

FIG. 4. Enhanced TPO-induced signaling pathway in DN-Lnk-expressing cells. After the EB cells, which were cultured for 7 days, had been plated and cultured on OP9 stromal cells for 14 days, and the hematopoietic cells were harvested as described in the Materials and Methods section. (a) Hematopoietic cells derived from *Neo*- or DN-Lnk-expressing cells were starved for 4–6 h in the absence of TPO, and the cells were then stimulated with 20 ng/mL of TPO. Total cell lysates were subjected to western blot analysis using the indicated antibodies. (b) After hematopoietic cells were collected, the rate of MPL-expressing cells was examined by flow cytometry. Representative results from 1 of 2 independent experiments performed are shown. TPO, thrombopoietin.

Discussion

In this report, we successfully generated and expanded hematopoietic cells, including immature hematopoietic cells, with colony-forming potential, from mouse ES and iPS cells by the suppression of an adaptor protein Lnk (Fig. 3). We also demonstrated that the expression levels of hematopoi-

etic transcription factors such as *Scl* and *Runx1* in DN-Lnk-expressing total EB cells were significantly increased in comparison with those in *Neo*-expressing total EB cells (Fig. 2c, d), and that cytokine response was augmented in DN-Lnk-expressing cells (Fig. 4). Therefore, the data obtained in this study suggest that Lnk inhibition by enforced expression of a *DN-Lnk* gene in ES and iPS cells would lead both to a promotion of mesodermal differentiation during EB formation and to an increase in the expansion potential of ES and iPS cell-derived hematopoietic cells on an OP9 coculture system, and thus Lnk inhibition could enhance the hematopoietic cell production.

In developing mouse embryos, Lnk is shown to be expressed in the aorta-gonad-mesonephros (AGM) region, the site of hematopoiesis [38]. It has also been reported that the production of CD45⁺ hematopoietic cells was severely impaired by the enforced expression of Lnk in AGM-derived cells, suggesting that Lnk suppresses hematopoietic commitment [38]. However, the function of Lnk in hematopoiesis is not fully understood. In the current study, we found that Lnk was highly expressed in Flk-1⁺ cells (Fig. 1b), which are known to be hemoangiogenic progenitor cells during ES cell differentiation [29]. Furthermore, it was of note that levels of expression of *Scl/Tal-1*, which is essential for hematopoietic commitment of hemoangiogenic progenitor cells derived from ES cells [34], were slightly upregulated in Flk-1⁺ cells by the inhibition of Lnk function (Fig. 2e). Thus, it is possible that Lnk might negatively regulate the hematopoietic commitment in Flk-1⁺ cells by modulating the expression of *Scl/Tal-1*. We also showed that the percentage of Flk-1⁺ cells was increased in DN-Lnk-expressing EB cells (Fig. 2b), and this could result in the elevated expression of other key hematopoietic transcription factors, such as *Runx1* and *Gata-1*, in DN-Lnk-expressing total EB cells compared with that in *Neo*-expressing total EB cells (Fig. 2c, d). This indicates that the functional Flk-1⁺ mesodermal cells would be efficiently generated from DN-Lnk-expressing ES and iPS cells. On the other hand, at earlier days of differentiation, the percentage of CD41⁺ cells, an early hematopoietic progenitor cells generated from pluripotent stem cells [39], in DN-Lnk-expressing EB cells was mostly equal to that in *Neo*-expressing EB cells (Supplementary Fig. S4). Taken together, the findings suggest that Lnk inhibition in ES and iPS cells could be effective for the generation of mesodermal cells with the potential for hematopoietic differentiation, but would not enhance the emergence of hematopoietic progenitor cells at earlier days of EB differentiation.

We examined the cytokine responses of iPS cell-derived hematopoietic cells, and observed the augmented phosphorylation of Erk and Akt in DN-Lnk-expressing cells (Fig. 4). This result is consistent with that of a previous report in which TPO-treated megakaryocytes derived from Lnk-deficient mice enhanced the extent of the activation of Erk and Akt [40]. By contrast, it was reported that Lnk-deficient adult HSCs or bone marrow-derived macrophages showed an enhanced Akt, but not Erk, activation after cytokine stimulation [16,41]. This difference in the activation of downstream molecules is most likely due to differences in cell populations. Because ES cell- and iPS cell-derived hematopoietic cells are heterogeneous, both Akt and Erk phosphorylation levels after cytokine treatment would be augmented in DN-Lnk-expressing cells relative to

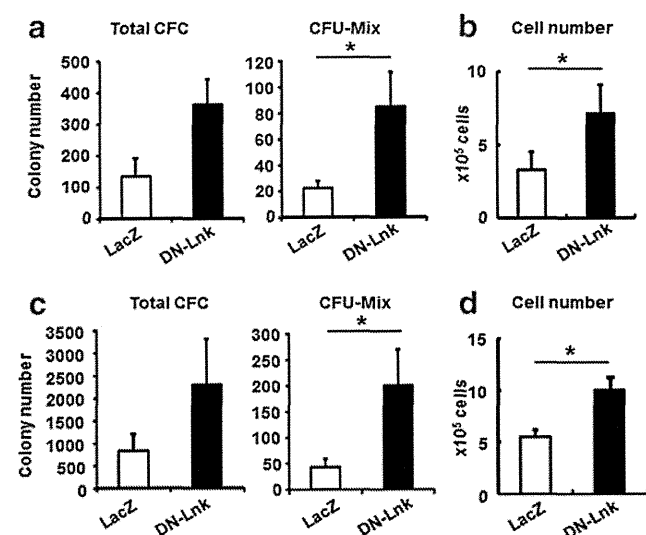


FIG. 5. Expansion of ES cell- and iPS cell-derived hematopoietic cells by the transient expression of DN-Lnk in EB cells. After ES cell- (a, b) or iPS cell- (c, d) derived EB cells, which were cultured for 7 days, had been transduced with Ad-LacZ or Ad-DN-Lnk at 3,000 VP/cell for 1.5 h, the cells were cultured on OP9 cells with cytokines for 7 days. Hematopoietic cells were collected, and then a colony assay was performed (a, c). The colony number was normalized to the total number of cells. We also counted the number of hematopoietic cells derived from Ad-LacZ- or Ad-DN-Lnk-transduced EB cells (b, d). The data were expressed as mean \pm SD ($n=3$); * $p < 0.05$ as compared with Ad-LacZ. Ad, adenovirus.

Neo-expressing cells. In general, Akt and Erk are known to be involved in cell survival and cell growth [42,43]. Hence, our data indicate that the suppression of Lnk by the ectopic expression of DN-Lnk in ES and iPS cells would lead to an increase in hematopoietic cell production through enhanced cytokine responses.

Recently, Dravid et al. reported the expression of Lnk in human ES cell-derived CD34⁺ hematopoietic progenitor cells, and they showed that the number of human ES cell-derived CD34⁺ cells was increased by Lnk knockdown using a lentivirus vector carrying the short-hairpin RNA against Lnk (shLnk) [44]. Their results are mostly consistent with our data, indicating the suppressive function of Lnk in hematopoietic cell generation in both mouse and human pluripotent stem cells. However, the generation of hematopoietic CFCs in shLnk-transduced cells and the molecular mechanisms associated with the generation of CD34⁺ cells by Lnk knockdown have not been addressed in detail. In this report, we clearly demonstrated that hematopoietic CFCs, including immature multipotent hematopoietic cells, were efficiently generated from mouse ES and iPS cells by Lnk inhibition, and these cells show potential for expansion on OP9 stromal cells (Fig. 3). In addition, we showed that the enhanced generation of hematopoietic cells in DN-Lnk-expressing cells was mediated by the promotion of mesodermal differentiation in EBs and augmented the sensitivity to cytokines in DN-Lnk-expressing cells as described above.

Another important finding of this study was that the transient inhibition of Lnk by Ad vector-mediated transduction of a *DN-Lnk* gene could also be an effective strategy for expanding hematopoietic cells (Fig. 5). Recently, the loss of Lnk and the mutation of Lnk have been reported to be associated with myeloproliferative diseases [45,46], indicating that oncogenesis may result from constitutive Lnk suppression in ES cell- and iPS cell-derived hematopoietic cells via the overexpression of a *DN-Lnk* gene or a lentivirus vector-mediated knockdown system, and such suppression would not be a directly applicable approach for clinical medicine. In this regard, our approaches using Ad vector-mediated transient Lnk inhibition are thought to be quite useful for the safe expansion of the ES cell- and iPS cell-derived hematopoietic cells. However, the number of ES cell- and iPS cell-derived hematopoietic cells in Ad-DN-Lnk-transduced cells was lower than that in the case of stably DN-Lnk-expressing cells (data not shown), possibly due to the low transduction efficiency of Ad vectors in EB cells. Therefore, it will still be necessary to establish methods for transiently inhibiting Lnk functionality using various types of Ad vectors [47] and short interference RNA.

In summary, we successfully developed efficient methods for differentiating mouse ES and iPS cells into hematopoietic cells by the suppression of the adaptor protein Lnk. Lnk functions downstream of multiple hematopoietic cytokine-signaling events, including those involving TPO, SCF, M-CSF, and erythropoietin [13,16,40,41,48,49], and Lnk-deficient mice show accumulation of pro-B cells in the bone marrow [48]. Therefore, various types of hematopoietic cells may be efficiently differentiated and expanded from ES and iPS cells by the inhibition of the Lnk function, when an appropriate cytokine is included in the culture; such work is currently ongoing in our laboratory.

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Author Disclosure Statement

The authors have no financial conflicts of interest.

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Supplementary Data

Supplementary Methods

Alkaline phosphatase staining and Oct-3/4 staining

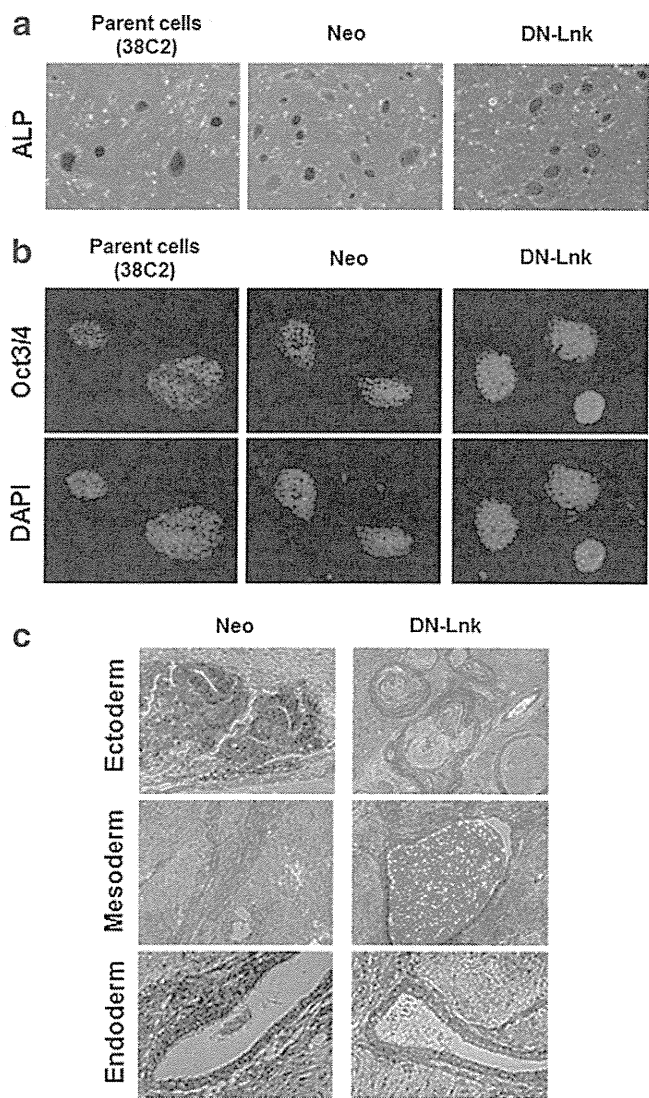
Mouse parent induced pluripotent stem (iPS) cells (38C2), *Neo*-expressing iPS cells, and dominant-negative mutant of the Lnk (DN-Lnk)-expressing iPS cells (1×10^4 cells) were plated on 12-well plates, and were then cultured for 3 days. Alkaline phosphatase staining was carried out using the Alkaline Phosphatase Detection Kit (Chemicon) according to the manufacturer's instructions. For Oct-3/4 staining, cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 15 min and blocked with PBS containing 2% bovine serum albumin for 30 min. After permeabilization with PBS containing 0.2% Triton X-100 for 5 min, the cells were incubated with mouse anti-Oct-3/4 antibody (Santa Cruz biotechnology, Inc.; diluted 1: 100) at

4°C overnight, followed by incubation with Alexa594-conjugated anti-mouse immunoglobulin G (Invitrogen) for 1 h. Nuclei were counterstained with Prolong Gold with DAPI (Invitrogen).

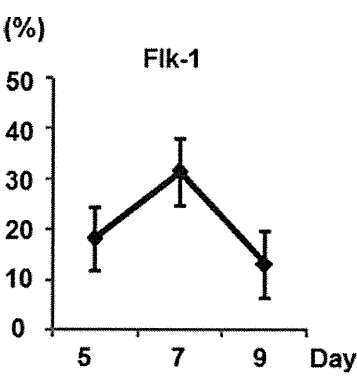
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Teratoma formation and histological analysis

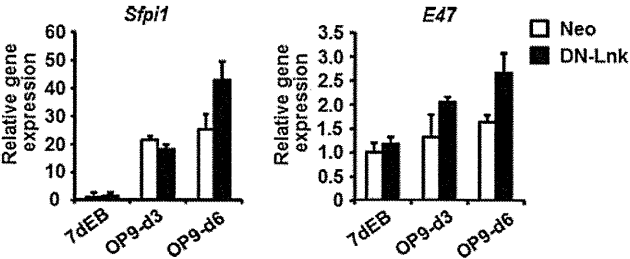
Neo- or DN-Lnk-expressing iPS cells were suspended in 1×10^7 cells/mL in PBS. Nude mice (8–10 weeks; Nippon SLC) were anesthetized with diethyl ether, and we injected 100 μ L of the cell suspension (1×10^6 cells) subcutaneously into their backs. Five weeks later, tumors were surgically dissected from mice. Samples were washed, fixed in 10% formalin, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated, and stained with hematoxylin and eosin. This sectioning and staining process was commissioned to the Applied Medical Research Laboratory.



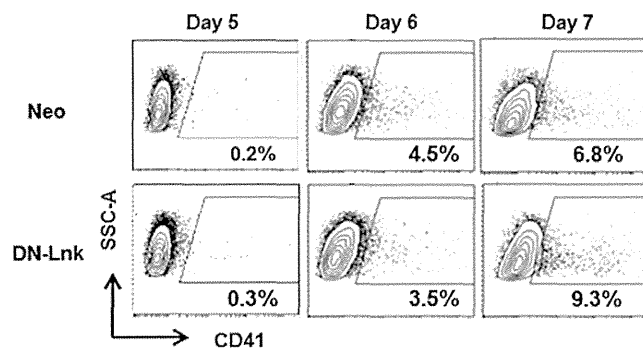
SUPPLEMENTARY FIG. S1. DN-Lnk-expressing iPS cells maintain the undifferentiated states in vitro and pluripotency in vivo. (a, b) The expression levels of undifferentiated markers, ALP (a) and Oct-3/4 (b), in Neo- or DN-Lnk-expressing iPS cells were similar to those in the parent iPS cells. (c) Neo- or DN-Lnk-expressing iPS cells were subcutaneously injected into the backs of nude mice. Paraffin sections of the teratomas derived from Neo- or DN-Lnk-expressing iPS cells were prepared, and the sections were stained with hematoxylin and eosin. DN-Lnk, dominant-negative mutant of the Lnk; iPS, induced pluripotent stem; ALP, alkaline phosphatase.



SUPPLEMENTARY FIG. S2. The proportion of Flk-1⁺ cells in EBs. After the EBs had been cultured for the indicated number of days, the cells were reacted with anti-Flk-1 antibody. Results shown are the mean of four independent experiments, with the indicated standard deviations. EBs, embryoid bodies.



SUPPLEMENTARY FIG. S3. The expression *Sfp1* and *E47* in DN-Lnk- or Neo-expressing hematopoietic cells. Seven-day-cultured EB cells (7-day EBs) were cultured on OP9 stromal cells for 3 or 6 days (OP9-d3 or OP9-d6, respectively). Total RNA was isolated from each cell, and the expression levels of *Sfp1* and *E47* were determined by real-time polymerase chain reaction.



SUPPLEMENTARY FIG. S4. The percentage of CD41⁺ early hematopoietic progenitor cells in DN-Lnk- or *Neo*-expressing EB cells. EBs, which were derived from DN-Lnk- or *Neo*-expressing iPS cells, were cultured for 5, 6, or 7 days, and then the percentage of CD41⁺ cells in the cells was determined by flow cytometry. Representative results from 1 of 3 independent experiments performed are shown.



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-positive 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

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; Hacey-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

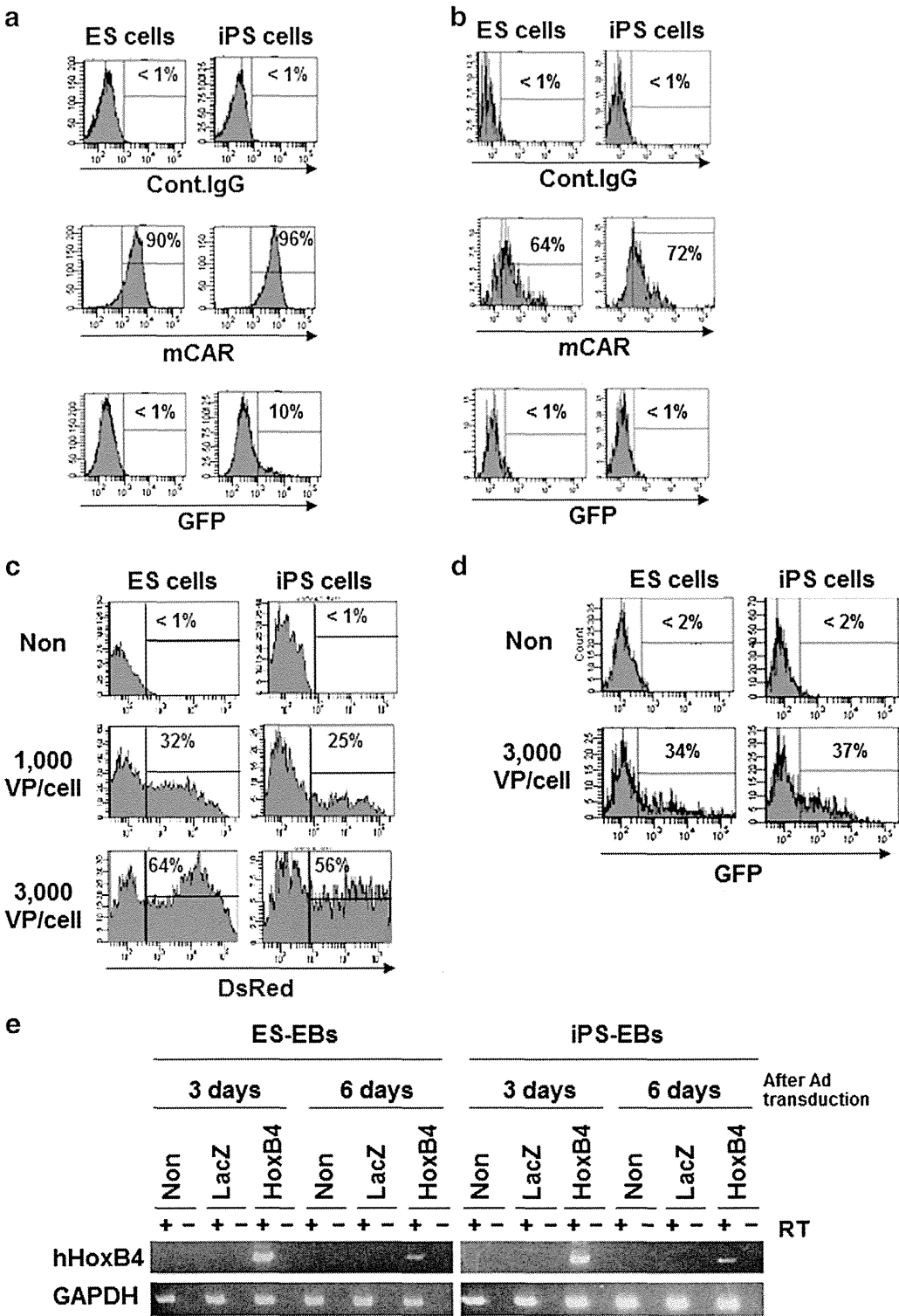
To examine whether hematopoietic immature cells with hematopoietic colony-forming potential could be generated from ES and iPS cells, ES cell-derived hematopoietic cells and iPS cell-derived hematopoietic cells, both of which were cultured on OP9 stromal cells for 10 days, were plated and cultured in 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



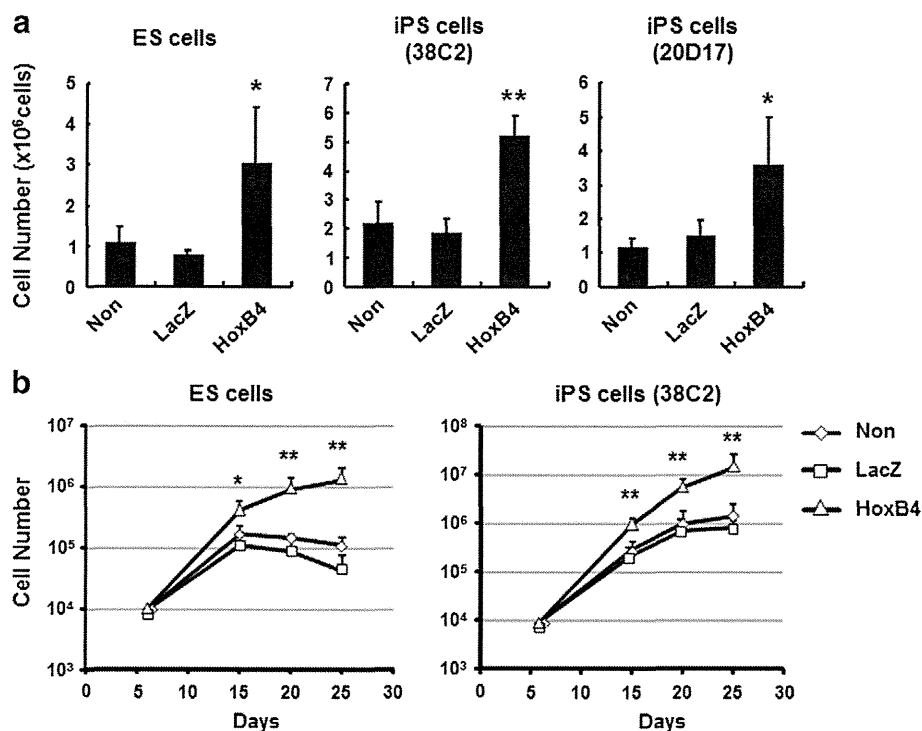


Figure 2 The number of ES cell- or iPS cell-derived hematopoietic cells was significantly increased in Ad-hHoxB4-transduced cells. (a, b) ES-EB- or iPS-EB-derived total cells (a) or CD41⁺c-kit⁺ cells (b) were transduced with Ad-LacZ or Ad-hHoxB4 at 3000 VPs/cell for 1.5 h, and the cells were then plated 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

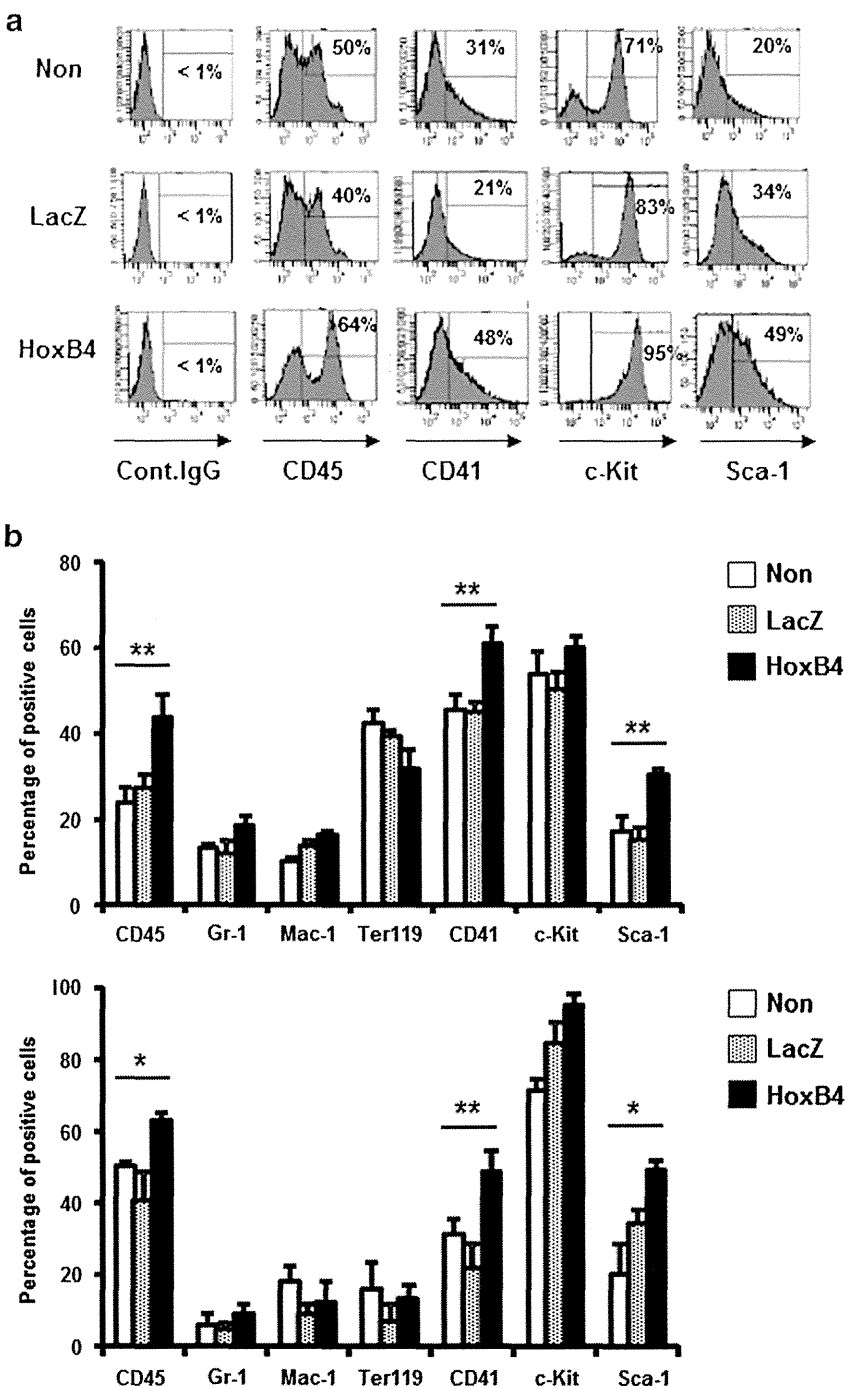


Figure 3 Expression of surface antigen and hematopoietic marker genes in mouse ES cell- or iPS cell-derived cells. (a, b) ES cell- or iPS cell line 38C2-derived cells were reacted with each antibody, and were then subjected to flow cytometric analysis. (a) Representative data from iPS cell line 38C2 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.

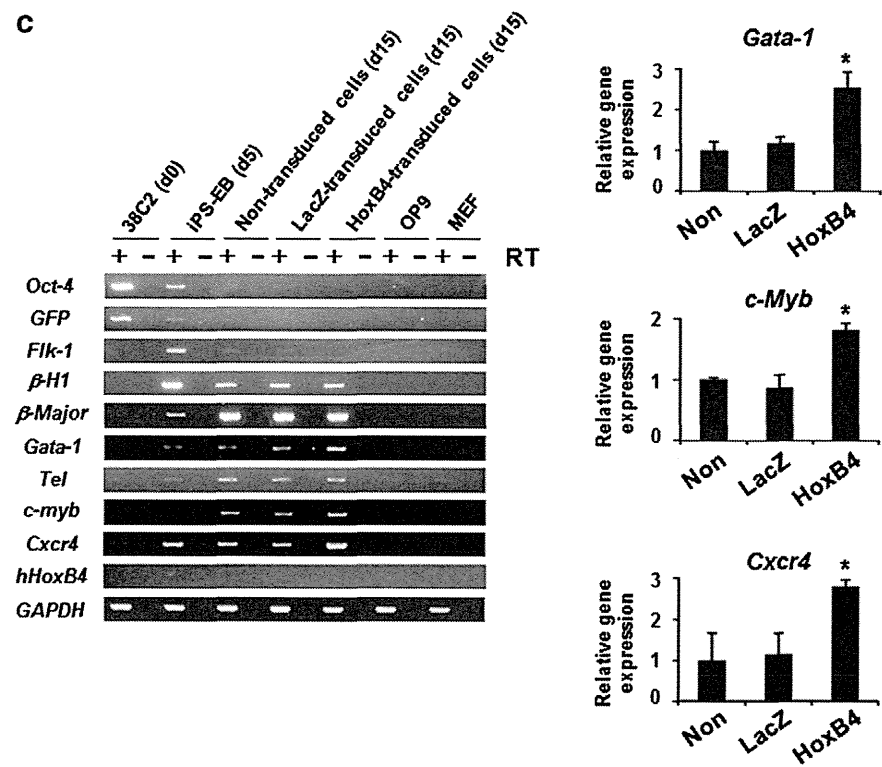


Figure 3 (continued).

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 (MWR30, 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 Biore-source 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

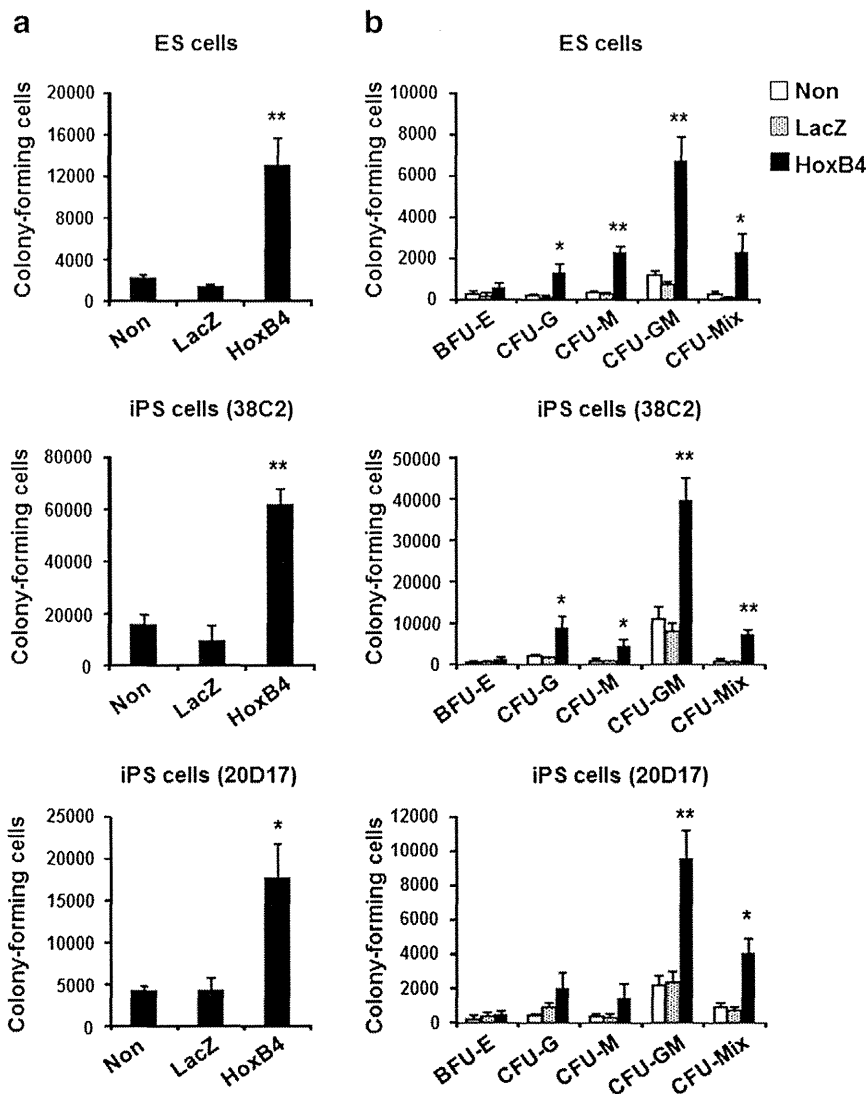


Figure 4 Significant increase of hematopoietic colony-forming cells in Ad-HoxB4-transduced hematopoietic cells. After ES-EB- or iPS-EB-derived cells were transduced with Ad-LacZ or Ad-hHoxB4, hematopoietic cells were generated by co-culturing with OP9 cells in the presence of hematopoietic cytokines 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.

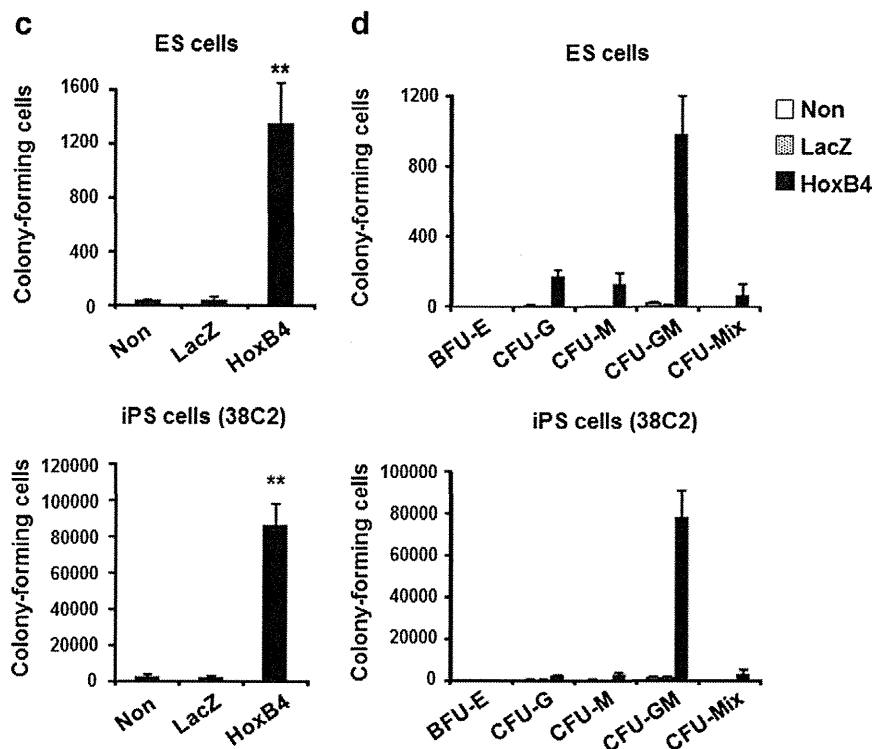


Figure 4 (continued).

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	TTCTTCTCCAGCTCCAAGA
Oct-3/4	Ms	GTTTGCCAAGCTGCTGAAGC	TCTAGCCCAAGCTGATTGGC
GFP	—	CACATGAAGCAGCACGACTT	TGCTCAGGTAGTGGTTGTCTG
Flk-1	Ms	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCCAACCACCC
Gata1	Ms	TTGTGAGGCCAGAGAGTGTG	TTCTCTGCTCTGGATTCCATC
Gata1 (real-time PCR)	Ms	GTCAGAACCGGCCTCTCATC	GTGGTCGTTTTGACAGTTAGTGCAT
Tel	Ms	CTGAAGCAGAGGAAATCTCGAATG	GGCAGGCAGTGATTATTCTCGA
c-myb	Ms	CCTCACCTCCATCTCAGCTC	GCTGGTGAGGCACTTTCTTC
β-H1	Ms	AGTCCCCATGGAGTCAAAGA	CTCAAGGAGACCTTTTGCTCA
β-Major	Ms	CTGACAGATGCTCTCTTGGG	CACAACCCAGAAACAGACA
CXCR4	Ms	GTCTATGTGGGCGTCTGGAT	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.

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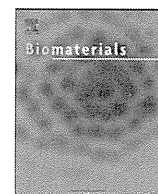
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3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing

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ABSTRACT

Although it is expected that hepatocyte-like cells differentiated from human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells will be utilized in drug toxicity testing, the actual applicability of hepatocyte-like cells in this context has not been well examined so far. To generate mature hepatocyte-like cells that would be applicable for drug toxicity testing, we established a hepatocyte differentiation method that employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a three-dimensional spheroid culture system using a Nanopillar Plate. We succeeded in establishing protocol that could generate more matured hepatocyte-like cells than our previous protocol. In addition, our hepatocyte-like cells could sensitively predict drug-induced hepatotoxicity, including reactive metabolite-mediated toxicity. In conclusion, our hepatocyte-like cells differentiated from human ES cells or iPS cells have potential to be applied in drug toxicity testing.

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1. Introduction

Hepatocyte-like cells that are generated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] are expected to be used in drug screening instead of primary [or cryopreserved] human hepatocytes (PHs). We recently demonstrated that stage-specific transient transduction of transcription factors, in addition to treatment with optimal growth factors and cytokines, is useful for promoting hepatic differentiation [3–6]. The hepatocyte-like cells, which have many hepatocyte characteristics (the abilities to uptake low-density lipoprotein and Indocyanine green, store glycogen, and synthesize urea) and drug metabolism capacity, were generated from hESCs/hiPSCs by

combinational transduction of FOXA2 and HNF1 α [6]. However, further maturation of the hepatocyte-like cells is required because their hepatic characteristics, such as drug metabolism capacity, are lower than those of PHs [6].

To promote further maturation of the hepatocyte-like cells, we subjected them to three-dimensional (3D) spheroid cultures. It is known that various 3D culture conditions (such as Algimatrix scaffolds [7], cell sheet technology [8], galactose-carrying substrata [9], and basement membrane substratum [10]) are useful for the maturation of the hepatocyte-like cells. Nanopillar Plate technology [11] used in the present study makes it easy to control the configuration of the spheroids. The Nanopillar Plate has an arrayed μ m-scale hole structure at the bottom of each well, and nanopillars were aligned further at the bottom of the respective holes. The seeded cells evenly drop into the holes, then migrate and aggregate on top surface of the nanopillars, thus likely to form the uniform spheroids in each hole. Not only 3D spheroid cultures [12] but also Matrigel overlay cultures [13] are useful for maintaining the hepatocyte characteristics of PHs. Therefore, we employed both 3D

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