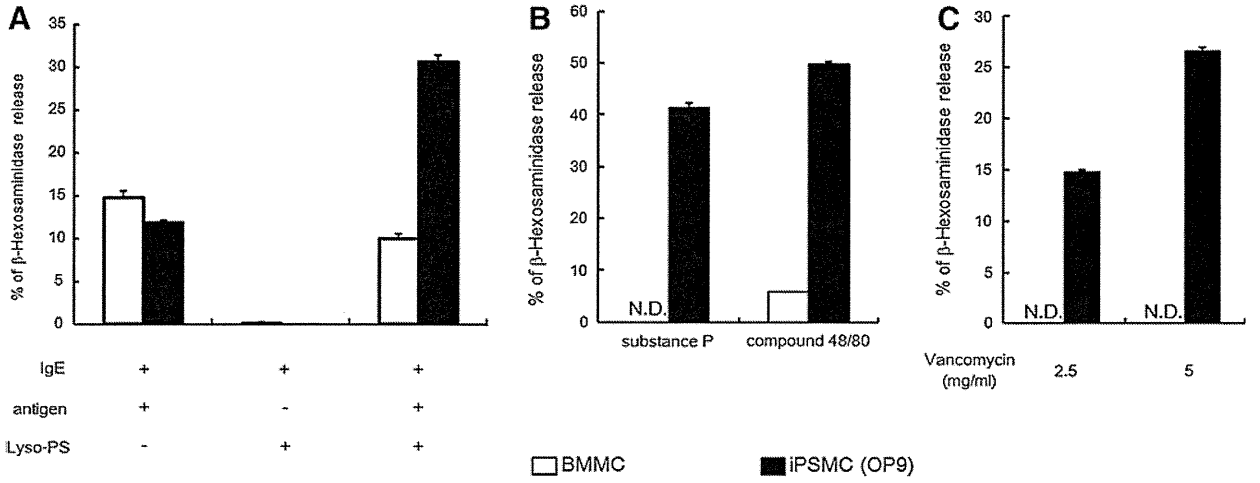

FIG. 4. β-hexosaminidase release from iPSMCs after stimulation with IgE/antigen, compound 48/80, substance P, and vancomycin. **(A)** The exocytotic response was determined by measuring the release of β-hexosaminidase. BMMCs (open bar) and the iPSMCs that were cocultured with OP9 cells (closed bar) were sensitized with anti-dinitrophenyl (DNP) IgE and stimulated with DNP human serum albumin (HSA) in the presence or absence of Lyso-PS as described in the Materials and Methods section. β-hexosaminidase enzymatic activity was measured in supernatants and cell pellets solubilized with 0.5% Triton X-100 in HEPES buffer. **(B)** BMMCs (open bar) and the iPSMCs that were cocultured with OP9 cells (closed bar) were stimulated with compound 48/80 or substance P. **(C)** BMMCs (open bar) and the iPSMCs that were cocultured with OP9 cells (closed bar) were stimulated with vancomycin. All data represent the means ± S.D. ($n = 3$).

Previously, Takano *et al.* demonstrated that *CD81*, a member of the tetraspanin superfamily, is one of the strikingly upregulated genes in BMMCs cocultured with Swiss 3T3 fibroblasts [8]. CD81 is also considered to be a marker of CTMCs. FACS analysis showed that expression of CD81 was elevated in the iPSMCs differentiated by either method as SF4 ▶ compared with that in BMMCs (Supplementary Fig. S4). In particular, our results revealed that the iPSMCs that were differentiated by coculture with OP9 cells were almost all CD81 positive and showed a homogeneous population.

Degranulation of iPSMCs

We compared FcεRI-mediated degranulation of BMMCs and iPSMCs by measuring the extracellular activity of β-hexosaminidase, a marker enzyme for histamine-containing F4 ▶ granules. As shown in Fig. 4A, the iPSMCs that were differentiated by coculture with OP9 cells displayed significantly less release of β-hexosaminidase than the BMMCs in response to IgE-mediated antigen stimulation. Similar results were obtained in iPSMCs that were derived from EB for- SF5 ▶ mation methods (Supplementary Fig. S5A). On the other hand, when the iPSMCs that were pretreated with anti-DNP monoclonal IgE were incubated with DNP-HSA in the presence of Lyso-PS, which is known to enhance IgE-mediated degranulation in rat peritoneal mast cells [27], the amount of β-hexosaminidase release was increased.

Responses to cationic secretagogues such as compound 48/80 and substance P are functional characteristics of CTMCs [4]. We next compared the IgE-independent responses between BMMCs and iPSMCs. Stimulation of iPSMCs with compound 48/80 resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of this enzyme was detected from stimulated BMMCs (Fig. 4B and Supplementary Fig. S5B). Similarly, β-hexosaminidase release from iPSMCs was much more markedly elevated by substance P treatment than β-hexosaminidase release from BMMCs (Fig. 4B and Supplementary Fig. S5B). In addition, stimulation of iPSMCs with vancomycin resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of β-hexosaminidase was detected from vancomycin-stimulated BMMCs (Fig. 4C and Supplementary Fig. S5C). These results indicate that the iPSMCs display a CTMC-like phenotype.

Comparison between iPSMCs differentiated by the OP9 coculture and EB formation protocols

We next compared the degree of differentiation between the iPSMCs that were differentiated by the OP9 coculture method and those differentiated by the EB formation method. The expression level of FcεRI was significantly lower in the iPSMCs that were differentiated by coculture with OP9 cells as compared with the iPSMCs that were differentiated by EB formation (Fig. 2). However, the number of Safranin O-positive cells was significantly greater in the iPSMCs that were cocultured with OP9 cells than in the iPSMCs that were derived from the EB formation method (Fig. 1B). In addition, the expression levels of HDC mRNA and CD81 protein were significantly higher in the iPSMCs that were cocultured with OP9 cells than in those that were derived from the EB formation method (Supplementary Figs. S3 and S4). These results showed that the iPSMCs that were cocultured with OP9 cells were more mature than the iPSMCs that were derived from the EB formation method. The iPSMCs that were derived from EB formation were more mature than BMMCs (Figs. 1–3). During the differentiation step, the iPSMCs that were derived from the EB formation method were designated as mast cells I (Fig. 5F). The iPSMCs that were differ- ◀ F5 entiated by coculture with OP9 cells were also designated as mast cells II.

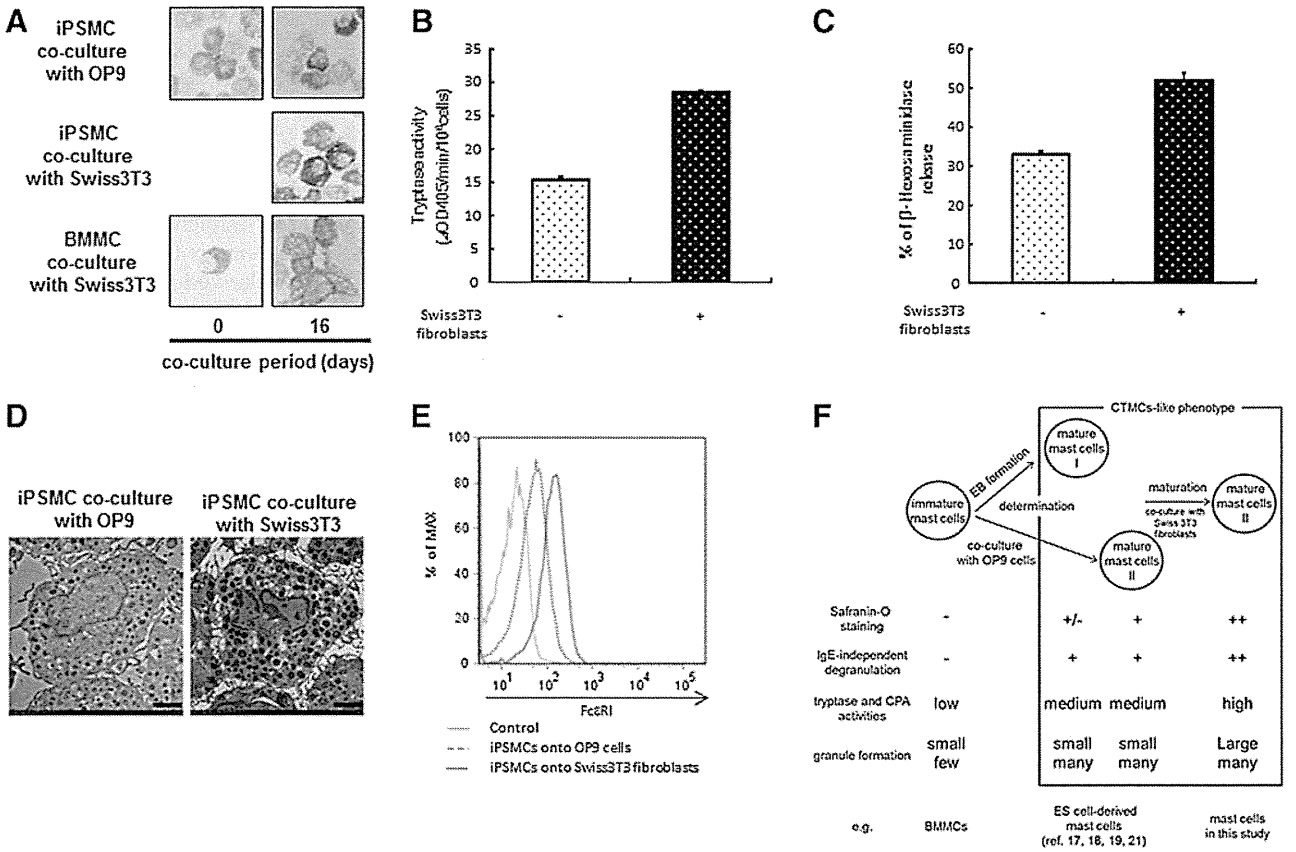

FIG. 5. Maturation of iPSMCs cocultured with Swiss 3T3 fibroblasts in the presence of stem cell factor. **(A)** Cytocentrifuged preparations of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts, and the BMMCs that were cocultured with Swiss 3T3 fibroblasts were stained with Alcian blue and Safranin O. **(B)** Granule protease activities of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were measured. **(C)** The exocytotic response was determined by measuring the release of β-hexosaminidase. The iPSMCs that were cocultured with OP9 cells (open bar) or Swiss 3T3 fibroblasts (closed bar) were stimulated with compound 48/80. **(D)** The iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were analyzed by transmission electron microscopy. Scale bar = 2.0 μm. **(E)** Suspensions of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were stained with FITC-labeled anti-FcεRI antibody for 45 min on ice. The stained cells were washed, resuspended in 1% FBS-PBS, and analyzed by flow cytometry. **(F)** Scheme of 2-step differentiation of mast cells from iPS cells. All data represent the means ± S.D. ($n = 3$).

Enhancement of maturation in iPSMCs by Swiss 3T3 fibroblasts

A previous study reported that coculture of BMMCs with Swiss 3T3 fibroblasts in the presence of SCF facilitated morphological and functional maturation toward a CTMC-like phenotype [8]. It is possible that Swiss 3T3 fibroblasts promote the maturation of the iPSMCs (mast cells II) that are generated on OP9 cells. Therefore, we expected to generate more mature iPSMCs by coculturing with Swiss 3T3 fibroblasts. We compared the degree of mast cell maturation of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts. Although the majority of BMMCs were Alcian blue positive and Safranin O negative, the percentage of Safranin O-positive mast cells was gradually increased up to ~80% on day 16 after coculturing with Swiss 3T3 fibroblasts (Fig. 5A). The staining intensity for Safranin O on the iPSMCs that were cocultured with Swiss 3T3 fibroblasts was stronger than the iPSMCs (mast cells II) (Fig. 5A). Therefore, the iPSMCs that were differentiated by coculture with Swiss 3T3 fibroblasts were found to be more mature than mast cells II and designated as mast cells III (Fig. 5F). We measured the tryptase activity in the iPSMCs (mast cells II) or iPSMCs (mast cells III), and found the elevated tryptase activity in the iPSMCs (mast cells III) relative to the iPSMCs (mast cells II) (Fig. 5B). Similarly, β-hexosaminidase release by compound 48/80 in the iPSMCs (mast cells III) was markedly elevated in comparison with that in the iPSMCs (mast cells II) (Fig. 5C). Electron microscopic analysis of mast cells revealed that the iPSMCs (mast cells III) contained more large granules (Fig. 5D). We performed flow cytometric analysis to examine the surface expression of c-kit and FcεRI on the iPSMCs (mast cells III). After coculturing, iPSMCs (mast cells III) and BMMCs still expressed similar levels of c-kit (data not shown). Remarkably, the expression level of FcεRI was elevated in the iPSMCs (mast cells III) (Fig. 5E). These results showed that there were 2 steps in iPSMC maturation process.

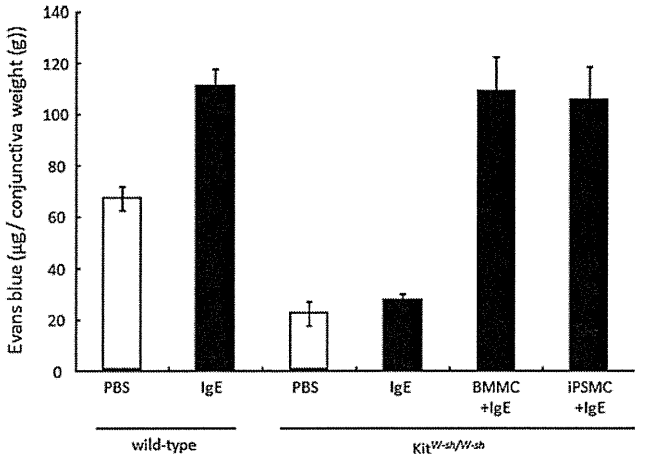

FIG. 6. IgE-dependent passive cutaneous anaphylaxis in mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with iPSMCs. BMMCs or iPSMCs were injected subcutaneously into the conjunctivae of mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. After 6 weeks, mice were subcutaneously sensitized with anti-DNP IgE or saline, followed by induction of passive cutaneous anaphylaxis. After 24 h, mice were intravenously injected with DNP-HSA along with 2% Evan's blue dye. Thirty minutes later, conjunctivae were excised, and Evan's blue dye was extracted. Extravasation of Evan's blue dye was quantified as described in the Materials and Methods section. Results are normalized to average conjunctivae weight and are expressed as mean ± S.D. ($n = 3$ mice per group).

Mast cell reconstitution and induction of passive cutaneous anaphylaxis

We assessed whether the iPSMCs (mast cells I) with a C57BL/6 background could exhibit passive cutaneous anaphylaxis 6 weeks after injection of iPSMCs or BMMCs into the conjunctiva of mast-cell-deficient ($Kit^{W\text{-}sh/W\text{-}sh}$) mice [28]. $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with BMMCs or iPSMCs exhibited passive cutaneous anaphylaxis reactions in the conjunctivae as measured by extravasation of Evan's Blue dye (Fig. 6). On the other hand, no passive cutaneous anaphylaxis reactions in the conjunctivae were observed for $Kit^{W\text{-}sh/W\text{-}sh}$ mice that were not reconstituted with mast cells. These results indicate that iPSMCs had the ability to respond to stimulation with IgE/antigen *in vivo*.

Discussion

In this study, we developed a protocol consisting of mesoderm induction (stage 1), mast cell specification (stage 2), determination of mast cells (stage 3), and maturation of mast cells (stage 4) for mast cell differentiation from iPS cells (Supplementary Figs. S1 and S6). BMMCs have been used extensively as a mast cell model. We compared the degree of maturation in BMMCs and iPSMCs. Expression level of CD81 was higher in the iPSMCs that were differentiated by both methods than in BMMCs (Supplementary Fig. S4). We also showed that IgE-mediated degranulation of iPSMCs was elevated in comparison with that in BMMCs in the presence of Lyso-PS (Fig. 4A and Supplementary Fig. S5A), demonstrating that iPSMCs were more mature than BMMCs. Therefore, both OP9 cells and EB-derived feeder cells might moderately promote the maturation of mast cells. The other possibility is that the maturation of iPSMCs might be accelerated by SCF. SCF is one of the most important cytokines for mast cell maturation [29]. BMMCs are in general generated without SCF. These are reasons why iPSMCs were more mature than BMMCs with respect to their phenotypes and functions.

Cytokines and feeder cells were required to induce the mast cell development from mouse iPS cells. As previously described, IL-3 is known to play an important role in mast cell specification. In contrast, BMMCs are generated without OP9 cells, suggesting that OP9 cells are not necessary for mast cell specification. OP9 cells might promote the maturation of mast cells, possibly by OP9 cell-derived factors, such as IL-4 [30–31], IL-6 [32–34], and nerve growth factor [34]. Therefore, cytokines and OP9 cells are all-essential and have distinct roles in the differentiation of mast cells from iPS cells.

We found a difference in FcεRI expression levels between iPSMCs (mast cell I) and iPSMCs (mast cell II) (Fig. 2). Surface expression level of FcεRI was lower in the iPSMCs (mast cells II), compared with that in the iPSMCs (mast cells I). More recently, Kovarova *et al.* reported that expression of FcεRIα mRNA was lower in human ES cell-derived mast cells that were cocultured with OP9 cells than in human ES cell-derived mast cells that were derived from the EB formation method [21]. These findings were fully consistent with our results. However, our results showed that iPSMCs (mast cells II) were more mature than iPSMCs (mast cells I). These results indicate that the expression levels of FcεRI are not completely correlated with the degree of mast cell differentiation, although the iPSMCs (mast cells III) showed a high level of FcεRI expression (Fig. 5E).

In the present study, we demonstrated that, as in the case of BMMCs, Swiss 3T3 fibroblasts could promote the maturation of iPSMCs (Fig. 5). A recent study has reported that cynomolgus monkey ES cells that are cocultured with the murine aorta-gonad-mesonephros-derived stromal cell line AGM-S1 cells are differentiated into CTMCs [20]. These results suggest that feeder cells, including AGM-S1 and Swiss3T3 fibroblasts, would promote mast cell maturation by similar mechanisms. These feeder cells might contribute

TABLE 1. PRIMER SEQUENCES (5′-3′) FOR QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Genes	*Sense primer*	*Antisense primer*
HDC	CGCTCCATTAAGCTGTGGTTTGTGATTCGG	AGACTGGCTCCTGGCTGCTTGATGATCTTC
FcεRIα	GAGTGCCACCGTTCAAGACA	GTAGATCACCTTGCGGACATTC
FcεRIβ	TGGTTGGTTTGATATGCCTTTGT	CACTGCACCCCAGAATGGATA
FcεRIγ	ATCTCAGCCGTGATCTTGTTCT	ACCATACAAAAACAGGACAGCAT

to the identification of factors that play a role in mast cell maturation.

While iPSMCs (mast cells II) were almost all Safranin O positive, iPSMCs (mast cells I) included both Safranin O-positive and negative populations, suggesting that immature cells were contained in iPSMCs (mast cells I). Expression levels of CD81 protein and HDC mRNA, and protease activities, were slightly elevated in the iPSMCs (mast cells II) as compared with the iPSMCs (mast cells I). Our results suggest that the iPSMCs (mast cells II) were more mature than iPSMCs (mast cell I). The iPSMCs (mast cells III) exhibited more mature phenotypes, such as large granules and high activity of protease. Taken together, the rank order of maturity in mast cells was the following: iPSMCs (mast cells III) > iPSMCs (mast cells II) > iPSMCs (mast cells I). The precise mechanisms of mast cell maturation process remain to be clarified. Classification of mast cells by using cell surface or internal marker can contribute to clarify the maturation mechanism of mast cells. Further studies are needed to find cell surface or internal marker that can clearly distinguish iPSMCs (mast cells I and II) and iPSMCs (mast cells III).

Our data demonstrate that iPSMCs could functionally respond to IgE stimulation *in vivo* (Fig. 6). There was no significant difference in Evan's blue extravasation in the $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with iPSMCs or BMMCs. Previously, Fukuda *et al.* demonstrated that conjunctiva reconstituted with BMMCs display a CTMC-like phenotype [28]. Therefore, passive cutaneous anaphylaxis reactions were comparable in $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with iPSMCs or BMMCs.

Galli's group first reported the identification of mast cell-committed progenitors (MCPs) in adult murine bone marrow [35]. They indicated that MCPs may be directly developed from multipotential progenitors independent of the myeloid pathway. In contrast, Arinobu *et al.* demonstrated that granulocyte/monocyte progenitors gave rise to MCPs [36]. The models of the developmental process in mast cells differed between these 2 reports. To analyze cells at each differentiation step, our differentiation protocol will be useful for clarifying the developmental process of mast cells.

Because of their pluripotency and self-renewal, ES cells and iPS cells are potential cell sources for regenerative medicine and other clinical applications, such as cell therapies, drug screening, toxicology, and investigation of disease mechanisms. Notably, iPS cell-based screening approaches might support the development of personalized medicine and tailor-made treatment plans. Vancomycin, an antibiotic to which methicillin-resistant *Staphylococcus aureus* (MRSA) is sensitive, frequently induces allergic reaction [37]. In this study, the stimulation of the iPSMCs with vancomycin resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of this enzyme was detected from BMMCs (Fig. 4C and Supplementary Fig. S5C). Therefore, iPSMCs would be potential cell sources for drug-allergy-screening system.

We developed a 2-step differentiation protocol of mast cells from iPS cells. In the conventional method, CTMC-like mast cells are produced from bone marrow cells after 45 days of culture. In contrast, the iPSMCs generated on OP9 cells in the present study were produced after 28 days of culture. Thus, homogeneous CTMC-like mast cells can be easily generated from iPS cells by the OP9 coculture method. On the other hand, Swiss 3T3 coculture methods have different advantage from OP9 coculture systems. The iPSMCs that were cocultured with Swiss 3T3 fibroblasts were more mature than the iPSMCs that were generated on OP9 cells. Because each of these methods has its advantages, the protocol should be chosen in accordance with the intended use.

We successfully developed a 2-step differentiation protocol for generating more mature mast cells from mouse iPS cells. The iPSMCs generated in this study exhibit many characteristics distinct from BMMCs. The iPSMCs possessed the characteristics of mature mast cells, including the heparin contents and degranulation, in response to cationic secretagogues and vancomycin. The iPSMCs serve as an excellent model for *in vitro* studies of CTMCs. Our results could facilitate clarification of the mechanisms that control the development of mast cells.

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Author Disclosure Statement

The authors have no financial conflict of interest.

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ORIGINAL RESEARCH REPORT

Inhibition of Lnk in Mouse Induced Pluripotent Stem Cells Promotes Hematopoietic Cell Generation

AU1 ▶ Katsuhisa Tashiro,[1,*] Miyuki Omori,[1,2,*] Kenji Kawabata,[1,3] Nobue Hirata,[1] Tomoko Yamaguchi,[1] Fuminori Sakurai,[2] Satoshi Takaki,[4] and Hiroyuki Mizuguchi[1,2,5]

AU2 ▶ Embryonic stem (ES) cell- and induced pluripotent stem (iPS) cell-derived hematopoietic stem/progenitor cells (HSPCs) are considered as an unlimited source for HSPC transplantation. However, production of immature hematopoietic cells, especially HSPCs, from ES and iPS cells has been challenging. The adaptor protein Lnk has been shown to negatively regulate HSPC function via the inhibition of thrombopoietin (TPO) and stem cell factor signaling, and Lnk-deficient HSPCs show an enhanced self-renewal and repopulation capacity. In this study, we examined the role of Lnk on the hematopoietic differentiation from mouse ES and iPS cells by the inhibition of Lnk using a dominant-negative mutant of the Lnk (*DN-Lnk*) gene. We generated mouse ES and iPS cells stably expressing a DN-Lnk, and found that enforced expression of a DN-Lnk in ES and iPS cells led to an enhanced generation of Flk-1-positive mesodermal cells, thereby could increase in the expression of hematopoietic transcription factors, including *Scl* and *Runx1*. We also showed that the number of both total hematopoietic cells and immature hematopoietic cells with colony-forming potential in DN-Lnk-expressing cells was significantly increased in comparison with that in control cells. Furthermore, Lnk inhibition by the overexpression of the *DN-Lnk* gene augmented the TPO-induced phosphorylation of Erk1/2 and Akt, indicating the enhanced sensitivity to TPO. Adenovirus vector-mediated transient *DN-Lnk* gene expression in ES and iPS cells could also increase the hematopoietic cell production. Our data clearly showed that the inhibition of Lnk in ES and iPS cells could result in the efficient generation and expansion of hematopoietic cells.

Introduction

SINCE EMBRYONIC STEM (ES) cells and induced pluripotent stem (iPS) cells can self-renew indefinitely and differentiate into all types of cells in the 3 germ layers, they are expected to have clinical applications in cell-based therapies [1–4]. For instance, ES cell- and iPS cell-derived hematopoietic cells are considered as an alternative source of adult hematopoietic cells for the treatment of hematological disorders and malignancies. Many groups have reported the differentiation of ES and iPS cells into mature hematopoietic cells, including erythrocytes, myeloid cells, and lymphoid cells [5–10]. However, previous reports have described the generation of only small numbers of mature hematopoietic cells, probably as a result of inefficient generation and expansion of immature hematopoietic cells derived from pluripotent stem cells. Therefore, the use of ES cell- and iPS cell-derived hematopoietic cells as a cell source for therapeutic applications depends on the efficient production of hematopoietic cells, especially immature hematopoietic cells, from pluripotent stem cells.

Recently, inhibitors of differentiation (*ID*) genes, which are negative regulators of E proteins (E2A, HEB, and E2-2) [11], were shown to negatively regulate the hematopoietic differentiation in ES and iPS cells [12]. The same study also showed that the suppression of the ID genes, *ID1* and *ID3* increased the number of ES and iPS cell-derived hematopoietic progenitor cells [12]. These data indicate that negative regulators play an important role in the hematopoietic differentiation process in ES and iPS cells, and that manipulation of the expression of negative regulators would be an effective strategy for the efficient generation of hematopoietic cells from ES and iPS cells.

An adaptor protein Lnk/SH2B3 (hereafter referred to Lnk) is shown to negatively regulate the thrombopoietin (TPO) and stem cell factor (SCF) signaling, both of which are crucial

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cytokine-signaling pathways involved in hematopoietic stem cell (HSC) self-renewal, since Lnk-deficient HSCs exhibit an augmented response to TPO and SCF stimulation, and thereby Lnk-deficient mice show the marked HSC expansion in the bone marrow [13–16]. In addition, Lnk is highly expressed in immature hematopoietic cells, particularly in HSCs [17], in contrast to ID genes, which are ubiquitously expressed in many tissues [11,18]. Therefore, we speculated that an inhibition of Lnk function in ES and iPS cells would lead to the efficient generation and expansion of immature hematopoietic cells. In the present study, we investigated the effects of Lnk inhibition on the hematopoietic differentiation of mouse ES and iPS cells, and we found that a suppression of Lnk function by the enforced expression of a dominant-negative mutant of the Lnk (*DN-Lnk*) gene in ES and iPS cells resulted in an increase in the number of both mesodermal cells with hematopoietic differentiation potential and immature hematopoietic cells. These findings indicate that the suppression of the Lnk would be useful for the efficient generation and expansion of ES cell- and iPS cell-derived hematopoietic cells.

Materials and Methods

Plasmid construction and adenovirus vectors

pEF-IRESneo, which contains internal ribosome entry sites (IRES) and a neomycin-resistant gene (*Neo*) downstream of the human elongation factor (EF)-1α promoter, was constructed by replacing the cytomegalovirus (CMV) promoter of pIRESneo (Clontech) with the EF-1α promoter, which is derived from pEF/myc/nuc (Invitrogen). Mouse DN-Lnk cDNA, derived from pMY-DN-Lnk [19], was inserted into pEFIRESneo, resulting in pEF-DNLnk-IRESneo. Adenovirus (Ad) vectors were constructed by an improved in vitro ligation method [20,21]. Mouse DN-Lnk cDNA was inserted into pHMCA5 [22], which contains the CMV enhancer/β-actin promoter with an β-actin intron (CA) promoter (a kind gift from Dr. J. Miyazaki, Osaka University) [23], resulting in pHMCA5-DN-Lnk. pHMCA5-DN-Lnk was digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested pAdHM4 [20], resulting in pAd-DN-Lnk. Ad-DN-Lnk and Ad-DsRed were generated and purified as described previously [24]. The CA promoter-driven β-galactosidase (LacZ)-expressing Ad vector, Ad-LacZ, and the CA promoter-driven DsRed-expressing Ad vector, Ad-DsRed, were generated previously [24,25]. The vector particle (VP) titer was determined using a spectrophotometric method [26].

Cell culture

The mouse ES cell line, BRC6 (Riken Bioresource Center), and the mouse iPS cell line, 38C2 (a kind gift from Dr. S. Yamanaka, Kyoto University) [27], were used in this study. DN-Lnk- or *Neo*-expressing mouse ES and iPS cell lines were generated as follows. The pEF-IRESneo and pEF-DNLnk-IRESneo were linearized and were then electroporated into mouse ES cells and iPS cells by using Gene Pulser Xcell (250 V, 500 μF; Bio-Rad Laboratory). pEF-IRESneo- or pEF-DNLnk-IRESneo-transfected ES cells and iPS cells were cultured in an ES cell medium containing 100 μg/mL G418 (for ES cells) or 200 μg/mL G418 (for iPS cells) for 10–14 days, and G418-resistant colonies were picked up and expanded. The expression of DN-Lnk was confirmed by conventional reverse transcription–polymerase chain reaction (RT-PCR). Mouse ES cells, iPS cells, and *Neo*- or DN-Lnk-expressing mouse ES and iPS cells were cultured in a leukemia inhibitory factor-containing ES cell medium (Millipore) on mitomycin C-treated mouse embryonic fibroblasts (MEFs) [28]. OP9 stromal cells were cultured in an α-minimum essential medium (α-MEM; Sigma) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), and non-essential amino acid (Invitrogen).

In vitro hematopoietic differentiation

For embryoid body (EB) differentiation, mouse ES and iPS cells were trypsinized and collected in an EB medium (EBM) containing the Dulbecco's modified Eagle's medium (Wako) supplemented with 15% FBS, non-essential amino acids (Millipore), penicillin/streptomycin (Invitrogen), 2 mM L-glutamine, and 100 μM β-mercaptoethanol (Nacalai Tesque), and they were plated on a culture dish for 30 min to allow the MEFs to adhere. Nonadherent cells were collected and plated on a round-bottom Lipidure-coated 96-well plate (Nunc) at 3×10^3 cells (ES cells) or 1×10^3 (iPS cells) cells per well. On day 5, half of the medium was exchanged for fresh EBM. EBs were collected on day 7, and a single-cell suspension was prepared by the use of trypsin/ethylenediaminetetraacetic acid. The EB-derived cells (4×10^5 cells) were plated on OP9 stromal cells in the wells of a 6-well plate and were then cultured with an OP9 medium containing recombinant hematopoietic cytokines [100 ng/mL mouse SCF, 100 ng/mL human Flt3-ligand, 20 ng/mL mouse TPO, 5 ng/mL mouse interleukin (IL)-3, and 5 ng/mL human IL-6] to induce and expand the hematopoietic cells. In the case of DN-Lnk transduction using the Ad vector, EB-derived cells were transduced with Ad-LacZ or Ad-DN-Lnk at 3,000 VP/cell for 1.5 h in a 15-mL tube before the transduced EB-derived cells plating on OP9 cells. Hematopoietic cells were collected as described previously [25]. In brief, the floating and loosely attached cells were collected by pipetting and were transferred to 15-mL tubes. The adherent hematopoietic cells were harvested by trypsin treatment, and were then incubated in a tissue culture dish for 30–60 min to eliminate the OP9 stromal 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

The following primary monoclonal antibodies (Abs), conjugated with fluorescein isothiocyanate, phycoerythrin, or allophycocyanin, were used for flow cytometric analysis: anti-CD45 (30-F11; eBioscience), anti-CD11b (M1/70; eBioscience), anti-Sca-1 (D7; eBioscience), anti-Ter119 (Ter-119; eBioscience), anti-CD34 (RAM34; eBioscience), anti-CXCR4 (2B11; BD Bioscience), anti-Gr-1 (RB6-8C5; eBioscience), anti-c-Kit (ACK2 or 2B8; eBioscience), and anti-CD41 (MWReg30; BD Bioscience). Purified rat anti-mouse c-Mpl/TPOR monoclonal Ab was obtained from IBL. Cells (1×10^5–5×10^5) were incubated with monoclonal Abs at 4°C for 30 min and washed twice with a staining buffer (phosphate-buffered saline/2% FBS). For detection of Mpl/TPOR, Dylight649-conjugated goat anti-rat IgG (BioLegend)

was used as a secondary Ab. After staining, the hematopoietic cells were analyzed and isolated by flow cytometry on an LSR II and FACSAria flow cytometer, respectively, using FACSDiva software (BD Bioscience).

Colony assay and May-Giemsa staining

The cells (5×10^4 cells) were cultured in a Methocult M3434 medium containing IL-3, IL-6, SCF, and erythropoietin (Stem-Cell Technologies, Inc.) for 10 days. The number of individual colonies was counted by microscopy. The colony number was normalized to the total number of hematopoietic cells. The multipotent hematopoietic progenitor cell-derived colonies (colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM)/CFU-Mix) were picked up, fixed on glass slides using a cytospin centrifuge (Cytospin 4; Thermo Shandon), and stained with May-Grünwald Stain solution (Sigma) and Giemsa solution (Wako).

Western blotting

The adherent hematopoietic cells and OP9 stromal cells were collected, and were then incubated in a new tissue culture dish for 40 min to eliminate adherent OP9 cells. Floating cells were harvested and were subsequently starved in an RPMI1640 medium containing 0.1% FBS and penicillin/streptomycin for 4–6 h. Cells were stimulated with 20 ng/mL TPO for 10 min (for Jak2) or 30 min (for Erk and Akt) before being lyzed in a lysis buffer [20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 10% glycerol] containing a protease inhibitor cocktail (Sigma) and a phosphatase inhibitor cocktail (Nacalai Tesque). Cell lysates were loaded onto polyacrylamide gels and were transferred to a polyvinylidene fluoride membrane (Millipore). After blocking, the membrane was exposed to mouse anti-phospho-Erk1/2 (Cell Signaling), rabbit anti-Erk1/2 (Sigma), mouse anti-phospho-Akt (Cell Signaling), rabbit anti-total Akt (Cell Signaling), rabbit anti-phospho-Jak2 (Tyr1007/1008; Cell Signaling), or rabbit anti-Jak2 (Cell Signaling), followed by horseradish peroxidase-conjugated secondary antibody. The band was visualized by ECL Plus Western blotting detection reagents (GE Healthcare) or Pierce Western Blotting Substrate Plus (Thermo Scientific), and the signals were read using an LAS-3000 imaging system (Fujifilm).

Reverse transcription–polymerase chain reaction

RT-PCR was carried out as described previously [25]. The sequences of the primers used in this study are listed in Table 1. ◀T1

Results

Expression of Lnk in mouse ES and iPS cells

We initially investigated Lnk expression in mouse ES cells, iPS cells, ES cell-derived EBs (ES-EBs), and iPS-EBs. As shown in Fig. 1a, Lnk was expressed in undifferentiated ES ◀F1 and iPS cells, and the expression levels of Lnk were significantly increased after EB formation. We further examined whether Lnk was expressed in Flk-1-positive ($^+$) cells, because hematopoietic cells were generated from Flk-1$^+$ cells, a common hemoangiogenic progenitor during ES cell differentiation [29–31]. Quantitative RT-PCR analysis after the purification of Flk-1$^+$ cells from ES-EBs and iPS-EBs revealed that Lnk was highly expressed in Flk-1$^+$ cells (Fig. 1b). These data suggest that Lnk plays some role in the hematopoietic differentiation process in ES and iPS cells.

Enhanced mesodermal differentiation in EB by the inhibition of Lnk

The data described above led to the expectation that hematopoietic cells, including hematopoietic progenitor cells, could be efficiently generated from ES cells and iPS cells by the suppression of Lnk. To inhibit the function of Lnk, we utilized the *DN-Lnk* gene, which was developed by Takizawa et al. [19]. DN-Lnk binds to Lnk, and forms a multimer complex by a homophilic interaction with the N-terminal domain, thereby inhibiting Lnk function [19]. DN-Lnk-expressing ES and iPS cells were generated by introducing a

Table 1. List of Primers Used for Reverse Transcription–Polymerase Chain Reaction

Gene name	*(5′) Sense primers (3′)*	*(5′) Antisense primers (3′)*
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
Flk-1	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCCAACCACCC
LnK	GCCACTTTCTGCAGCTCTTC	GTCCAGGGAGTCAGTGCTTC
LnK (for real-time)	AGCCACTTTCTGCAGCTCTTC	GTAGAGGTTGTCAGGCATCTCC
DN-LnK	GGGCTACCAGTGACACCAAT	CACTGTCCACGCTCTGTGAG
Oct-3/4	GTTTGCCAAGCTGCTGAAGC	TCTAGCCCAAGCTGATTGGC
Nanog	ATGGTCTGATTCAGAAGGGC	TTCACCTCCAAATCACTGGC
β-H1	AGTCCCCATGGAGTCAAAGA	CTCAAGGAGACCTTTGCTCA
β-major	CTGACAGATGCTCTCTTGGG	CACAACCCCAGAAACAGACA
Scl/Tal-1	AACAACAACCGGGTGAAGAG	GGGAAAGCACGTCCTGTAGA
Runx1	CTTCCTCTGCTCCGTGCTAC	GACGGCAGAGTAGGGAACTG
Gata1	TTGTGAGGCCAGAGAGTGTG	TTCCTCGTCTGGATTCCATC
Gata2	TAAGCAGAGAAGCAAGGCTCGC	ACAGGCATTGCACAGGTAGTGG
Fli1	CCAACGAACGGAGAGTCATT	ATTCCTTGCCATCCATGTTC
Erg	GGAGCTGTGCAAGATGACAA	GATTAGCAAGGCGGCTACTG
Erg (for real-time)	GGAGTGCAACCCTAGTCAGG	TAGCTGCCGTAGCTCATCC
Sfpi1	CCATAGCGATCACTACTGGGATTT	TGTGAAGTGGTTCTCAGGGAAGT
E47	ATACAGCGAAGGTGCCCACT	CTCAAGGTGCCAACACTGGT

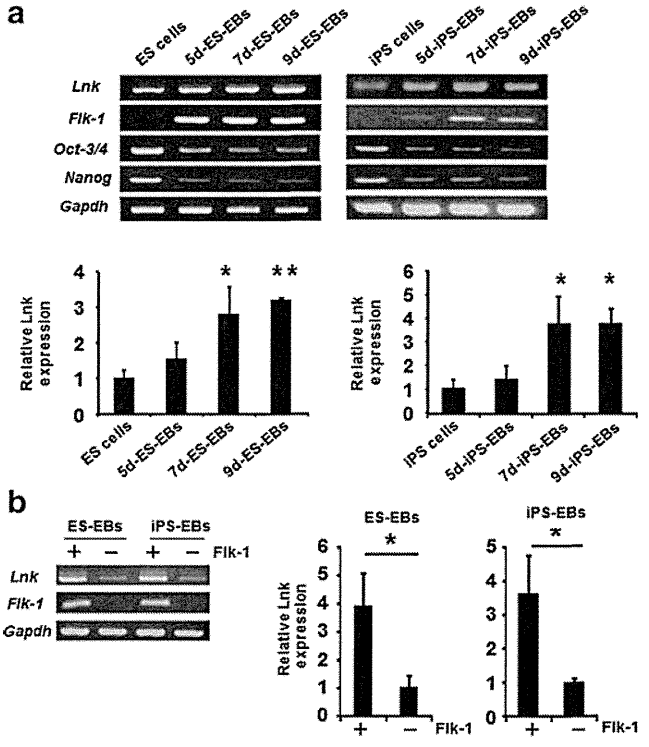

FIG. 1. Lnk is expressed in mouse ES cells, iPS cells, and Flk-1$^+$ hemoangiogenic progenitor cells. **(a)** Total RNA was extracted from undifferentiated ES cells, ES cell-derived EBs cultured for 5, 7, or 9 days (5-day ES-EBs, 7-day ES-EBs, 9-day ES-EBs, respectively), undifferentiated iPS cells, 5-day iPS-EBs, 7-day iPS-EBs, or 9-day iPS-EBs. Then, conventional (above) and quantitative (below) RT-PCR analysis was carried out. Results shown were the mean of 3 independent experiments with indicated SD. $*p<0.05$, $**p<0.01$ as compared with undifferentiated ES cells or iPS cells. **(b)** Flk-1$^+$ and Flk-1$^-$ cells were sorted from 7-day ES-EBs or 7-day iPS-EBs using FACSAria. The purity of the Flk-1$^+$ and Flk-1$^-$ cells exceeded 90% and 95%, respectively (data not shown). Total RNA was extracted from both types of cell, and the expression of Lnk was examined by conventional (*left*) and quantitative (*right*) RT-PCR analysis. The data were expressed as mean ± SD ($n=3$); $*p<0.05$ as compared with Flk-1$^-$ cells. ES, embryonic stem; EBs, embryoid bodies; iPS, induced pluripotent stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription–polymerase chain reaction, SD, standard deviation.

DN-Lnk-expressing plasmid, and *DN-Lnk* mRNA expression was confirmed by RT-PCR (Fig. 2a). In this report, we present data from one DN-Lnk-expressing ES and iPS cell clone, because the same results were obtained from other DN-Lnk-expressing clones. Notably, the expression levels of wild-type Lnk in DN-Lnk-expressing cells were similar to those in *Neo*-expressing cells and their parent cells (Fig. 2a). DN-Lnk-expressing iPS cells maintained the undifferentiated state in culture and possessed pluripotency, as demonstrated by alkaline phosphatase staining, immunostaining, and teratoma formation (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Hence, ectopic expression of the *DN-Lnk* gene in ES and iPS cells would not affect their function.

Next, we generated EBs to induce mesodermal cells from DN-Lnk- or *Neo*-expressing ES and iPS cells. EBs were cultured for 7 days, because the proportion of Flk-1$^+$ cells in EBs increased to a peak on day 7, and decreased over the next 2 days in our culture conditions (Supplementary Fig. S2). We found that DN-Lnk-expressing cells on day 7 of the EB culture yielded a modest increase in the number of Flk-1$^+$ hemangiogenic progenitor cells relative to that of *Neo*-expressing cells (Fig. 2b). Interestingly, elevated expression of *Scl/Tal-1*, *Runx1*, and *Gata-1* was observed in DN-Lnk-expressing total EB cells (Fig. 2c). Besides the expression levels of these genes, those of other key transcription factors of blood stem/progenitor cells, including *Gata-2*, *Fli-1*, and *Erg* [32], in DN-Lnk-expressing cells were also upregulated in comparison with those in *Neo*-expressing cells (Fig. 2d). To examine whether increased expression of these transcription factors in DN-Lnk-expressing cells was due to the increased generation of Flk-1$^+$ cells, we performed the gene expression analysis after purification of Flk-1$^+$ cells from DN-Lnk- or *Neo*-expressing total EB cells (Fig. 2e). No difference in the expression of *Runx1*, *Gata-1*, *Gata-2*, *Fli-1*, or *Erg* was observed between DN-Lnk-expressing cells and *Neo*-expressing cells, indicating that elevated expression of these hematopoietic genes in DN-Lnk-expressing EB cells would be largely because of the increased population of Flk-1$^+$ cells. On the other hand, DN-Lnk-expressing Flk-1$^+$ cells showed a 2-fold increase in the expression of *Scl/Tal-1*, an essential transcription factor for the hematopoietic development [33,34], compared with *Neo*-expressing Flk-1$^+$ cells. The increased *Scl/Tal-1* expression thus suggests that an inhibition of Lnk in Flk-1$^+$ cells might contribute to enhance the production of hematopoietic progenitor cells. Taken together, these results raise the possibility that mesodermal cells with a hematopoietic differentiation potential would be efficiently generated in DN-Lnk-expressing cells during EB formation.

Inhibition of Lnk function increases the production of hematopoietic cells

To induce hematopoietic cells, EB-derived cells were cultured on OP9 stromal cells in the presence of hematopoietic cytokines. During culture, cobblestone-forming cells were more frequently observed in DN-Lnk-expressing cells than in *Neo*-expressing cells (Fig. 3a), indicating that DN-Lnk-expressing cells were immature hematopoietic cells with expansion potential. In support of this observation, DN-Lnk-expressing cells showed a significant increase in the number of hematopoietic cells compared to that of *Neo*-expressing cells (Fig. 3b). Importantly, compared to *Neo*-expressing cells, DN-Lnk-expressing cells could more efficiently proliferate on OP9 stromal cells for a period exceeding 14 days (Fig. 3b). Therefore, the proliferation of hematopoietic cells could be augmented by the inhibition of Lnk.

To investigate whether primitive and definitive hematopoiesis could occur in DN-Lnk- or *Neo*-expressing cells, we measured the expression levels of red cell globin by RT-PCR analysis. In both DN-Lnk- and *Neo*-expressing hematopoietic cells, the expression levels of the embryonic globin, *β-H1*, and the adult globin, *β-major*, were decreased and increased, respectively, after culturing on OP9 stromal cells in comparison with those in total EB cells (Fig. 3c). This indicates that DN-Lnk- or *Neo*-expressing cells can show the primitive

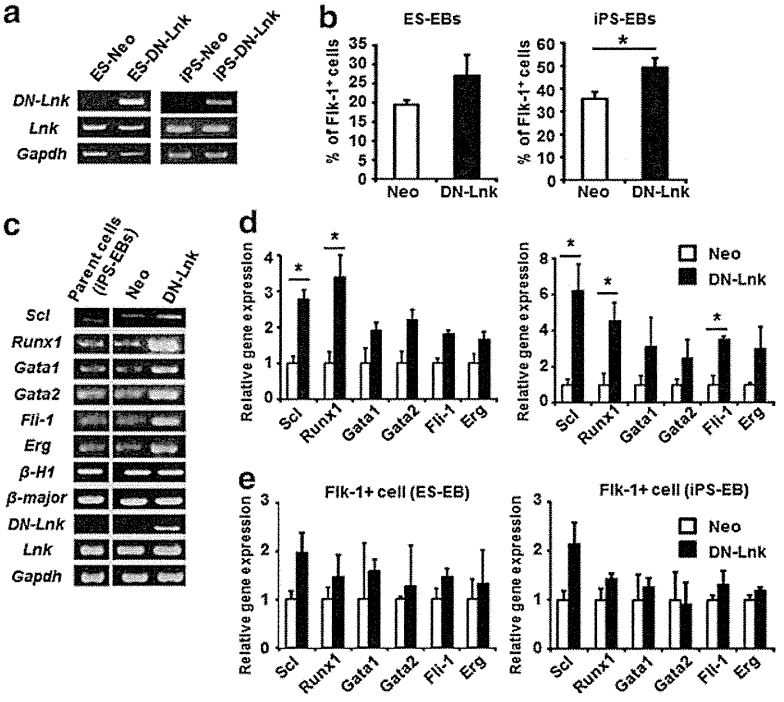

FIG. 2. Increased expression of hematopoietic transcription factors in DN-Lnk-expressing EB cells. **(a)** DN-Lnk-expressing ES cells and iPS cells were generated as described in the Materials and Methods section. DN-Lnk expression was confirmed by RT-PCR. **(b)** EB cells, which were cultured for 7 days, were stained with anti-mouse Flk-1 antibody, and were then subjected to flow cytometric analysis. The data were expressed as the mean ± SD ($n = 3$). **(c)** The expression level of hematopoietic marker genes in 7-day iPS-EBs were investigated by semi-quantitative RT-PCR analysis. The *left panel* indicates the parent iPS cell (38C2)-derived 7-day EBs. **(d)** Gene expression analysis of the key transcription factors of hematopoietic stem/progenitor cells in total 7-day ES-EBs (*left*) and 7-day iPS-EBs (*right*). The data were expressed as mean ± SD ($n = 3$); $^*p < 0.05$ as compared with *Neo*. **(e)** After Flk-1$^+$ cells were sorted from *Neo*- or DN-Lnk-expressing 7-day EB cells, quantitative RT-PCR analysis was performed. *Left*, ES-EB-derived Flk-1$^+$ cells; *right*, iPS-EB-derived Flk-1$^+$ cells. *DN-LnK*, dominant-negative mutant of the Lnk.

hematopoiesis followed by definitive hematopoiesis under our culture conditions.

We next examined the colony-forming potential of DN-Lnk-expressing cells. As shown in Fig. 3d, DN-Lnk-expressing cells showed a significant increase in the total colony-forming cell (CFC) number and CFU–granulocyte, macrophage number. Note that the number of CFU-GEMM/CFU-Mix, the most immature multipotent hematopoietic cells, in DN-Lnk-expressing cells was ~5 times as much as that in *Neo*-expressing cells (Fig. 3d). May-Giemsa staining after picking up the colonies revealed that mixed colonies derived from DN-Lnk-expressing cells contained the erythroblasts, granulocytes, macrophages, and megakaryocytes (Fig. 3e), thus confirming the generation of multipotent hematopoietic cells. An elevated CFU-Mix number in DN-Lnk-expressing cells might have been due to the fact that Lnk is highly expressed in immature hematopoietic cells, especially in hematopoietic stem/progenitor cells [13,17]. We also analyzed surface antigen expression in DN-Lnk- or *Neo*-expressing cells by flow cytometry, and found that DN-Lnk-expressing cells showed a higher percentage of CD34$^+$ cells and CD41$^+$ cells (Fig. 3f), suggestive of an increased number of immature hematopoietic cells. In addition, the proportion of CD45$^+$ cells, CD11b$^+$ cells, Gr-1$^+$ cells, or CXCR4$^+$ cells was also increased in DN-Lnk-expressing cells (Fig. 3f). By contrast, a lower percentage of Ter119$^+$ cells were observed in DN-Lnk-expressing cells (Fig. 3f). Consistent with this flow cytometric analysis, we found an increased expression of *Sfpi1* (encoding Pu.1) and *E47*, which are the key factors responsible for hematopoiesis, and a decreased expression of *β-major* globin in DN-Lnk-expressing cells after the cultivation on OP9 stromal cells (Fig. 3c and Supplementary Fig. S3). These results clearly showed that Lnk inhibition promoted the production of hematopoietic cells, including multipotent immature hematopoietic cells and myeloid cells, from mouse ES and iPS cells.

SF3 ▶

Inhibition of Lnk function in pluripotent stem cell-derived hematopoietic cells augments TPO-mediated signaling

It was previously shown that Lnk negatively regulates various types of hematopoietic cytokine signaling, such as TPO [16]. To investigate whether the increased production of hematopoietic cells from DN-Lnk-expressing cells, described above, is due to the enhanced TPO-mediated signaling, we analyzed protein phosphorylation after TPO stimulation using DN-Lnk-expressing cells. Hematopoietic cells were starved and subsequently stimulated with 20 ng/mL of TPO before the preparation of the cell lysates. The results showed the elevated phosphorylation of Jak2, Erk1/2, and Akt, all of which are downstream of TPO signaling, in DN-Lnk-expressing cells (Fig. 4a). We also found almost no difference in the percentage of Mpl/TPOR-positive cells between DN-Lnk-expressing cells and *Neo*-expressing cells (Fig. 4b), indicating that enhanced TPO signaling in DN-Lnk-expressing cells does not result from the increased percentage of Mpl/TPO-expressing cells. Thus, our data suggest that Lnk inhibition by *DN-Lnk* gene transduction would augment the activation of signaling molecules upon stimulation with cytokines, and thus Lnk inhibition would promote the production of hematopoietic cells in DN-Lnk-expressing cells.

◀ F4

Increased generation of hematopoietic progenitor cells from mouse pluripotent stem cells by transient transduction of a DN-Lnk gene

Our groups have shown that Ad vector-mediated transient, but not constitutive, transduction of differentiation-related genes in pluripotent stem cells could result in the efficient generation of functional cells, such as adipocytes, osteoblasts, hepatocytes, and hematopoietic cells [25,28,35–37]. We expected that the transient inhibition of Lnk in iPS cells could also

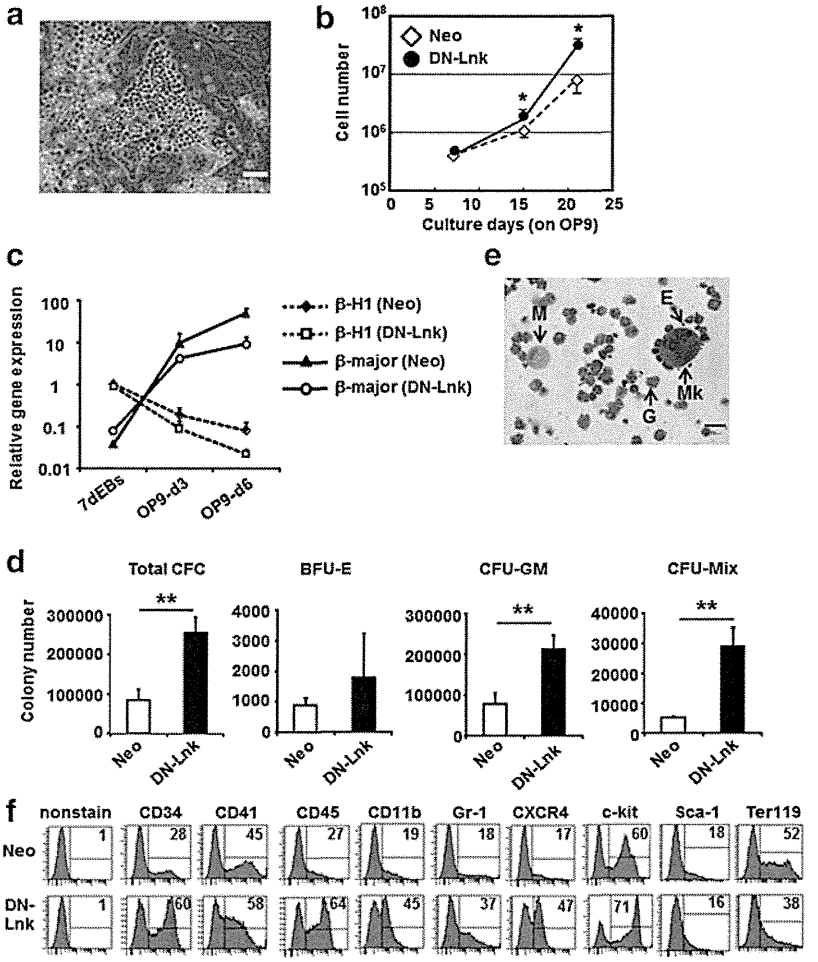

FIG. 3. Efficient generation of hematopoietic cells from iPS cells by overexpression of the *DN-Lnk* gene. EBs derived from *Neo*- or DN-Lnk-expressing iPS cells were cultured for 7 days, and were then plated and cultured on OP9 cells with hematopoietic cytokines to induce and expand the hematopoietic cells. **(a)** Morphology of cobblestone-forming cells derived from DN-Lnk-expressing cells on OP9 stromal cells. Scale bar indicates 100 μm. **(b)** The number of hematopoietic cells was counted on days 7 and 14, after the EB cells were plated on OP9 cells. The data were expressed as mean ± SD ($n = 3$); $^*p < 0.05$ as compared with *Neo*. **(c)** Seven-day-cultured EB cells (7-day EBs) were cultured on OP9 cells for 3 or 6 days (OP9-d3 or OP9-d6, respectively). Total RNA was extracted from each cell, and the expression levels of the embryonic *β-H1* globin and the adult *β-major* globin in the cells were measured by real-time PCR. **(d)** After the EB cells had been cultured on OP9 stromal cells for 7 days, the hematopoietic cells were cultured in a methylcellulose-containing medium with hematopoietic cytokines. Ten days later, the number of hematopoietic colonies was then determined using light microscopy. The number of total colonies or subdivided colonies (by morphological subtypes BFU-E, CFU-GM, and CFU-Mix) is shown. The colony number was normalized to the total number of cells. The data were expressed as mean ± SD ($n = 3$); $^*p < 0.05$ as compared with *Neo*. **(e)** Cytospin preparation from a DN-Lnk-expressing cell-derived CFU-Mix obtained from the cultures described in **(d)**. E, erythrocyte; G, granulocyte; M, macrophage; Mk, megakaryocyte. Scale bar indicates 30 μm. **(f)** After the EB cells were cocultured with OP9 stromal cells for 14 days, the hematopoietic cells were collected as described in the Materials and Methods section. Hematopoietic cells derived from *Neo*- or DN-Lnk-expressing iPS cells were stained with each antibody, and were then subjected to flow cytometric analysis. The proportion of antigen-positive cells is indicated in the histograms. Representative results from 1 of 3 independent experiments performed are shown. CFC, colony-forming cell; BFU-E, burst-forming unit; CFU-GM, colony-forming unit–granulocyte and monocyte; CFU-Mix/CFU-GEMM, CFU–granulocyte, erythrocyte, monocyte, and megakaryocyte.** ◀AU4

accelerate the hematopoietic differentiation. To test this expectation, we generated a DN-Lnk-expressing Ad vector, Ad-DN-Lnk, and examined the effects of transient Lnk inhibition on hematopoietic cell differentiation. The transduction efficiency in EBs, which was transduced with a DsRed-expressing Ad vector, was approximately 40%, as determined by flow cytometry (data not shown). A colony assay after transduction with Ad vectors revealed that the number of total colonies and mixed colonies in the cells transduced with Ad-DN-Lnk was slightly increased in comparison with that in the cells transduced with Ad-LacZ (control vector) (Fig. 5a, c). Moreover, the ◀F5 number of hematopoietic cells increased in Ad-DN-Lnk-transduced cells after 7-day cultivation on OP9 stromal cells (Fig. 5b, d). Thus, our data indicate that the transient inhibition of Lnk also enhances the differentiation and proliferation of hematopoietic cells derived from pluripotent stem cells.

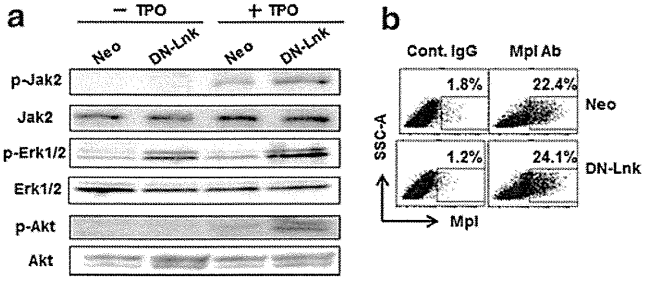

FIG. 4. Enhanced TPO-induced signaling pathway in DN-Lnk-expressing cells. After the EB cells, which were cultured for 7 days, had been plated and cultured on OP9 stromal cells for 14 days, and the hematopoietic cells were harvested as described in the Materials and Methods section. **(a)** Hematopoietic 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 hematopoietic 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.

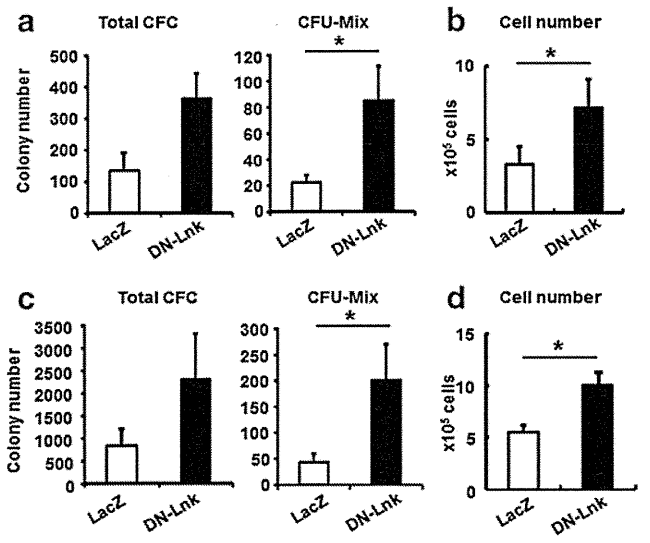

FIG. 5. Expansion of ES cell- and iPS cell-derived hematopoietic cells by the transient expression of DN-Lnk in EB cells. After ES cell- **(a, b)** or iPS cell- **(c, d)** derived EB cells, which were cultured for 7 days, had been transduced with Ad-LacZ or Ad-DN-Lnk at 3,000 VP/cell for 1.5 h, the cells 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 ± SD ($n = 3$); $^*p < 0.05$ as compared with Ad-LacZ. Ad, adenovirus.

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 $Flk\text{-}1^+$ 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 $Flk\text{-}1^+$ cells by the inhibition of Lnk function (Fig. 2e). Thus, it is possible that Lnk might negatively regulate the hematopoietic commitment in $Flk\text{-}1^+$ cells by modulating the expression of *Scl/Tal-1*. We also showed that the percentage of $Flk\text{-}1^+$ 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 $Flk\text{-}1^+$ 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

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, AU3 ▶ 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

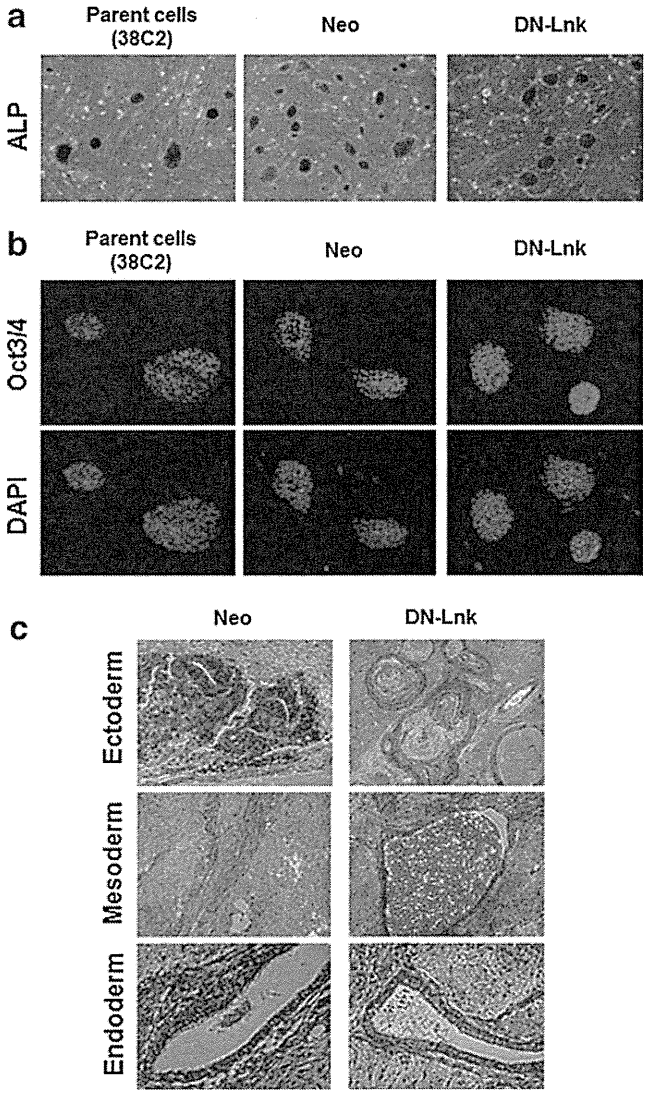
Supplementary Methods

Alkaline phosphatase staining and Oct-3/4 staining

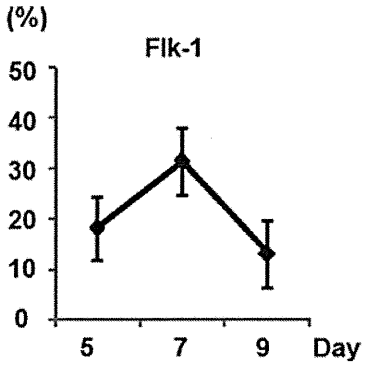
Mouse parent induced pluripotent stem (iPS) cells (38C2), *Neo*-expressing iPS cells, and dominant-negative mutant of the Lnk (DN-Lnk)-expressing iPS cells (1×10^4 cells) were plated on 12-well plates, and were then cultured for 3 days. Alkaline phosphatase staining was carried out using the Alkaline 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). ◀AU5

Teratoma formation and histological analysis

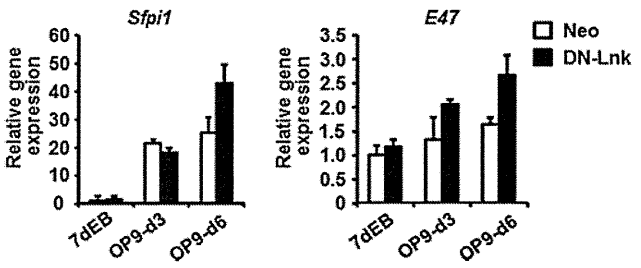
Neo- or DN-Lnk-expressing iPS cells were suspended in 1×10^7 cells/mL in PBS. Nude mice (8–10 weeks; Nippon SLC) were anesthetized with diethyl ether, and we injected 100 μL of the cell suspension (1×10^6 cells) subcutaneously into their backs. Five weeks later, tumors were surgically dissected from 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.



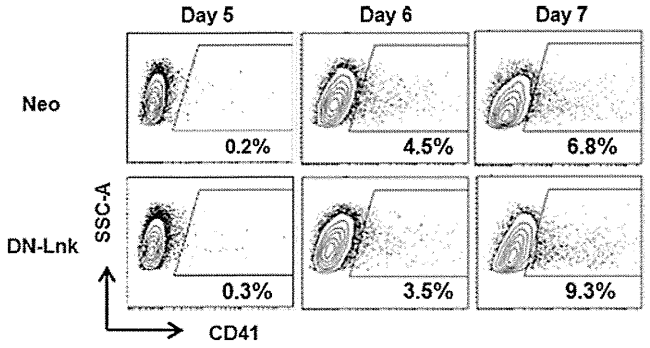
SUPPLEMENTARY FIG. S1. DN-Lnk-expressing iPS cells maintain the undifferentiated states in vitro and pluripotency in vivo. **(a, b)** The expression levels of undifferentiated markers, ALP **(a)** and Oct-3/4 **(b)**, in Neo- or DN-Lnk-expressing iPS cells were similar to those in the parent iPS cells. **(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 *Sfpi1* 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 Sfpi1 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.

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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-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.

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, PPARγ, 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, $CD41^+c\text{-}kit^+$ 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 $CD41^+c\text{-}kit^+$ 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 $CD41^+c\text{-}kit^+$ cells, although the expression levels of CAR in $CD41^+c\text{-}kit^+$ 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 $CD41^+c\text{-}kit^+$ 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 $CD41^+c\text{-}kit^+$ 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 $CD41^+c\text{-}kit^+$ 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