and methods

Antibodies

The following primary monoclonal antibodies (Abs), conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or PE-Cy7, were used for flow cytometric analysis: anti-CD45 (30-F11, eBioscience, San Diego, CA), anti-CD11b (M1/70, eBioscience), anti-Sca-1 (D7, eBioscience), anti-Ter-119 (Ter-119, eBioscience), anti-Gr-1 (RB6-8C5, eBioscience), anti-c-Kit (ACK2 or 2B8, eBioscience), anti-CD41 (MWRReg30, BD Bioscience San Jose, CA). Purified rat anti-coxsackievirus and adenovirus receptor (CAR) was kindly provided from Dr. T. Imai (KAN Research Institute, Hyogo, Japan). For detection of CAR, the PE-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West

Grove, PA) or DyLight649-conjugated goat anti-rat IgG (BioLegend, San Diego, CA) was used as secondary Abs.

Cell cultures

The mouse ES cell line E14 and two mouse iPS cell lines, 38C2 and 20D17, both of which were generated by Yamanaka and his colleagues (Okita et al., 2007), were used in this study. 38C2 was kindly provided by Dr. S. Yamanaka (Kyoto University, Kyoto, Japan), and 20D17 was purchased from Riken Bioresource Center (Tsukuba, Japan). In the present study, we mainly used 38C2 iPS cells except where otherwise indicated. Mouse ES and iPS cells were cultured in leukemia inhibitory factor-

containing medium on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts (MEF) as described previously (Tashiro et al., 2009). OP9 stromal cells were cultured in α -minimum essential medium (α MEM: Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and non-essential amino acid (Invitrogen).

Ad vectors

Ad vectors were constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). The shuttle

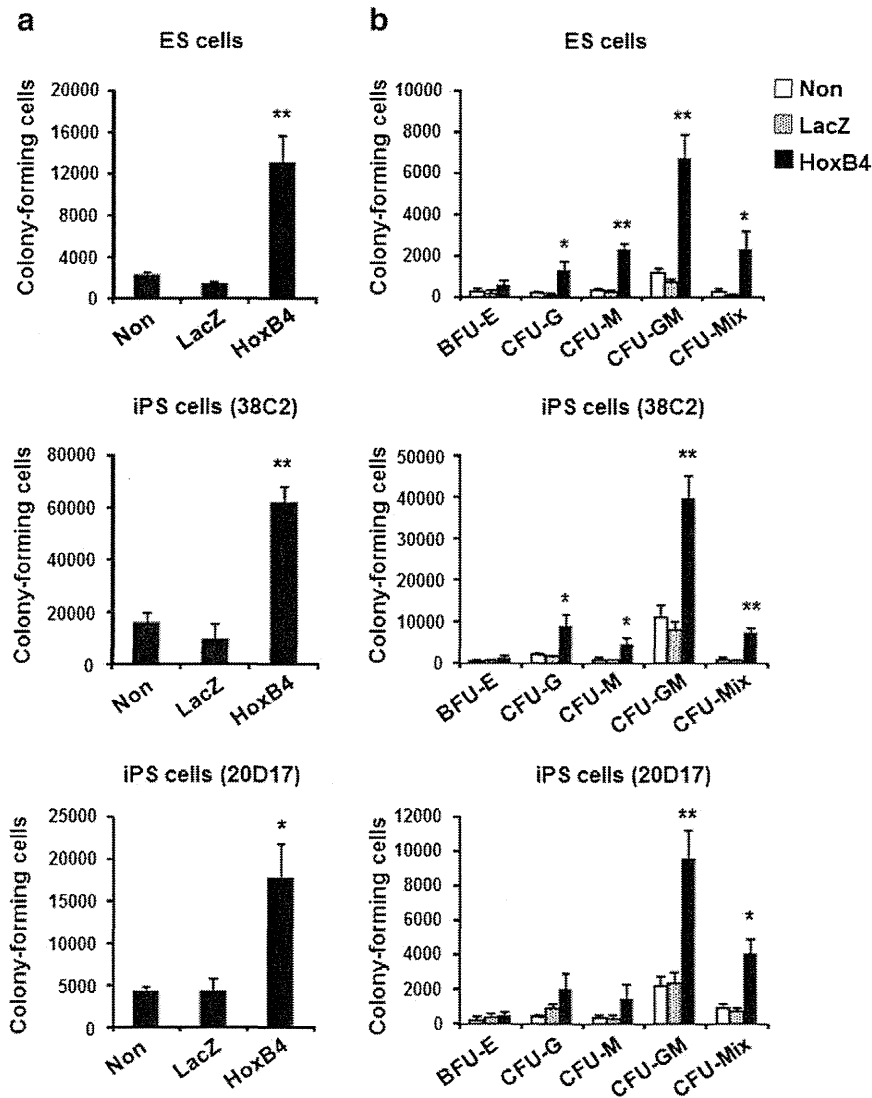


Figure 4 Significant increase of hematopoietic colony-forming cells in Ad-HoxB4-transduced hematopoietic cells. After ES-EB- or iPS-EB-derived cells were transduced with Ad-LacZ or Ad-hHoxB4, hematopoietic cells were generated by co-culturing with OP9 cells in the presence of hematopoietic cytokines for 10 days (a, b) or 20 days (c, d). A colony-forming assay was performed using methylcellulose medium, and the number of hematopoietic colonies was then counted under light microscopy. The number of total colonies (a, c) or subdivided colonies by morphological subtype (BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-Mix) (b, d) generated from ES cells (E14) or iPS cells (38C2 and 20D17) was shown. Colony number was normalized to total number of the cells. Results shown were the mean of three (c, d) or four (a, b) independent experiments with indicated standard deviations. * $p < 0.05$, ** $p < 0.01$ as compared with non-transduced or Ad-LacZ-transduced cells. Abbreviation: BFU-E, burst-forming unit; CFU-G, colony-forming unit-granulocyte; CFU-M, CFU-monocyte; CFU-GM, CFU-granulocyte, monocyte; CFU-GEMM/CFU-Mix, CFU-granulocyte, erythrocyte, monocyte, megakaryocyte.

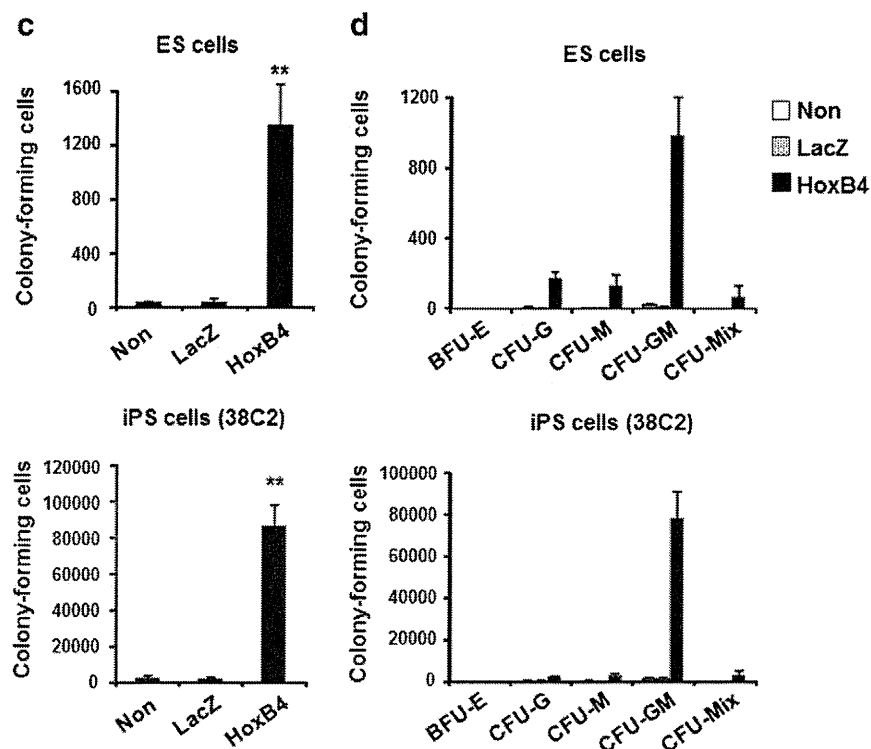


Figure 4 (continued).

plasmid pHMCA5, which contains the CMV enhancer/ β -actin promoter with β -actin intron (CA) promoter (a kind gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan) (Niwa et al., 1991), was previously constructed (Kawabata et al., 2005). The human HoxB4 (hHoxB4)-expressing plasmid, pHMCA-hHoxB4, and DsRed-expressing plasmid, pHMCA-DsRed, were generated by inserting a hHoxB4 cDNA (a kindly gift from Dr. S. Karlsson, Lund University Hospital, Lund, Sweden) and a DsRed cDNA (Clontech, Mountain View, CA), respectively, into pHMCA5. pHMCA-hHoxB4 or pHMCA-DsRed were digested with *I-CeuI*/*PI-SceI* and ligated into *I-CeuI*/*PI-SceI*-digested pAdHM4 (Mizuguchi and Kay, 1998), resulting in pAd-hHoxB4 or pAd-DsRed, respectively. Ad-hHoxB4 and Ad-DsRed were generated and purified as described previously (Tashiro et al., 2008). The CA promoter-driven β -galactosidase (LacZ)-expressing Ad vector, Ad-LacZ, and the CA promoter-driven GFP-expressing Ad vector, Ad-CA-GFP, were generated previously (Tashiro et al., 2008). The vector particle (VP) titer was determined by using a spectrophotometrical method (Maizel et al., 1968).

In vitro differentiation

Prior to embryoid body (EB) formation, mouse ES or iPS cells were suspended in differentiation medium (Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 15% FBS, 0.1 mM 2-mercaptoethanol (Nacalai tesque, Kyoto, Japan), 1 \times non-essential amino acid (Specialty Media, Inc.), 1 \times nucleosides (Specialty Media, Inc.), 2 mM L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen)) and cultured on a culture dish at 37 °C for 45 min to remove MEF layers. Mouse ES cell- or iPS cell-derived EBs (ES-EBs or iPS-

EBs, respectively) were generated by culturing ES or iPS cells on a round-bottom low cell binding 96-well plate (Lipidure-coat plate; Nunc) at 1 \times 10³ cells per well. ES-EBs or iPS-EBs were collected on day 5, and a single cell suspension was prepared by trypsin/EDTA treatment (Invitrogen) at 37 °C for 2 min. ES-EB- or iPS-EB-derived CD41⁺c-kit⁺ cells were sorted by FACSaria (BD Bioscience). The purity of the CD41⁺c-kit⁺ cells was greater than 90% based on flow cytometric analysis (Supplemental Fig. 1). Cells were then transduced with an Ad vector at 3000 vector particles (VPs)/cell for 1.5 h in a 15 ml tube. After transduction, total cells (2 \times 10⁵) or CD41⁺c-kit⁺ cells (1 \times 10⁴) were cultured on OP9 feeder cells in a well of a 6-well plate in α MEM supplemented with 20% FBS, 2 mM L-glutamine, non-essential amino acid, 0.05 mM 2-mercaptoethanol, and hematopoietic cytokines (50 ng/ml mouse stem cell factor (SCF), 50 ng/ml human Flt-3 ligand (Flt-3L), 20 ng/ml thrombopoietin (TPO), 5 ng/ml mouse interleukin (IL)-3, and 5 ng/ml human IL-6 (all from Peprotec, Rocky Hill, NJ)). After culturing with OP9 stromal cells, both non-adherent hematopoietic cells and adherent hematopoietic cells were collected as follows. The non-adherent hematopoietic cells were collected by pipetting and were transferred to 15 ml tubes. The adherent hematopoietic cells were harvested with the use of trypsin/EDTA, and then incubated in a tissue culture dish for 30 min to eliminate the OP9 cells. Floating cells were collected as hematopoietic cells and transferred to the same 15 ml tubes. These hematopoietic cells were kept on ice for further analysis.

Flow cytometry

Cells (1 \times 10⁵ to 5 \times 10⁵) were incubated with monoclonal Abs at 4 °C for 30 min and washed twice with staining buffer

Table 1 List of primers used for RT-PCR.

Gene name	Species	(5') Sense primers (3')	(5') Antisense primers (3')
GAPDH	Ms	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
HoxB4	Hs	AGAGGCGAGAGAGCAGCTT	TTCCTTCTCCAGCTCCAAGA
Oct-3/4	Ms	GTTTGCCAAGCTGCTGAAGC	TCTAGCCCAAGCTGATTGGC
GFP	–	CACATGAAGCAGCAGCAGCTT	TGCTCAGGTAGTGGTTGTCCG
Flk-1	Ms	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCACACCACCC
Gata1	Ms	TTGTGAGGCCAGAGAGTGTG	TTCCTCGTCTGGATTCCATC
Gata1 (real-time PCR)	Ms	GTCAGAACCGGCCTCTCATC	GTGGTCGTTTGACAGTTAGTGCAT
Tel	Ms	CTGAAGCAGAGGAAATCTCGAATG	GGCAGGCAGTGATTATTCTCGA
c-myb	Ms	CCTCACCTCCATCTCAGCTC	GCTGGTGAGGCACTTTCTTC
β -H1	Ms	AGTCCCCATGGAGTCAAAGA	CTCAAGGAGACCTTTGCTCA
β -Major	Ms	CTGACAGATGCTCTTTGGG	CACAACCCAGAAACAGACA
CXCR4	Ms	GTCTATGTGGCGCTCTGGAT	GGCAGAGCTTTTGAACCTGG

(PBS/2%FBS). Dead cells were excluded from the analysis by 7-amino actinomycin D (7-AAD, eBioscience). Analysis was performed on an LSRFortessa flow cytometer by using FACS-Diva software (BD Bioscience). For detection of transgene expression by Ad vectors, EB-derived total cells or CD41⁺c-kit⁺ cells were transduced with Ad-DsRed or Ad-CA-GFP, respectively, for 1.5 h. At 48 h of incubation with the hematopoietic cytokines as described above, transgene expression in the cells was analyzed by flow cytometry.

Colony assay

A colony-forming assay was performed by plating ES cell-derived hematopoietic cells or iPS cell-derived hematopoietic cells into methylcellulose medium M3434 (Stem Cell Technologies, Vancouver, BC, Canada). After incubation at 37 °C and 5% CO₂ for 10 to 14 days in a humidified atmosphere, colony numbers were counted. The morphology of colonies was observed using an inverted light microscope.

RT-PCR

Total RNA was isolated with the use of ISOGENE (Nippon Gene, Tokyo, Japan). cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT) primer. Semi-quantitative PCR was performed with the use of TaKaRa ExTaq HS DNA polymerase (Takara, Shiga, Japan). The PCR conditions were 94 °C for 2 min, followed by the appropriate number of cycles of 94 °C for 15 s, 55 °C for 30 s with 72 °C for 30 s and a final extension of 72 °C for 1 min, except for the addition of 5% dimethyl sulfoxide in the case of hHoxB4 cDNA amplification. The product was assessed by 2% agarose gel electrophoresis followed by ethidium bromide staining. Quantitative real-time PCR was performed using StepOnePlus real-time PCR system with FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The sequences of the primers used for in this study are listed in Table 1.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.09.001.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgments

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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

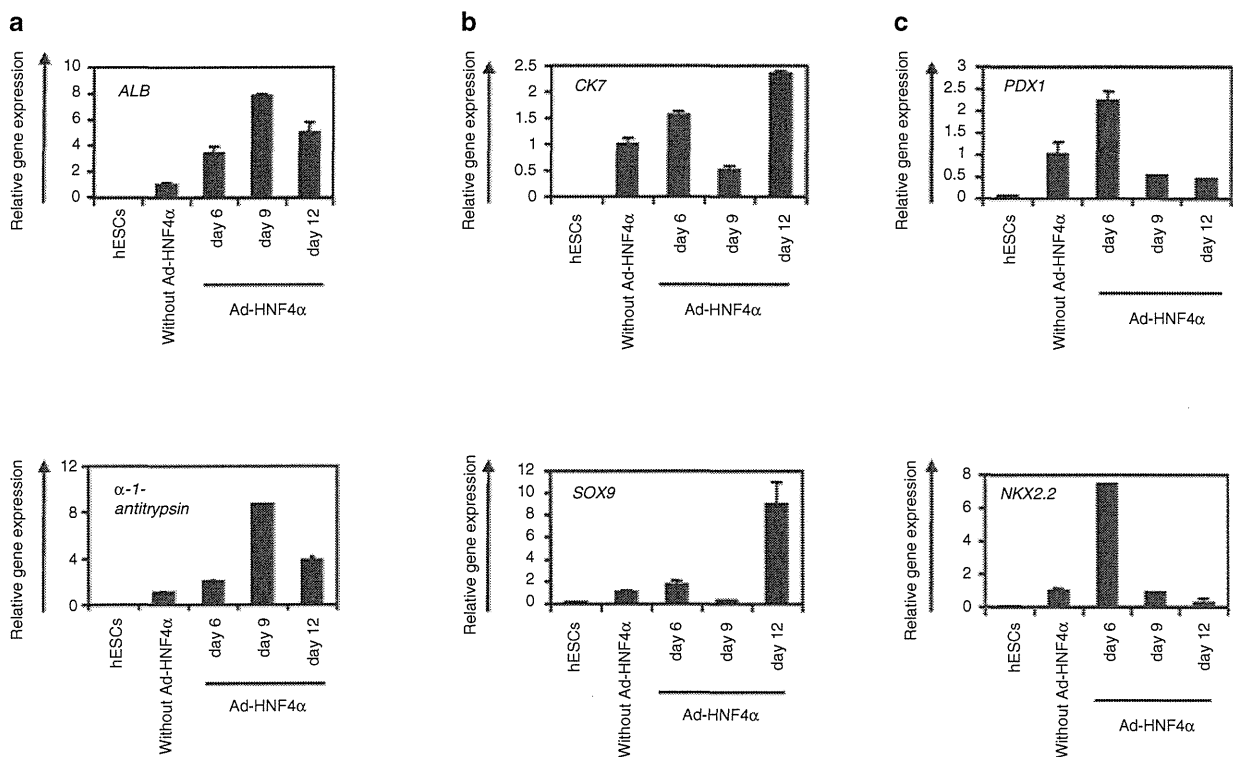


Figure 1 Transduction of HNF4 α into hepatoblasts promotes hepatic differentiation. (a–c) The human ESC (H9)-derived cells, which were cultured for 6, 9, or 12 days according to the protocol described in Figure 2a, were transduced with 3,000 vector particles (VP)/cell of Ad-HNF4 α for 1.5 hours and cultured 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

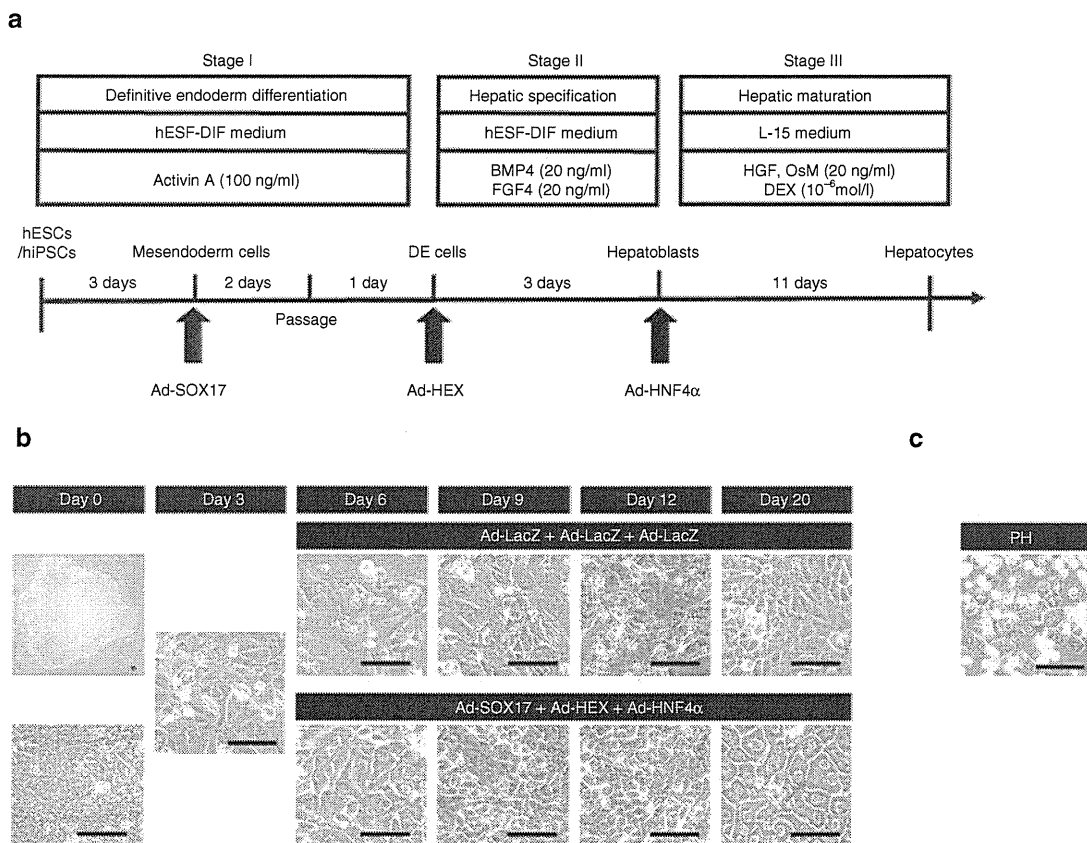


Figure 2 Hepatic differentiation of human ESCs and iPSCs transduced with three factors. **(a)** The procedure for differentiation of human ESCs and iPSCs into hepatocytes via DE cells and hepatoblasts is presented schematically. The hESF-DIF medium was supplemented with 10 μ g/ml human recombinant insulin, 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.

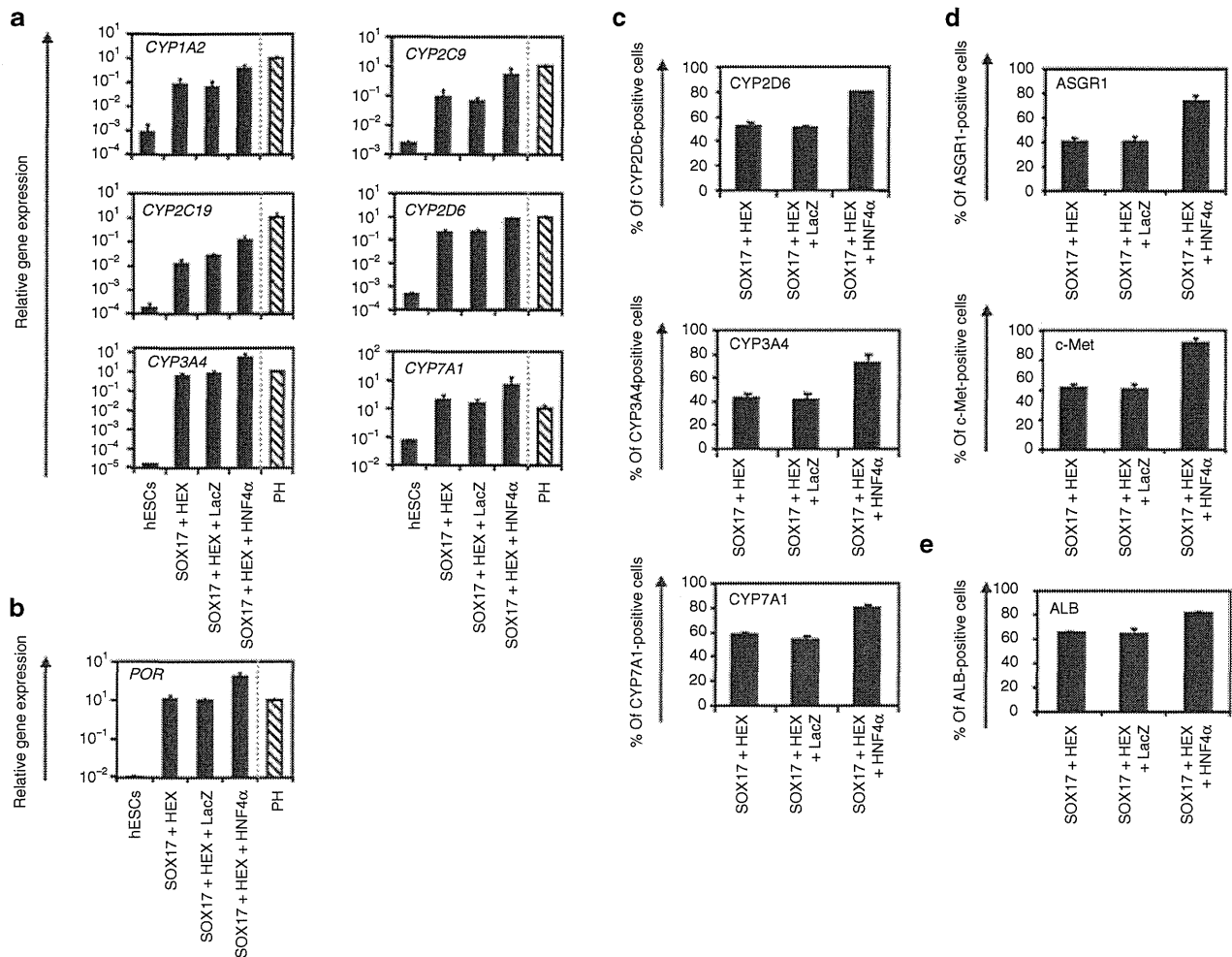


Figure 3 Transduction of HNF4 α promotes hepatic maturation from human ESCs and iPSCs. **(a,b)** The human ESCs were differentiated into hepatocytes according to the protocol described in **Figure 2a**. On day 20 of differentiation, the gene expression levels of **(a)** CYP enzymes (*CYP1A2*, *CYP2C9*, *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 iPSC 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 iPSC 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 iPSC cell lines. That is, two of the human iPSC cell lines (Tic and Dotcom) were more committed to the hepatic lineage than another human iPSC 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 \sim 80% in the three factors-transduced cells, while they were \sim 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 \sim 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).

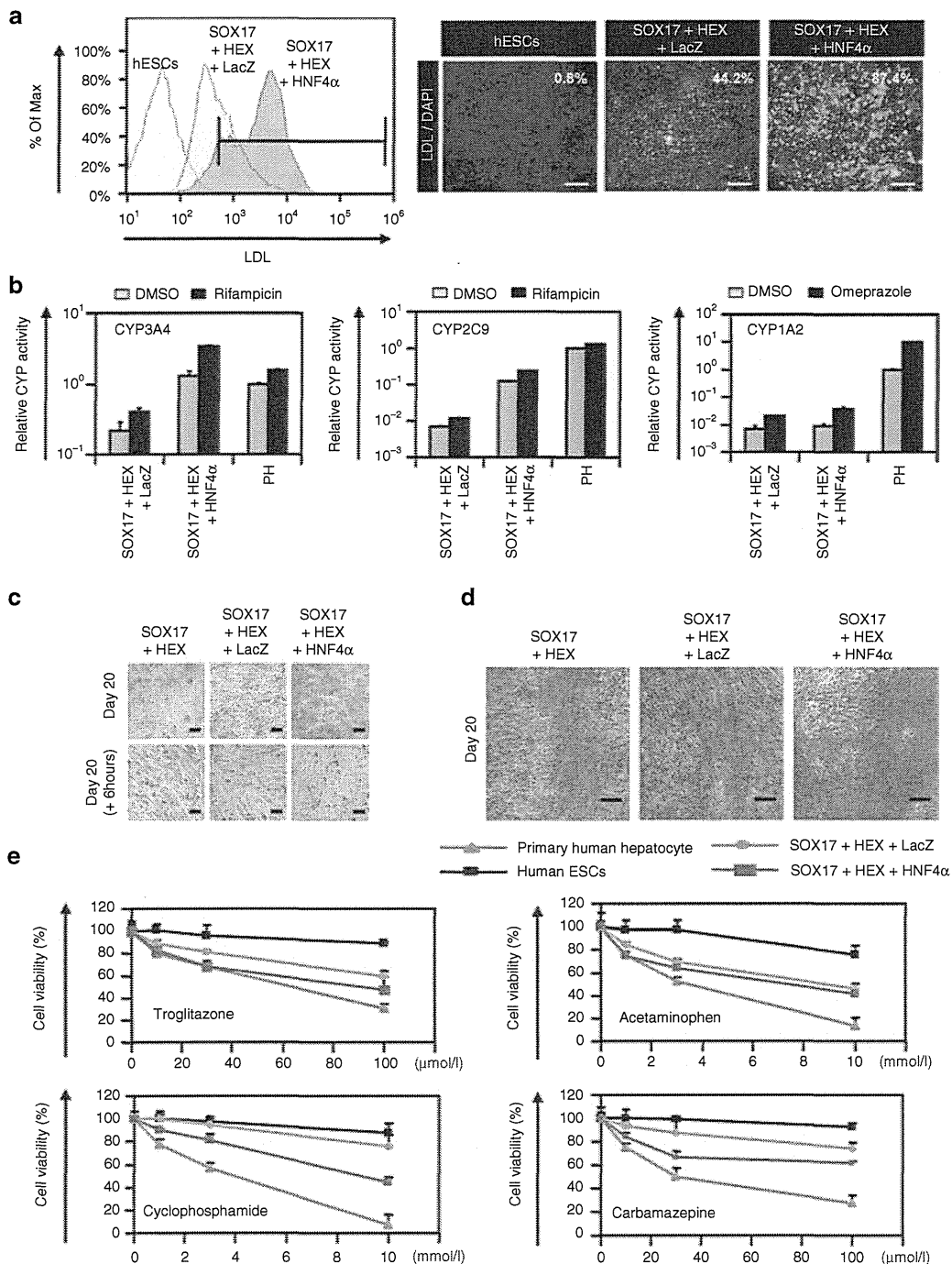


Figure 4 Transduction of the three factors enhances hepatic functions. The human ESCs were differentiated into hepatoblasts and transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hours and cultured until day 20 of differentiation according to the protocol described in **Figure 2a**. The hepatic 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.

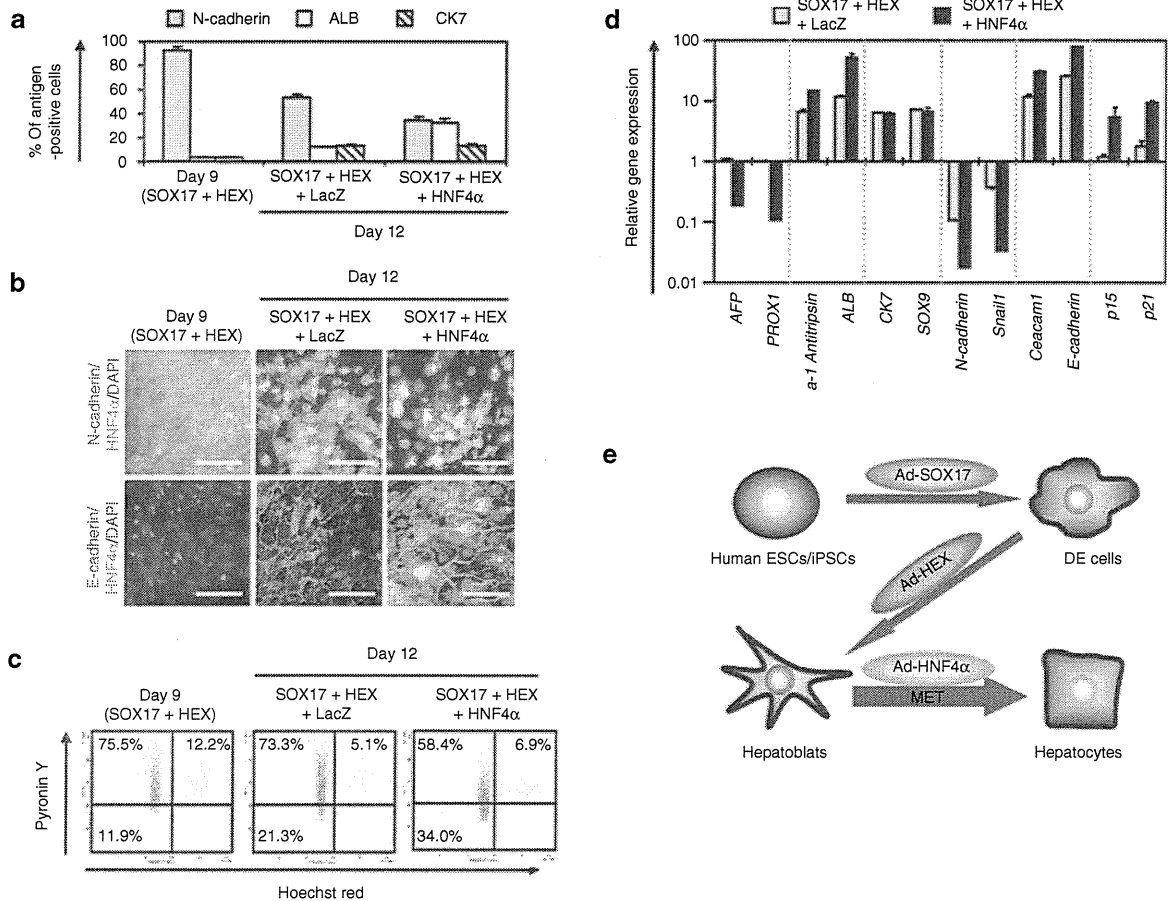


Figure 5 HNF4 α promotes hepatic differentiation by activating MET. Human ESCs were differentiated into hepatoblasts according to the protocol described in **Figure 2a**, and then transduced with 3,000 VP/cell of Ad-LacZ or Ad-HNF4 α for 1.5 hours, and finally cultured until day 12 of differentiation. **(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