

In vitro generation of HSC-like cells from murine ESCs/iPSCs by enforced expression of LIM-homeobox transcription factor *Lhx2*

Kenji Kitajima,¹ Ken-ichi Minehata,¹ Kenji Sakimura,² Toru Nakano,³ and Takahiko Hara¹

¹Stem Cell Project Group, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan;

²Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan; and ³Department of Stem Cell Pathology, Medical School and Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

Identification of genes involved in in vitro differentiation induction of embryonic stem cells (ESCs) into hematopoietic stem cells (HSCs) has been challenged during last decade. To date, a homeobox transcription factor *Hoxb4* has been only demonstrated to possess such an effect in mice. Here, we show that HSC-like cells were efficiently induced from mouse ESCs by enforced expression of *Lhx2*, a LIM-homeobox transcrip-

tion factor. Transduction of *Lhx2* into ESC-derived mesodermal cells resulted in robust differentiation of c-Kit⁺/Sca-1⁺/Lineage⁻ (KSL) cells in vitro. The KSL cell induction frequency was superior to the case of *Hoxb4*. Furthermore, transplantation of *Lhx2*-transduced hematopoietic cells into lethally irradiated mice resulted in multilineage repopulation of hematopoietic cells over 4 months. Transduction of *Lhx2*

into induced pluripotent stem cells (iPSCs) was also effective in generating KSL cells in vitro, as well as HSC-like activities in vivo. These results demonstrate that ectopic expression of *Lhx2* confers an in vivo engrafting capacity to ESC/iPSC-derived hematopoietic cells and in vivo behavior of iPSC-derived hematopoietic cells is almost identical to that of ESC-derived cells. (*Blood* 2011;117(14):3748-3758)

Introduction

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can be differentiated into various types of cells in the body.¹ Establishment of human iPSCs has led to new clinical strategies using iPSCs as universal sources for regeneration therapy for damaged organs and tissues.² On the other hand, vigorous self-renewal of iPSCs could pose a risk for uncontrolled cell behavior in vivo, potentially resulting in teratoma formation. Therefore, lineage-directed in vitro differentiation of iPSCs into a desirable cell type is critical for their use in therapeutic applications.

Among the various methods for in vitro differentiation of ESCs/iPSCs, reliable protocols for hematopoietic cell differentiation have already been established, for example, embryoid body formation,³ culture on type-IV collagen-coated plates,⁴ coculture with OP9 stromal cells,^{5,6} and combinations of these approaches.⁷

In mammals, hematopoiesis is supported by a small number of hematopoietic stem cells (HSCs) from which more than 8 distinct lineages of mature blood cells are continuously produced.⁸ HSCs possess a capacity for self-renewal, which makes it possible to sustain hematopoiesis throughout life. Experimentally, HSCs are defined as those cells exhibiting the long-term repopulating (LTR) activity of multilineage hematopoietic cells when transplanted into lethally irradiated adult mice. LTR-HSCs give rise to short-term repopulating HSCs followed by the production of lineage-restricted hematopoietic progenitor cells (HPCs).

During in vitro ESC differentiation to the hematopoietic lineage, Flk1⁺ mesodermal cells, including hemangioblasts, are differentiated from ESCs.⁹ HPCs are then generated from these Flk1⁺ cells. Although primitive erythrocytes and HPCs are serially emerged, LTR-HSCs are not efficiently generated from ES cells without genetic manipulation, that is, overexpression of *Hoxb4*.¹⁰

To amplify the iPSC-derived HPCs, we focused on the *Lhx2* (*LH2*) gene, which encodes a LIM-homeobox transcription factor, because enforced expression of *Lhx2* has been reported to facilitate ex vivo expansion of adult bone marrow HSCs and to immortalize ESC-derived HPCs.^{11,12} Studies of *Lhx2*-deficient mice have revealed that *Lhx2* is involved in cell fate determination in a wide variety of organs and tissues.¹³⁻¹⁵ In particular, *Lhx2* is required for the development and preservation of hair follicle stem cells.¹⁶

Here, we show that the enforced expression of *Lhx2* conferred HSC-like ability to mouse ESC/iPSC-derived hematopoietic cells. These HSC-like cells could colonize bone marrow and continuously produce multilineage hematopoietic cells for more than 4 months when transplanted into lethally irradiated recipient mice. These findings suggest that *Lhx2* might become a novel molecular tool to amplify HSCs/HPCs derived from ESCs/iPSCs.

Methods

Cell culture

Two mouse ESC lines, C57BL/6-derived RENKA and 129/Ola-derived E14tg2a, were used in this study. RENKA ESCs and iPSCs were maintained on mitomycin C (Sigma-Aldrich)-treated mouse embryonic fibroblasts (MMC-MEFs) in DMEM-based ES-medium that contained DMEM (Sigma-Aldrich) supplemented with 15% FCS (JRH biosciences), 0.1mM nonessential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM 2-mercaptoethanol (Sigma-Aldrich), 50 µg/mL penicillin, 75 µg/mL streptomycin, and ESGRO (Invitrogen). E14tg2a ESCs were cultured on 0.1% gelatin-coated plates in GMEM-based ES-medium in which Glasgow modified essential medium (GMEM; Sigma-Aldrich) and leukemia inhibitory factor (LIF) were used instead of DMEM and ESGRO,

Submitted July 26, 2010; accepted January 26, 2011. Prepublished online as *Blood* First Edition paper, February 22, 2011; DOI 10.1182/blood-2010-07-298596.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2011 by The American Society of Hematology

respectively. LIF was prepared from the supernatant of CHO-LIF cells expressing exogenous LIF. OP9 stromal cells were maintained in OP9-medium containing α -MEM (Invitrogen) supplemented with 20% FCS, nonessential amino acids, penicillin, and streptomycin. The PLAT-E retrovirus packaging cell line was maintained in DMEM supplemented with 10% FCS, penicillin, and streptomycin. All microphotographic images were obtained using an Olympus Provis microscope with a UPlan APO 40 \times /0.85 numeric aperture objective lens, an Olympus DP70 camera, and DC controller software ViewFinder 7.1 (all from Olympus) under ambient conditions. The images were processed using Adobe Photoshop element 6 (Adobe Systems).

In vitro hematopoietic differentiation of mouse ESCs/iPSCs

In vitro differentiation of hematopoietic cells from ESCs/iPSCs was performed by coculturing with OP9 stromal cells.¹⁷ In some experiments, day-5 differentiated cells were used for retroviral transduction experiments. Thereafter, $1-2 \times 10^5$ of day-5 differentiated cells were subcultured on OP9 cells in each well of 6-well plates in the presence of appropriate cytokines. To expand the KSL cells, 10 ng/mL murine IL-6 (PeproTech) and 50 ng/mL murine SCF (kindly provided by Kirin Brewery, Takasaki, Japan) were added to the culture. To induce erythroid cells, megakaryocytes, and myeloid cells, 2 U/mL human erythropoietin (EPO; Kirin), 10 ng/mL human thrombopoietin (TPO; Kirin), 10 ng/mL human macrophage colony stimulating factor (M-CSF; Morinaga milk), 10 ng/mL murine GM-CSF (PeproTech), or murine IL-3 were added, beginning on day 5 of differentiation. IL-3 was produced from the X63 melanoma cell line, a kind gift from Dr H. Karasuyama (Tokyo Medical and Dental University, Tokyo, Japan). Between day 8 and day 10 of the in vitro differentiation, floating and loosely attached cells were collected by vigorous pipetting and reseeded onto OP9 cells.

FACS analyses and colony assays

The in vitro differentiated hematopoietic cells were harvested by gentle pipetting. The cells were stained with various antibodies (supplemental methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). After the multicolor staining, the hematopoietic cells were analyzed by FACSCalibur or FACSaria (BD Bioscience). Optical filter sets were installed to detect EGFP and EYFP simultaneously (535AF LP mirror/520DRLP BP filter for EYFP and 510AF LP mirror/502LP BP filter for EGFP). The flowcytometric data were analyzed using FlowJo Version 4.6 software (TreeStar).

The cells (10^4) were cultured in Methocult M3434 containing IL-6, IL-3, SCF, and EPO (StemCell Technologies) supplemented with TPO for 7 days. For the colony formation assays, *Lhx2*-transduced hematopoietic cells were sorted from the bone marrow of recipient mice 12 weeks after transplantation, and 2 thousand cells were used in each assay.

The cells were adhered to slides using Cytospin 4 (Thermo Shandon), followed by staining with Diff-Quick solution (Sysmex). Images were analyzed using an inverted microscope (FSX-100; Olympus), equipped with a $\times 40$ objective lens at room temperature in air. The images were processed using Adobe Photoshop element 6.

PCR and Western blotting

Total RNA was recovered using a RNeasy mini kit (QIAGEN) and examined by RT-PCR using the ThermoScript RT-PCR system (Invitrogen). In some experiments, cDNAs were serially diluted and amplified. *Gapdh* or β -*actin* were used as a template control. To analyze immunoglobulin rearrangement, 4×10^5 cells were lysed in buffer containing 60 μ g/mL proteinase K (Nacalai Tesque), 0.1% Triton X-100, and 10mM Tris-HCl (pH 8.0); incubated at 55°C for 60 minutes; and denatured at 95°C for 12 minutes. Genomic PCR was performed using rTaq polymerase (TOYOBO). The gene fragments for the immunoglobulin heavy chain (*IgH*) and light chain (*IgL*) were amplified using primers for *VH γ 3.8* and *JH4* and *VL1* and *JL1,3*, respectively.¹⁸ All of the primer sequences are listed in supplemental Table 1. The amplified DNA were visualized by ethidium bromide staining. Western blotting was carried out as previously described.¹⁹

Plasmids

The retroviral vector for *Lhx2* was constructed by inserting mouse *Lhx2* cDNA, a kind gift from Dr R. A. Maurer (Oregon Health & Sciences

University), into pMY.IRES.EGFP vector (kind gifts from Dr T. Kitamura, University of Tokyo Institute of Medical Science). The *FLAG* tag was inserted into N-terminal terminus of *Lhx2* to construct pMY.FLAG.Lhx2.IRES.EGFP. The retroviral vector pMY.IRES.EYFP was constructed by replacing the EGFP gene of pMY.IRES.EGFP with an EYFP gene purchased from Clontech. Mouse *Hoxb4* cDNA, a kind gift from Dr A. Iwama (Chiba University), was inserted into pMY.IRES.EYFP. Retroviral vectors for expressing the 4 factors described by the Yamanaka group²⁰ (pMXs-Oct3/4, pMXs-Sox2, pMXs-Klf4, and pMXs-c-Myc) were purchased from Addgene.

Retroviral transduction

The retroviral vectors were transfected into PLAT-E cells using Lipofectamine 2000 (Invitrogen) or FuGene HD (Roche), according to the manufacturers' instructions. ESC/iPSC-derived differentiated cells were inoculated by spin-infection. Five to 10×10^5 of day 5 differentiated cells were suspended in 2 mL of filtered virus supernatant in the presence of 8 μ g/mL polybrene (Sigma-Aldrich) and transferred into 1 well of a 24-well plate. The plate was then centrifuged at 1100g for 2 hours at 25°C and the cells were seeded onto OP9 cells.

Establishment of mouse iPSCs

We established 2 new iPSC lines (clone 24 and clone 2) from E13.5 MEFs harvested from C57BL/6 mice (Nihon SLC). Introduction of the 4 factors described by the Yamanaka group was carried out as previously described.²⁰ Alkaline phosphatase staining was carried out using a leukocyte alkaline phosphatase kit (Sigma-Aldrich).

Transplantation experiments

Floating and lightly adherent hematopoietic cells were collected by mild pipetting. The remaining hematopoietic cells underneath the OP9 stromal cells were collected by 0.1% trypsin. The cells were mixed, centrifuged and re-seeded onto gelatin-coated dishes for 30 minutes to remove the OP9 cells. The supernatant was recovered, passed through a cell strainer (BD Bioscience), and resuspended in PBS(-). One to 2×10^6 cells were injected into the lateral tail vein of lethally (10.4 Gy of X-ray) irradiated Ly5.1-C57BL/6 mice (10-16 weeks old). Secondary transplantation experiments were carried out using EGFP⁺ cells sorted from the bone marrow of primary recipient mice 12 weeks after the transplantation. Cells (3×10^5) were transplanted into each recipient. All animal protocols were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Medical Science.

Results

In vitro hematopoietic induction from mouse ESCs

In this study, we have used the OP9 coculture induction method to differentiate mouse ESCs/iPSCs into the hematopoietic lineage.¹⁷ This coculture method has been widely used for hematopoietic induction. When ESCs were put onto OP9 stromal layer, mesodermal cell clusters emerged around day 5 of the induction (Figure 1A). Essentially all of the ESC-derived cells on day 5 were Flk1⁺ (Figure 1B). These cells were harvested and subcultured on fresh OP9 cells. Around days 6 to 8, HPC-like cells emerged. These cells were differentiated into mature hematopoietic cells around days 10-14 (Figure 1A) and subsequently underwent apoptotic cell death.

The expression of *Lhx2* mRNA was next examined. The undifferentiated ESCs expressed a lower amount of endogenous *Lhx2* mRNA (Figure 1C). The level of *Lhx2* mRNA gradually increased as the cells differentiated into mesodermal cells over 5 days, and *Lhx2* expression was undetected in hematopoietic cells on day 8 (Figure 1C).

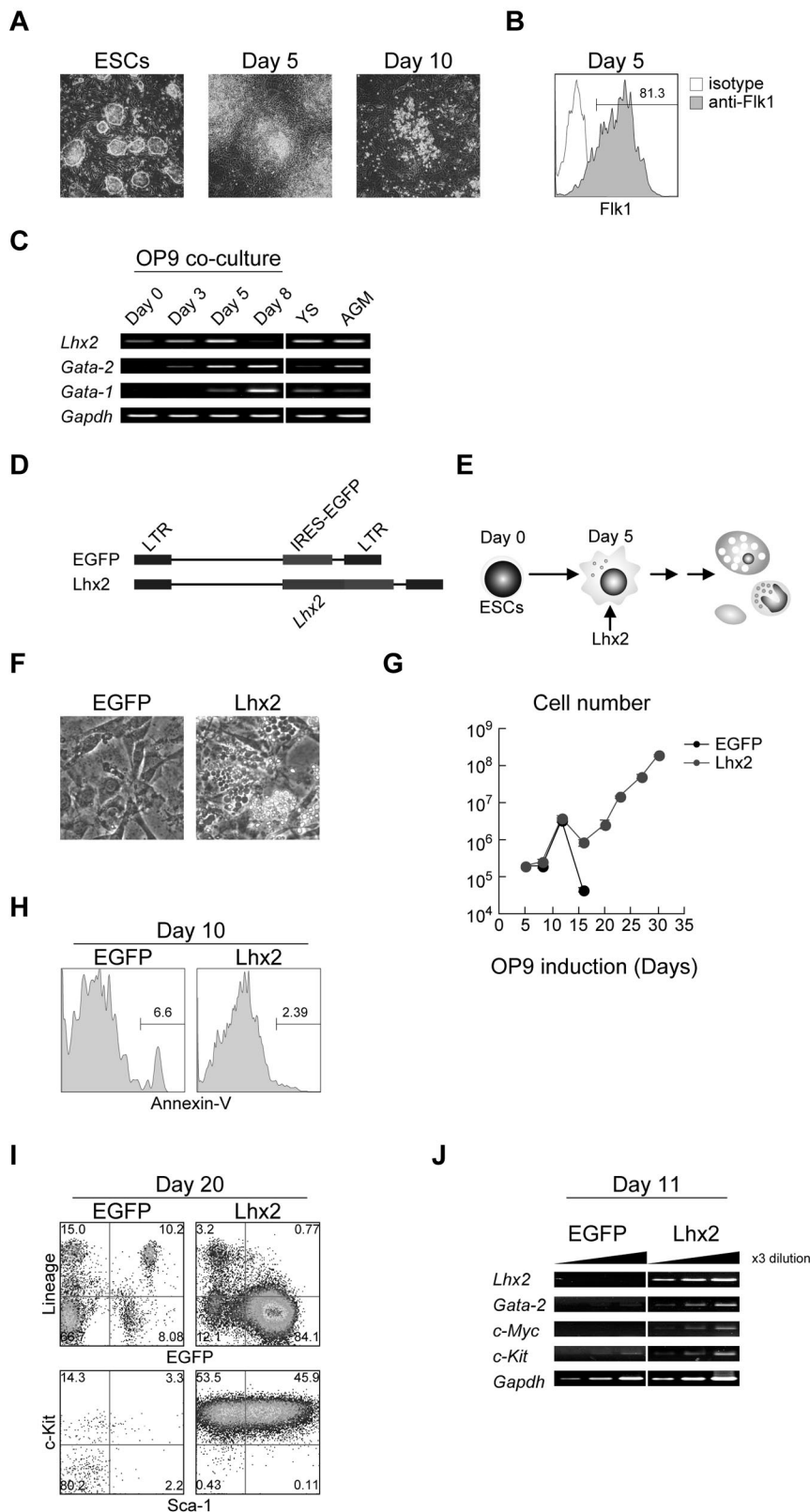


Figure 1. Robust production of HPCs from mouse ESCs by enforced expression of *Lhx2*. (A) In vitro hematopoietic differentiation of ESCs by OP9 coculture. Undifferentiated ESCs, day 5-, and day 10-induced cells were shown. Original magnification: $\times 4$. (B) Fik1 expression on day 5. (C) The RT-PCR analysis of *Lhx2*, *Gata-1* and *Gata-2* expression during ESC differentiation and at hematopoietic sites. Ys and AGM indicate yolk sac and aorta/gonad/mesonephros region at E10.5, respectively. (D) Retroviral vectors used in this study. (E) Study design. *Lhx2* was transduced on day 5. (F) Microscopic analysis of EGFP- and *Lhx2*-transduced cells. Original magnification: $\times 4$. (G) Growth curves of hematopoietic cells from ESCs transduced with EGFP or *Lhx2*. (H) Annexin-V staining. EGFP⁺ cells were shown. (I) FACS analysis of KSL cells derived from RENKA ESCs. The relative frequencies (%) are shown in the corners. (J) The RT-PCR analysis *Lhx2*, *Gata-2*, *c-Myc*, and *c-Kit*.

In vitro induction of KSL cells from ESCs by enforced expression of *Lhx2*

Differentiating ESCs were infected with a retroviral vector carrying *Lhx2* (*Lhx2*) and an empty vector (EGFP) on day 5, and the cells were then further cultured on OP9 in the presence of SCF and

IL-6 (Figure 1D-E), since *Lhx2*-transduced, ESC-derived HPCs have been shown to preferentially grow in the presence of SCF and IL-6.²¹ In control, almost all hematopoietic cells were fully differentiated and subsequently disappeared by completing terminal differentiation by 2 weeks of the differentiation induction.

Consequently, only OP9 cells were present in culture on day 20 (Figure 1F). In contrast, the *Lhx2* transduction gave rise to cells that continued to proliferate. Some cells grew underneath OP9 stromal cells, which was known as pseudoemperipolesis, indicating that these cells were immature hematopoietic cells (Figure 1F). In control, the differentiated cells stopped increasing by day 14 (Figure 1G). In contrast, the *Lhx2*-transduced cells were continuously increased (Figure 1G). The *Lhx2* transduction did not affect cell death (Figure 1H), suggesting that the continued cell growth by *Lhx2* would largely result from the enhanced cell proliferation.

Next, the increasing cells were analyzed. FACS analyses revealed that almost all *Lhx2*-transduced EGFP⁺ cells were lineage negative (Lin⁻; Figure 1I). Strikingly, approximately 50% of the Lin⁻ fraction consisted of c-Kit⁺/Sca-1⁺ cells (designated KSL cells) and the rest were c-Kit single positive (KL cells; Figure 1I). These results are contradictory, however, to a previous report by Pinto do et al showing that *Lhx2*-transduced HPC cell lines derived from ESCs expressed c-Kit but not Sca-1.¹² Presumably, environmental factors provided by OP9 cells could contribute to this difference, as Pinto do et al used an embryoid body formation protocol.¹² Similar results were obtained when E14tg2a ESCs were used (supplemental Figure 1A). The molecular analysis showed that the *Lhx2*-transduced cells expressed *Gata-2*, *c-Myc*, and *c-Kit*, all of which are known to be expressed in immature hematopoietic cells (Figure 1J).

Establishment of C57BL/6 mouse-derived iPSCs

Because iPSCs are an important cell source for regenerative medicine, we have challenged whether the *Lhx2* transduction would be effective in iPSCs. Mouse iPSC lines were newly established from MEFs from C57BL/6 strain using the 4 factors described by Takahashi et al, *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*.²² We confirmed that established iPSCs were positive for representative ESC markers (supplemental Figure 2A-B). In addition, the iPSCs were capable of producing various lineages of hematopoietic cells in vitro (supplemental Figure 2C-E). Next, the established iPSCs were differentiated in vitro and transduced with *Lhx2*. Very similar to the ESCs, on day 20, most of the *Lhx2*-transduced cells displayed a KSL/KL phenotype (supplemental Figure 2F) and the iPSC-derived KSL cells were morphologically blastic (supplemental Figure 2G). Approximately 10⁷ KSL/KL cells were obtained from 10⁴ iPSCs by *Lhx2* transduction and 20 days of the differentiation induction culture.

Comparison between *Lhx2* and *Hoxb4*

Previously, it has been reported that over-expression of *Hoxb4* induced HSCs from ESCs.¹⁰ Therefore, we carried out side-by-side comparison between *Hoxb4* and *Lhx2* under our experimental conditions. The expression of exogenous *Lhx2* and *Hoxb4* was monitored by EGFP and EYFP fluorescence, respectively (Figure 2A).

On day 15, more than half of the hematopoietic cells derived from *Hoxb4*-transduced cells were Lin⁺, whereas almost no cells were Lin⁺ after *Lhx2* transduction (Figure 2B). The frequency of KSL cells in *Hoxb4*-transduced cells was gradually decreased during cultivation (Figure 2C). In contrast, the population of KSL cells was relatively constant in the case of *Lhx2* (Figure 2C).

We next evaluated whether *Lhx2* would regulate *Hoxb4* expression and candidate *Hoxb4* target genes or not. The enforced expression of *Lhx2* did not up-regulate *Hoxb4* mRNA, and *Hoxb4*

was also ineffective in inducing *Lhx2* expression in differentiating ESCs (Figure 2D). *Hemgn* and *Laptm4b*, which have been shown to be *Hoxb4* target genes in primary murine Lin⁻ bone marrow cells and hematopoietic progenitor cells line EML, respectively,^{23,24} were not up-regulated by *Hoxb4* in the ESC differentiation induction (Figure 2E). The expression of *c-Mpl* which has been shown to be up-regulated in embryoid bodies with inducible *Hoxb4* expression²⁵ was also unaffected (Figure 2E). These data suggest that trans-activation targets of *Hoxb4* were cell context dependent. Notably, *Laptm4b* was up-regulated by *Lhx2* (Figure 2E), suggesting some roles of *Lhx2* on *Laptm4b* regulation.

We next examined whether there was synergism between *Lhx2* and *Hoxb4*. Cells cotransduced with the *Lhx2* and *Hoxb4* retroviral vectors were separated using EGFP and EYFP, respectively (Figure 2F). In the case of cells expressing either *Lhx2* or *Hoxb4* alone, 99.0% and 18.2% of the ESC-derived hematopoietic cells were Lin⁻, respectively (Figure 2G). ESCs transduced with both genes exhibited an intermediate phenotype, that is, 75.7% were Lin⁻ (Figure 2G). In addition, the frequency of KSL cells was decreased in the cotransduced cells compared with the *Lhx2*-transduced cells (Figure 2G). Therefore, the KSL cell induction activities of *Lhx2* and *Hoxb4* are not synergistic.

Differentiation ability of *Lhx2*-transduced hematopoietic cells in vitro

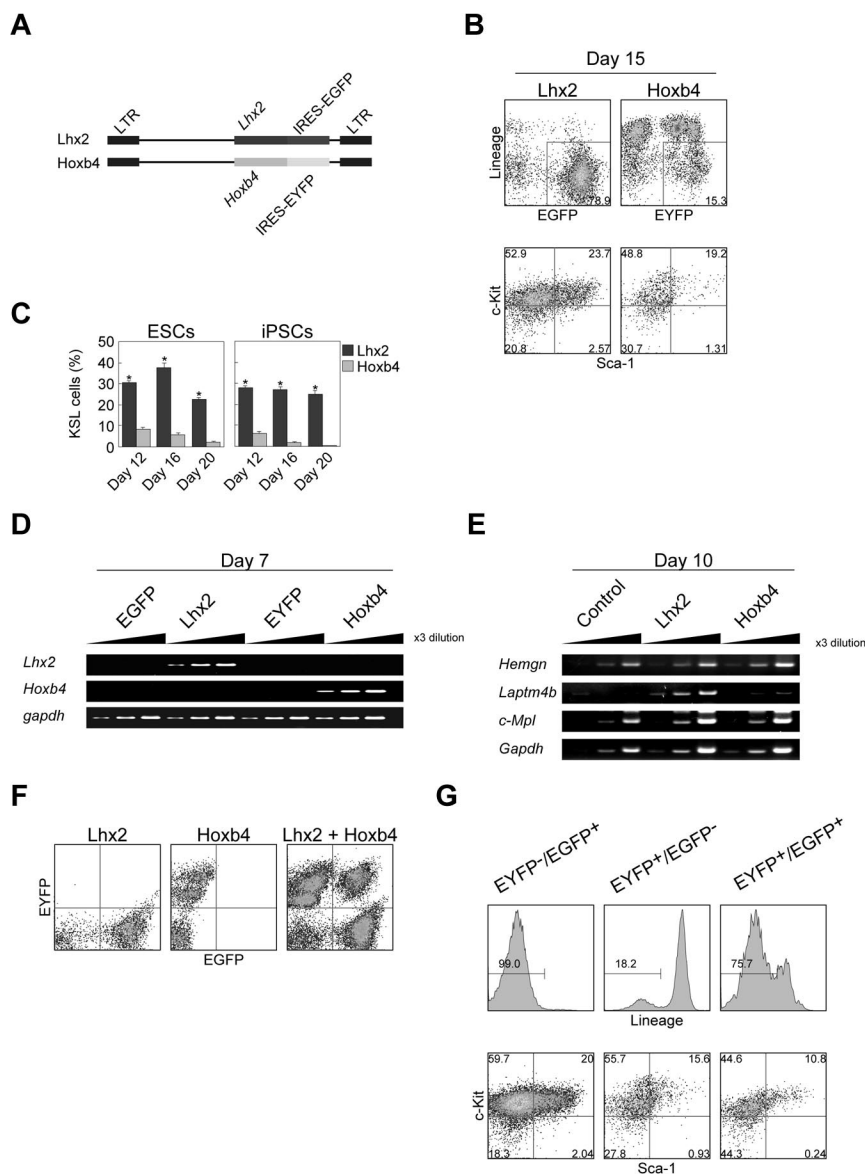
Differentiation potentials of *Lhx2*-transduced hematopoietic cells were next analyzed. It has been reported that bone marrow KL cells can be subdivided into lineage-restricted HPCs, common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs), and megakaryocyte/erythrocyte progenitors (MEPs).²⁶ The *Lhx2*-transduced KL cells derived from ESCs contained these HPCs (Figure 3A).

The *Lhx2*-transduced cells were next cultured in semi-solid media supplemented with EPO, TPO, IL-3, IL-6, and SCF. A much larger number of myeloid or mixed colonies were formed by *Lhx2* transduction (Figure 3B-C). In addition, the *Lhx2*-transduced KSL cells were sorted and cultured on OP9 cells in the presence of EPO, TPO, or GM-CSF for 7 days. FACS and morphologic analyses confirmed the successful derivation of erythrocytes, megakaryocytes, and neutrophils (Figure 3D-E). Therefore, *Lhx2* transduction did not interfere with the differentiation of these hematopoietic cell lineages.

We next examined whether primitive and definitive erythropoiesis would be affected by *Lhx2* or not, since *Hoxb4* has been known to affect primitive erythropoiesis. OP9 differentiation induction of ESCs produced primitive erythrocytes (EryP; CD71^{high}/Ter-119⁻) and definitive erythrocytes (EryD; CD71^{high}/Ter-119⁺) on day 8 and day 11, respectively (Figure 3F). In the presence of *Lhx2*, EryP and EryD could be detected, although their frequencies were decreased (Figure 3F). On day 8 and day 11, the cells expressed embryonic globin (β H1) and adult globin (β maj), respectively (Figure 3F), which confirmed that these erythroid cells were EryP and EryD, respectively. These data showed that EryP and EryD were produced in the presence of *Lhx2*.

Differentiation and repopulation abilities of *Lhx2*-transduced hematopoietic cells in vivo

We next evaluated the differentiation potential of the *Lhx2*-transduced hematopoietic cells by transplantation experiments. The C57BL/6-derived RENKA ESCs were differentiated and transduced with *Lhx2* (Figure 4A). Then the *Lhx2*-transduced cells

**Figure 2. Comparison between *Lhx2* and *Hoxb4*.**

(A) Retroviral vectors. (B-C) Frequencies of KSL cells on day 15 (B) and between day 12 and day 20 (C). The data were shown as mean \pm SD ($n = 6$). Asterisks indicate significant differences ($P < .01$ by t test). (D-E) The RT-PCR analysis of *Lhx2* and *Hoxb4* expression (D), and *Hoxb4* target genes (E). (F) Verification of the separation of EGFP/EYFP fluorescence. (G) KSL frequency of *Lhx2* (EYFP⁻/EGFP⁺), *Hoxb4* (EYFP⁺/EGFP⁻)-transduced and cotransduced (EYFP⁺/EGFP⁺) cells. Cells within Lin⁻ compartments were shown in bottom panels.

($1-2 \times 10^6$; CD45.2⁺) were intravenously injected into lethally irradiated congenic CD45.1⁺ mice. The donor-derived cells were CD45.2⁺/EGFP⁺, while recipient hematopoietic cells were CD45.1⁺/EGFP⁻ (Figure 4B).

Four weeks after transplantation, EGFP⁺ mononuclear cells were detected at 20% to 34% of the total peripheral blood cells of recipient mice (Figure 4C). In the bone marrow and spleen, EGFP⁺ cells contributed to the Mac-1⁺ myeloid cells, Ter-119⁺ erythroid cells, GPIIb⁺ megakaryocytes (Figure 4D). B220⁺ or CD19⁺ B-lymphoid cells of the donor origin were also detected (Figure 4E). More importantly, donor-derived KSL cells were present in the bone marrow (Figure 4F).

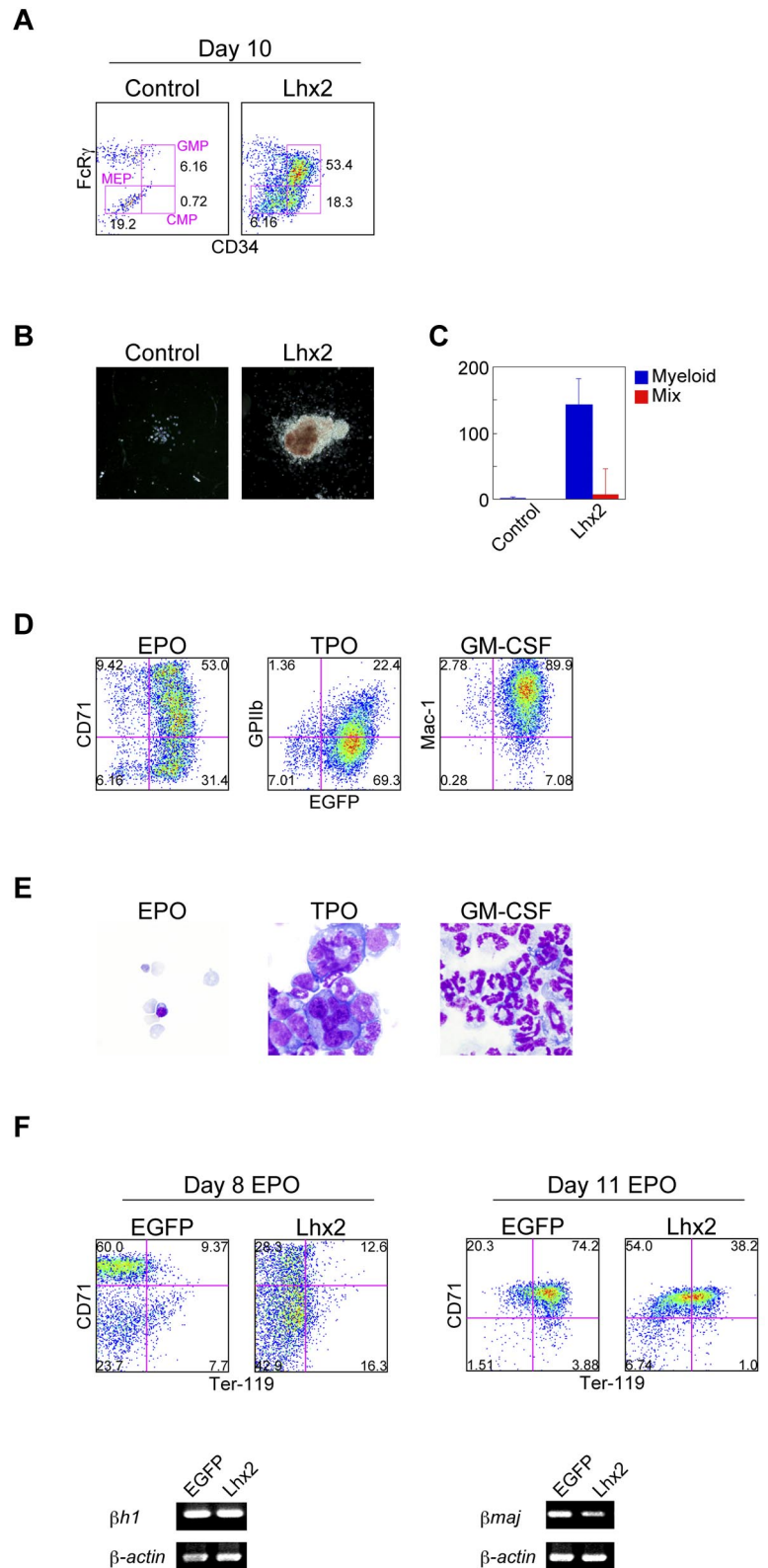
Next, LTR activity was assessed. Sixteen weeks after transplantation, approximately 30% of the hematopoietic cells in the bone marrow and spleen were donor origin in the representative transplanted mouse (supplemental Figure 3A-B). They contributed to myeloid, erythroid, megakaryocyte, and B-lymphoid populations, indicating the multilineage, long-term engraftment capacity of the ESC-derived hematopoietic cells by *Lhx2* transduction. On the other hand, no donor cells were detected in the thymus

(supplemental Figure 3C). The LTR activities were observed in multiple recipient mice (supplemental Table 2 and supplemental Figure 4A).

It would be predicted that long in vitro culture of cells might exhaust LTR activity. Therefore, we compared LTR activity of *Lhx2*-transduced cells between days 18 and 31 of the culture. Approximately 100-fold of amplification was seen between days 18 and 31 (supplemental Table 2). Donor cell chimerism in the transplanted mice was analyzed and the results were similar in the both cases (supplemental Figure 4B), indicating that the LTR activity was sustained by *Lhx2* at least until day 31 of culture.

Next, we evaluated the functional activity of transplanted hematopoietic cells. EGFP⁺ cells were sorted from the bone marrow of primary recipient mice 12 weeks after transplantation and subjected to colony formation assays and secondary transplantation experiments (Figures 5A-B). These cells were capable of producing hematopoietic colonies (Figure 5C). Furthermore, EGFP⁺ cells contained secondary LTR activity (Figure 5D-E). The secondary LTR activity was seen in the multiple primary recipients. These data clearly excluded a possibility that the secondary engraftment

Figure 3. Composition and differentiation of *Lhx2*-transduced hematopoietic cells. (A) FACS analysis of lineage-restricted HPCs derived from ESCs. (B-C) Morphology of representative colonies (B) and colony forming units per 10⁴ cells (C) of uninfected and *Lhx2*-transduced cells derived from ESCs. Original magnification: ×4. The data were shown as mean ± SD (n = 5). (D-E) FACS analysis (D) and May-Grünwald-Giemsa staining (E) of the cells differentiated from *Lhx2*-transduced KSL cells derived from ESCs. Original magnification: ×40. (F) Effects of *Lhx2* on erythroid production from ESCs and on β-globin expression.



Downloaded from http://ashpublications.net/blood/article-pdf/117/14/3748/1338230/zh801411003748.pdf by guest on 08 June 2024

ability could be because of retroviral integrative changes of the *Lhx2*-transduced cells.

No signs of myeloid proliferative disorder were observed in the recipient mice in clear contrast to the results observed in mice transplanted with *Lhx2*-transduced adult HSCs.²⁷

In vivo nature of iPSC-derived hematopoietic cells

We next carried out the transplantation experiments using iPSCs. iPSCs were differentiated, hematopoietic cells were amplified by *Lhx2*, and transplanted (Figure 6A). Essentially similar data were

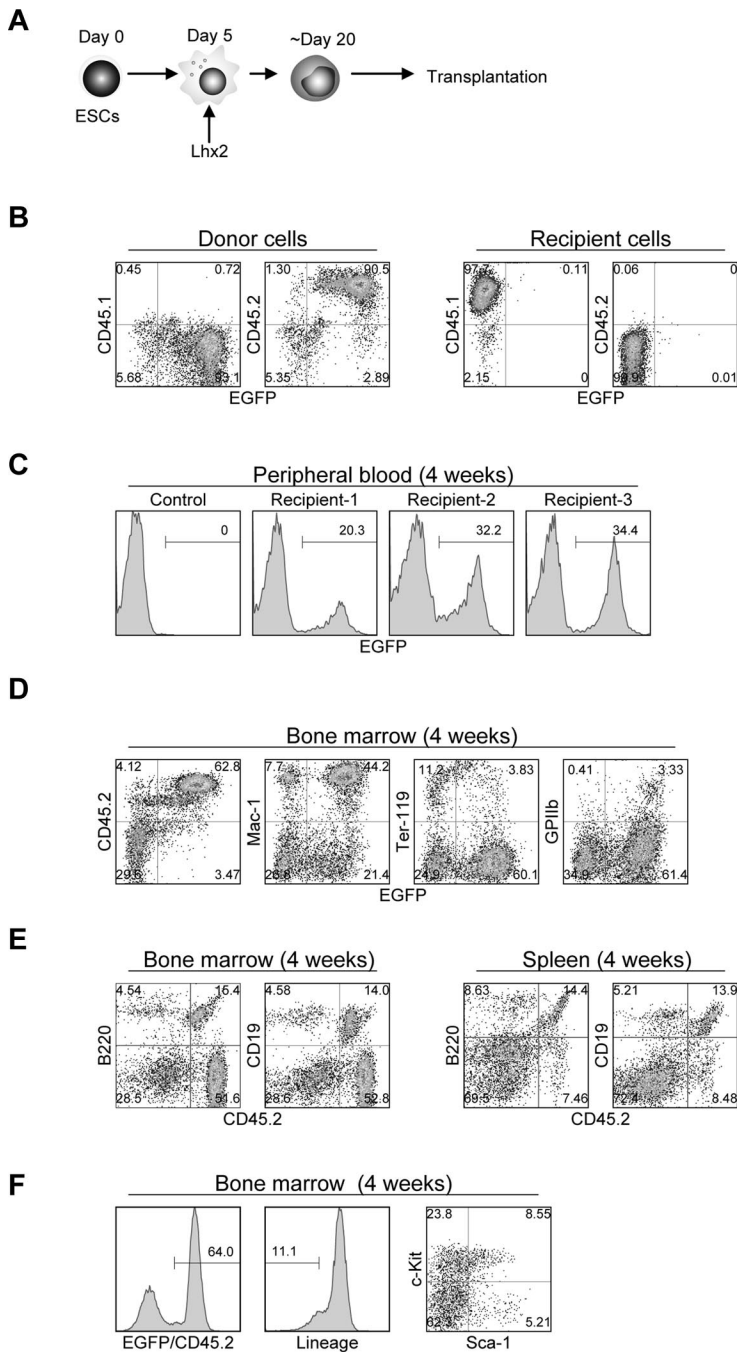


Figure 4. In vivo repopulation by *Lhx2*-transduced hematopoietic cells derived from ESCs. (A) Study design. (B) CD45 isotype expression of donor and recipient cells. (C) Detection of the donor cells in peripheral blood. (D-F) FACS analysis of bone marrow and spleen of recipient mice. Representative data were shown.

obtained; the iPSC-derived hematopoietic cells contributed multilineage of hematopoietic cells at 6 weeks and 12 weeks after transplantation (Figure 6B-F, supplemental Table 2, and supplemental Figure 4A).

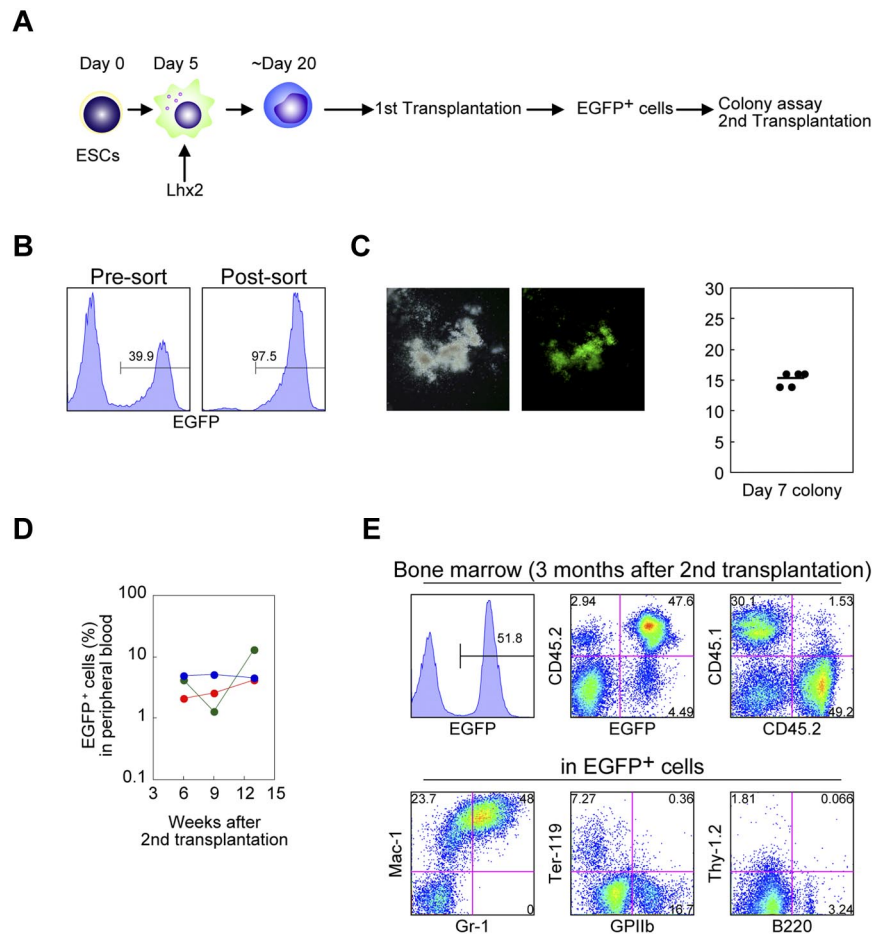
The iPSC-derived mature hematopoietic cells were next analyzed. In the recipients transplanted with iPSC-derived cells, EGFP⁺ erythrocytes and platelets were abundantly observed (Figure 6G). The EGFP⁺/Ter-119⁺ cells in the peripheral blood were enucleated erythrocytes and the EGFP⁺/Gr-1⁺ cells in the bone marrow were segmented neutrophils (Figure 6H). Thus, the *Lhx2*-transduced hematopoietic cells derived from iPSCs underwent terminal differentiation and the mature hematopoietic cells were morphologically normal. Furthermore, EGFP⁺/B220⁺ cells in spleen had passed through immunoglobulin gene rearrangement

(Figure 6I). Taken together, these results demonstrate that iPSC-derived mature hematopoietic cells could be obtained by the *Lhx2* transduction and transplantation.

Comparison between ESC-derived and iPSC-derived hematopoietic cells in vivo

We next compared nature of iPSC-derived cells with that of ESC-derived cells. First, the frequency of KSL cells induced by *Lhx2* transduction were compared and no differences were seen (Figure 1I, supplemental Figure 2F). In the case of *Hoxb4* transduction, the population of KSL cells from iPSCs was also comparable with that from ESCs (Figure 2C). Next, LTR activity was compared. After transplantation of *Lhx2*-transduced HSC-like cells derived from iPSC clone 24, donor-derived EGFP⁺ cells were

Figure 5. Functional analysis of the *Lhx2*-transduced donor cells derived from ESCs. (A) Study design. (B) Cell sorting of the donor cells from the primary recipients. (C) Colony assays. EGFP⁺ cells in were cultured in semisolid media for 1 week. Representative colony was shown. The colony numbers per 2000 cells are shown as dots. The bar indicates the mean value. (D) Frequency of donor cells in peripheral blood of secondary recipients. (E) FACS analysis of bone marrow of secondary recipients. Representative data were shown.



detected for more than 20 weeks (Figure 6J). Essentially similar results were obtained in the case of ESC-derived HSC-like cells, although ESC-derived hematopoietic cells exhibited slightly higher chimerism (Figure 6J, supplemental Figure 4A). The transplantation of iPSC clone 2-derived HSC-like cells resulted in successful engraftment but the contribution was lower (Figure 6J), suggesting a clonal variation of iPSCs.

We did not detect donor-derived T-lymphoid cells in the recipient mice transplanted with *Lhx2*-transduced HSC-like cells derived from iPSCs (Figure 6F). The similar results were observed in the case of ESCs (supplemental Figure 3C), indicating that the failure of T-lymphoid contribution was not caused by the nature of iPSCs. It would be expected that *Lhx2* might inhibit T-lymphoid differentiation.

Lhx2 expression in vivo

Finally, we confirmed the expression of exogenous *Lhx2* in vivo, since it would be postulated that integrative proviral construct itself would act as modulators of transcription factors or epigenetic modulators. The *Lhx2* mRNA could be detected in the EGFP⁺ differentiated cells of transplanted mice (Figure 7A), but not in control mice, indicating that endogenous *Lhx2* mRNA was not expressed in any hematopoietic cell fractions examined (Figure 7B). To know protein expression, FLAG-tagged *Lhx2* (FLAG.*Lhx2*) was transduced into ESC-derived cells, and the transduced HSC-like cells were transplanted. The donor-derived differentiated cells were purified, and analyzed by Western blotting (Figure 7C). The FLAG.*Lhx2* fusion protein was specifically detected in EGFP⁺ differentiated hematopoietic cells, indicating that the retrovirally transduced *Lhx2* was being translated in vivo.

Discussion

In this study, we demonstrated that *Lhx2* conferred LTR capacity to ESC/iPSC-derived hematopoietic cells, with the exception of the T-lymphoid lineage. The new system we developed, that is, the *Lhx2* transduction and subculture on OP9 stromal cells, may be useful for investigating the molecular mechanisms by which HSCs develop the pathways underlying lineage specification from pluripotent stem cells.

The multidifferentiation potentials of *Lhx2*-transduced hematopoietic cells

Our data presented here demonstrated that the *Lhx2*-transduced HSC-like cells were multipotent for the long term, raising a question of whether the maintenance of HSC-like cells by *Lhx2* would be caused by the inhibition of hematopoietic differentiation or not. The data in the literature may provide some clues to the inhibitory actions. *Lhx2* binds to Ldb1 (NLI), a LIM-domain binding protein,²⁸ a known member of the Gata-1/2 multiprotein complex, which is composed of Gata-1/2, Ldb1, Scl, E47, and Lmo2.²⁹ We have previously shown that *Gata-1* knockout erythroid cells acquired multidifferentiation potentials.³⁰ The data raise a possibility that multipotentials of the *Lhx2*-transduced HSC-like cells might be related to the function of *Gata-1*. However, the *Lhx2*-transduced HSC-like cells could produce mature hematopoietic cells in vitro and in vivo. As revealed by the data assessing the effects of *Lhx2* expression on EryD production, the inhibitory

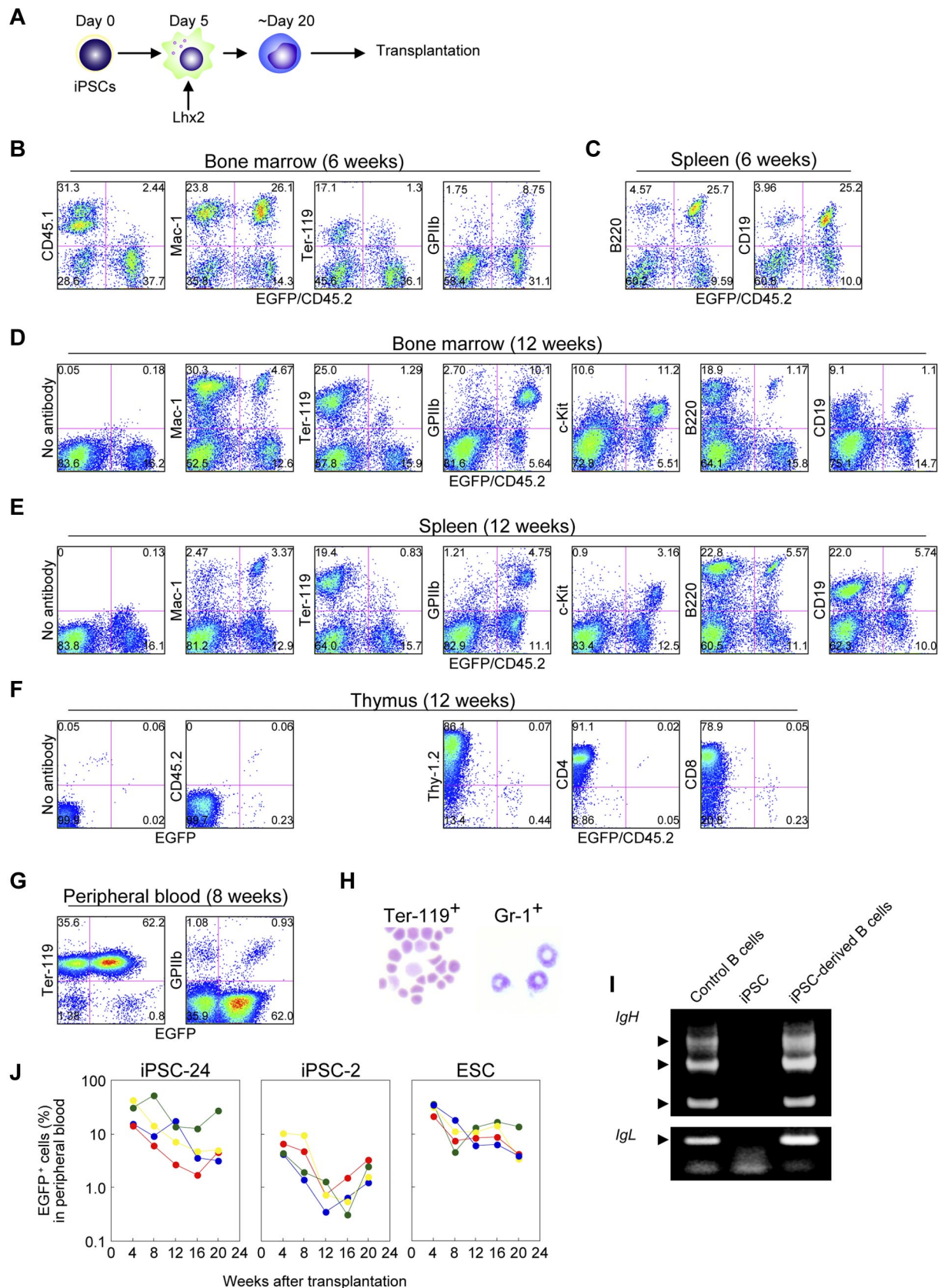
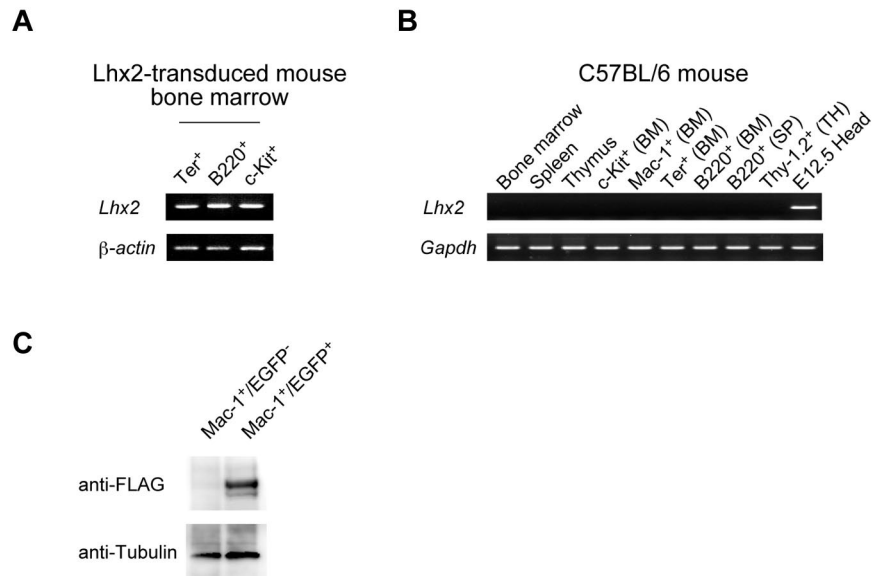


Figure 6. Transplantation analysis of iPSC-derived hematopoietic cells amplified by *Lhx2*. (A) Study design. (B and C) FACS analysis at 6 weeks after transplantation of iPSC-derived hematopoietic cells. (D-F) Long-term hematopoiesis repopulation by *Lhx2*-transduced hematopoietic cells derived from iPSCs (clone 24). (G) FACS analysis of peripheral blood of recipients transplanted with iPSC-derived cells. (H) May-Grünwald-Giemsa staining EGFP⁺/Ter-119⁺ cells and EGFP⁺/Gr-1⁺ cells. Original magnification: $\times 30$. (I) Rearrangement of immunoglobulin gene loci in iPSC-derived B-lymphoid cells. EGFP⁺/B220⁺ cells were sorted from recipient mice transplanted with iPSC-derived hematopoietic cells and analyzed. (J) Long-term monitoring of chimerism in the peripheral blood of recipient mice transplanted with *Lhx2*-transduced hematopoietic cells derived from iPSC clone 24, clone 2, and RENKA ESCs.

Figure 7. Expression of *Lhx2* in vivo. (A) Expression of *Lhx2* mRNA in the donor-derived differentiated cells. The donor-derived erythroid, B-lymphoid and progenitor cells were purified from a recipient transplanted with ESC-derived cells and subjected to RT-PCR analyses. (B) The *Lhx2* expression in normal mouse. BM, SP, and TH indicate bone marrow, spleen, and thymus, respectively. Head region dissected from E12.5 mouse embryos was a positive control. (C) Western blot of FLAG.*Lhx2* expression in donor-derived differentiated cells. ESC-derived HSC-like cells were amplified by *Flag.Lhx2* and transplanted. The donor-derived Mac-1⁺ cells and control Mac-1⁺ cells were purified and analyzed.



action of *Lhx2* on hematopoietic differentiation would be partial. Investigation of the interactions between *Lhx2* and key hematopoietic regulators may clarify the molecular mechanisms underlying *Lhx2*-mediated HSC-like cell induction with maintaining their multipotentials.

Comparison between *Lhx2* and *Hoxb4*

Hoxb4 has been known to induce engraftable HSCs from mouse ESCs¹⁰ and has also been used to produce iPSC-derived engraftable hematopoietic cells which were effective for treatment of a mouse model of sickle cell anemia.³¹ Like *Hoxb4*, *Lhx2* also induced HSC-like cells from ESCs/iPSCs, suggesting that *Lhx2* might be also theoretically useful for regenerative medicine. However, one study has demonstrated that *HOXB4* is dispensable for inducing hematopoiesis from human ESCs.³² Therefore, whether or not *LHX2* could induce HSC-like cells from human ESCs/iPSCs remains to be an important issue.

Understanding of the molecular function of *Lhx2* as well as *Hoxb4* in mice might be helpful for development of effective HSC-like cell induction protocol from human iPSCs. To gain a clue for understanding the *Lhx2* and *Hoxb4* functions, we carried out side by side comparison between these 2 factors. When *Hoxb4* was used, approximately 14.6%, and 3.3% were Lin⁻/c-Kit⁺ and Lin⁻/Sca-1⁺ cells on day 15 in the present study. These values were consistent with the data reported by others; Sca-1⁺ cells were 5.4%,¹⁰ c-Kit⁺ cells and Sca-1⁺ cells were 4.1% and 1.2%, respectively,³³ and c-Kit⁺/Sca-1⁺ cells were 12.1%.³⁴ Therefore, we were able to reproduce the *Hoxb4* effects. compared with *Hoxb4*, the KSL cell induction efficiency of *Lhx2* was superior. Notably, the *Hoxb4* coexpression reduced KSL cell population in the *Lhx2*-transduced cells. The data raise 2 possibilities; *Hoxb4* might inhibit *Lhx2* activity or *Hoxb4* might induce differentiation. *Hoxb4* has been reported to preferentially induce myeloid differentiation from ESC-derived and yolk sac-derived reprogrammed HSCs,^{35,10} implying that later possibility seems likely.

Failure of T-lymphoid repopulation by *Lhx2*-transduced hematopoietic cells

Several papers have been reported that the *Hoxb4*-induced HSCs have low B/T-lymphoid repopulation activity in vivo.^{34,35} In

contrast, the *Lhx2*-transduced HSC-like cells could be contributed into B-lymphoid cells even in the nonimmunocompetent recipient mice. Therefore, the *Lhx2* transduction would be useful for investigating iPSC-derived B-lymphoid cells in vivo. On the other hand, the failure of the *Lhx2*-transduced HSC-like cells to differentiate into T-lymphoid cells raises the possibility that *Lhx2* might disturb T cell differentiation. In *Drosophila*, *Apterous*, a *Lhx2* homolog, has been shown to inhibit the function of the Gata factor *Panier*.³⁶ Thus, one could speculate that *Lhx2* might inhibit the function of *Gata-3*, a master regulator of T cell differentiation.³⁷ Interestingly, both *Lhx2* and *Gata-3* have been implicated in stem cell maintenance in hair follicles^{16,38} and in eye development,^{14,39,40} suggesting a functional interaction between *Lhx2* and *Gata-3*.

Investigation of hematopoietic cells derived from iPSCs by comparing with those from ESCs

Using the combination of OP9 differentiation induction and the *Lhx2* transduction, we could investigate nature of hematopoietic cells derived from iPSCs by comparing with that from ESCs. All assays we carried out showed that the iPSC-derived hematopoietic cells displayed essentially identical behavior to the ESC-derived cells. The *Lhx2*-transduced HSC-like cells derived from both of ESCs and iPSCs conferred LTR ability. However, compared with ESC-derived cells, iPSC-derived hematopoietic cells exhibited slightly less contribution. The lower activities of the iPSC-derived cells might be caused by clonal variation rather than innate differences between ESC-derived and iPSC-derived cells, since the RENKA ESC line was selected based on its higher germ line transmission efficiency. In contrast, our iPSC clones were just established based on their ESC-like morphology.

Using *Lhx2*, we could investigate iPSC-derived cells in vivo. Thus, *Lhx2* could be served as a novel tool for analyzing hematopoietic cells derived from various iPSCs established from different tissues, reprogrammed by different defined factors, and derived from genetically modified mice.

Notably, no teratomas have been observed to date in mice transplanted with *Lhx2*-transduced HSC-like cells derived from ESCs and iPSCs (4 months after transplantation). This suggests that a 2 step in vitro differentiation procedure (lineage-directed differentiation induction and selective amplification) could substantially eliminate the risk of tumorigenesis caused by undifferentiated ESCs/iPSCs.

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Authorship

Contribution: K.K. conceived ideas, performed most of the ESC and iPSC experiments, and analyzed the results; K.M. assisted with the transplantation experiments; K.S. provided the C57BL/6-

derived ESC system; T.N. supervised the ES experiments; and K.K. and T.H. codirected the project and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Kenji Kitajima, PhD, Stem Cell Project Group, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan; e-mail: kitajima-kj@igakuken.or.jp; or Takahiko Hara, PhD, Stem Cell Project group, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan; e-mail: hara-tk@igakuken.or.jp.

References

- Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 1981;292(5819):154-156.
- Yamanaka S, Blau HM. Nuclear reprogramming to a pluripotent state by three approaches. *Nature*. 2010;465(7299):704-712.
- Keller G, Kennedy M, Papayannopoulou T, Wiles MV. Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol Cell Biol*. 1993; 13(1):473-486.
- Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development*. 1998;125(9):1747-1757.
- Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science*. 1994;265(5175):1098-1101.
- Nakano T, Kodama H, Honjo T. In vitro development of primitive and definitive erythrocytes from different precursors. *Science*. 1996;272(5262): 722-724.
- Perlingeiro RC, Kyba M, Daley GQ. Clonal analysis of differentiating embryonic stem cells reveals a hematopoietic progenitor with primitive erythroid and adult lymphoid-myeloid potential. *Development*. 2001;128(22):4597-4604.
- Metcalfe D. On hematopoietic stem cell fate. *Immunity*. 2007;26(6):669-673.
- Jaffredo T, Nottingham W, Liddiard K, Bollerot K, Pouget C, de Bruijn M. From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp Hematol*. 2005;33(9):1029-1040.
- Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell*. 2002;109(1):29-37.
- Pinto do OP, Richter K, Carlsson L. Hematopoietic progenitor/stem cells immortalized by Lhx2 generate functional hematopoietic cells in vivo. *Blood*. 2002;99(11):3939-3946.
- Pinto do OP, Kolterud A, Carlsson L. Expression of the LIM-homeobox gene LHX2 generates immortalized steel factor-dependent multipotent hematopoietic precursors. *EMBO J*. 1998;17(19): 5744-5756.
- Mangale VS, Hirokawa KE, Satyaki PR, et al. Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. *Science*. 2008;319(5861):304-309.
- Porter FD, Drago J, Xu Y, et al. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development*. 1997;124(15):2935-2944.
- Wandzioch E, Kolterud A, Jacobsson M, Friedman SL, Carlsson L. Lhx2-/- mice develop liver fibrosis. *Proc Natl Acad Sci U S A*. 2004; 101(47):16549-16554.
- Rhee H, Polak L, Fuchs E. Lhx2 maintains stem cell character in hair follicles. *Science*. 2006; 312(5782):1946-1949.
- Kitajima K, Tanaka M, Zheng J, Sakai-Ogawa E, Nakano T. In vitro differentiation of mouse embryonic stem cells to hematopoietic cells on an OP9 stromal cell monolayer. *Methods Enzymol*. 2003; 365:72-83.
- Hanna J, Markoulaki S, Schorderet P, et al. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell*. 2008;133(2): 250-264.
- Kitajima K, Masuhara M, Era T, Enver T, Nakano T. GATA-2 and GATA-2/ER display opposing activities in the development and differentiation of blood progenitors. *EMBO J*. 2002;21(12):3060-3069.
- Takahashi K, Okita K, Nakagawa M, Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc*. 2007;2(12):3081-3089.
- Dahl L, Richter K, Häggglund AC, Carlsson L. Lhx2 expression promotes self-renewal of a distinct multipotential hematopoietic progenitor cell in embryonic stem cell-derived embryoid bodies. *PLoS One*. 2008;3(4):e2025.
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006; 126(4):663-676.
- Jiang J, Yu H, Shou Y, et al. Hemgn is a direct transcriptional target of HOXB4 and induces expansion of murine myeloid progenitor cells. *Blood*. 2010;116(5):711-719.
- Lee HM, Zhang H, Schulz V, Tuck DP, Forget BG. Downstream targets of HOXB4 in a cell line model of primitive hematopoietic progenitor cells. *Blood*. 2010;116(5):720-730.
- Matsumoto K, Isagawa T, Nishimura T, et al. Stepwise development of hematopoietic stem cells from embryonic stem cells. *PLoS ONE*. 2009;4(3):e4820.
- Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000; 404(6774):193-197.
- Richter K, Pinto do OP, Hügglund AC, Wahlin A, Carlsson L. Lhx2 expression in hematopoietic progenitor/stem cells in vivo causes a chronic myeloproliferative disorder and altered globin expression. *Haematologica*. 2003;88(12):1336-1347.
- Jurata LW, Gill GN. Functional analysis of the nuclear LIM domain interactor NLI. *Mol Cell Biol*. 1997;17(10):5688-5698.
- Wadman IA, Osada H, Grütz GG, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J*. 1997;16(11):3145-3157.
- Kitajima K, Zheng J, Yen H, Sugiyama D, Nakano T. Multipotential differentiation ability of GATA-1-null erythroid-committed cells. *Genes Dev*. 2006;20(6): 654-659.
- Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007;318(5858):1920-1923.
- Wang L, Menendez P, Shojaei F, et al. Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *J Exp Med*. 2005;201(10): 1603-1614.
- Chan KM, Bonde S, Klump H, Zavazava N. Hematopoiesis and immunity of HOXB4-transduced embryonic stem cell-derived hematopoietic progenitor cells. *Blood*. 2008;111(6):2953-2961.
- Wang Y, Yates F, Naveiras O, Ernst P, Daley GQ. Embryonic stem cell-derived hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 2005;102(52): 19081-19086.
- Rideout WM III, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell*. 2002;109(1):17-27.
- Ramain P, Khechumian R, Khechumian K, Arbogast N, Ackermann C, Heitzler P. Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning. *Mol Cell*. 2000; 6(4):781-790.
- Ting CN, Olson MC, Barton KP, Leiden JM. Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature*. 1996;384(6608): 474-478.
- Kaufman CK, Zhou P, Pasolli HA, et al. GATA-3: an unexpected regulator of cell lineage determination in skin. *Genes Dev*. 2003;17(17):2108-2122.
- Kolterud A, Alenius M, Carlsson L, Bohm S. The Lim homeobox gene Lhx2 is required for olfactory sensory neuron identity. *Development*. 2004; 131(21):5319-5326.
- Maeda A, Moriguchi T, Hamada M, et al. Transcription factor GATA-3 is essential for lens development. *Dev Dyn*. 2009;238(9):2280-2291.