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Reprogramming of Mouse Embryonic Fibroblasts to Mouse Hematopoietic Progenitor Cells

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Abstract

Hematopoietic stem cells (HSCs) show great potential for clinical applications, although obtaining matched HSCs for specific applications and efficiently expanding HSCs *in vitro* are difficult. Recently, reprogramming of somatic cells into hematopoietic lineage has been reported by introduction of several transcription factors. Transduction of hematopoietic transcription factors induced conversion of fibroblast to hematopoietic progenitors. In this study, mouse embryonic fibroblasts (MEFs) were reprogrammed into hematopoietic progenitor cells (HPCs) in long-term HSC culture medium by co-infecting the MEFs with pMXs-Oct4/Sox2/Klf4/c-Myc/Lmo2/c-Fos/c-Myb (pMXs-7TF MEFs). Furthermore, the expanded pMXs-7TF-MEFs could be differentiated into lymphoid and myeloid lineages performing specialized functions using specific cytokines *in vitro*. The *in vivo* transplantation of these pMXs-7TF-MEFs lethally irradiated mice resulted in the complete reconstitution of the hematopoietic system by these pMXs-7TF-MEFs. Taken together, our results demonstrated that reprogrammed cells expressing HSC markers can be differentiated into the lymphoid and myeloid lineages and that these cells exhibit reconstitutive capacities.

Keywords: MEFs; Reprogram; Hematopoietic progenitor cells; Hematopoiesis; Lymphoid; Myeloid

Introduction

Due to their capacity for self-renewal and their potential to differentiate into all blood cell lineages, hematopoietic stem cells (HSCs) are considered to be the exclusive source of the hematopoietic system [1-5]. Although HSCs can be used to treat blood disorders and malignant diseases [6], their clinical application has been limited, primarily because HSCs represent only a small percentage of bone marrow (BM) cells and because it is difficult to maintain their self-renewal capacity *in vitro* and to avoid the immune rejection of HSCs during transplantation [2,3].

Induced pluripotent stem (iPS) cells, which can be produced via the over expression of specific factors [7-9], exhibit the capacity for self-renewal and the potential to differentiate into any cell type; therefore, these cells are of great interest for disease treatment [10,11], drug screening [12], toxicology and regenerative medicine [13]. However, the generation of iPS cells is time-consuming and inefficient (0.001-0.1%) and, most importantly, involves several safety concerns [14,15]. To overcome these issues, investigators have developed methods for the direct conversion of cells [16-18]. According to recent reports, these reprogramming processes generate a variety of intermediate cell types; for instance, iPS cells are one of the products of somatic reprogramming. However, the final results of these reprogramming processes depend on many specific conditions [18,19]. Bhatia's group reported that human fibroblasts could be converted into multi-lineage blood progenitors via the binding of transcription factors (TFs) to hematopoietic regulatory regions [19], and their results indicate that Oct4 acts as a lineage-specific TF [18].

In addition, it was recently reported that a combination of four transcription factors, Gata2, Gfi1b, c-Fos and Etv6, induces hematopoietic cell formation from endothelial-like precursor cells. These cells exhibit a $CD45^+cKit^+CD150^+CD48^-$ phenotype that is similar to that of LT-HSCs [20]. However, the hematopoietic potential of these reprogrammed cells is limited. Another report showed that the combination of Run1t1, Hlf, Lmo2, Prdm5, Pbx1 and Zfp37 promotes the conversion of blood cells to HSCs, suggesting that specific factors regulate the gene networks of HSCs [21]. However, as blood cells are a distinct cell type, the conversion of MEF cells to HSCs remains a challenge. More recently, the expression of FOSB, GFI1, RUNX1 and SPI1 in human umbilical vein endothelial cells and human adult dermal microvascular endothelial cells was found to induce the generation of hematopoietic cells displaying long-term MPP activity [22], although obtaining human endothelial cells is difficult. In contrast, MEFs are easily obtainable for use in reprogramming processes. Lacaud's group also demonstrates that hematopoietic transcription factors induced conversion of fibroblasts to hematopoietic progenitors. However long-term *in vivo* engraftment capacity was not shown [23].

An in-depth understanding of the hematopoietic system and recent findings on somatic cell reprogramming motivated us to examine whether mouse somatic cells can be reprogrammed into hematopoietic progenitor cells (HPCs) using a combination of HSC-specific factors and pluripotency-related genes and then culturing these transduced cells under HSC-specific culture conditions. In this study, we were able to generate lineage-$c\text{-}Kit^+Sca\text{-}1^+$ (LKS), long-term HSC (LT-HSC; $CD34^-Flk2^-CD150^+CD48^-$ LKS), short-term HSC (ST-HSC; $CD34^+Flk2^-CD150^+CD48^-$ LKS) and MPP ($CD34^+Flk2^+CD150^-CD48^-$ LKS) cell populations [24,25] by transfecting mouse embryonic fibroblasts (MEFs) with pMXs-Oct4/Sox2/Klf4/c-Myc/Lmo2/c-Fos/c-Myb (pMXs-7TF MEFs). Furthermore, pMXs-7TF MEFs were successfully differentiated

into immune cells both *in vitro* and *in vivo*. It is highly likely that the successful generation of HPCs will resolve the primary difficulties associated with the use of matched HSCs in individual patients and with the *in vitro* expansion of HSCs for clinical use.

Materials and Methods

Mice

C57BL/6J mice and congenic CD45.1$^+$ C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, http://www.jax.org), and Rag2-/-Il2γg-/- mice (C57BL/6J X C57BL/10SgSnAi) were purchased from Taconic Farms [26]. The hCD34 transgenic mice (C57B6) carrying a PAC clone containing the entire hCD34 gene and 12.8 kb of 5' and 25.6 kb of 3' flanking sequences were constructed as previously reported [27]. All mice were maintained under specific pathogen-free conditions, and we used 8 to 12-week-old male mice. All experiments were conducted in accordance with the guidelines of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Institutional Animal Care and Use Committee (KRIBB-AEC-13013).

Cell culture

HSCs (Lin-c-Kit$^+$Sca-1$^+$; LSK) or Lin-c-Kit$^+$ (LK) cells were isolated from mouse BM using a FACSAria flow cytometer (BD Biosciences). pMXs-7TF MEFs and HSCs were cultured in Myelocult long-term culture medium (ST05350, STEMCELL Technologies, Vancouver, British Columbia, Canada) supplemented with 2 μg/ml indomethacin, 20 μg/ml gentamicin, 30 ng/ml SCF, 50 ng/ml Flt3L, 20 ng/ml TPO and 20 ng/ml IL-6. All cytokines were purchased from PeproTech (Rocky Hill, NJ, USA). MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone).

MEF preparation

MEFs were prepared from 13.5-day pregnant mice and were used for 1-2 passages. The heads and internal organs of the mice were removed, and the torsos were minced and dispersed in 0.1% trypsin (HyClone) for 30 min at 37°C. The cells were cultured for two population doublings (considered as one passage) and then viably frozen. These MEFs were used for all subsequent experiments. The MEFs were maintained in DMEM containing 10% fetal bovine serum (HyClone) and were sub cultured at a dilution of 1:3 upon reaching confluence. MEFs cultured for fewer than three passages were used for pMXs-7TF MEFs generation and feeder cell preparation.

Retroviral infection

Oct 3/4, Sox2, Klf4, c-Myc, LIM domain only 2 (Lmo2), c-Fos, c-myeloblastosis (c-Myb) and c-Jun were transduced using the pMXs vector. The pMXs plasmids expressing Oct 3/4, Sox2, Klf4 and c-Myc were obtained from Addgene. The Lmo2, c-Fos, c-Myb and c-Jun fragments were obtained from the 21C Frontier Human Gene Bank (KRIBB). To generate pMXs-expressing Lmo2, c-Fos, c-Myb and c-Jun plasmids, c-Fos, c-Myb and c-Jun were introduced into the BamHI/HindIII sites of the pMXs plasmid; Lmo2 was introduced into the BglII/HindIII sites of pMXs. Platinum-E retroviral packaging cells (Plat-E cells, Cell Biolabs Inc., [28]) were seeded on six-well plates at 6×10^5 cells per well. The next day, these cells were transfected with the pMXs retroviral vectors using Lipofectamine and PLUS reagents (Invitrogen) according to the manufacturer's instructions. After four hours, the medium was replaced with 5 ml DMEM containing 10% FBS, and the cells were incubated at 37°C in an incubator. After 48 hours, the medium was collected as the first virus-containing supernatant and was replaced with fresh medium. After 24 hours, the second virus-containing supernatant was collected. The viruses were filtered using a 0.45-μm cellulose filter (Millipore), and equal amounts of virus were mixed and transferred to the MEFs in the presence of 4 g/ml Polybrene (Sigma-Aldrich). This infection process was repeated every 12 hours for a total of three times.

Induction of HPCs

To generate HPCs, MEFs were co-infected with pMXs-Oct 4/Sox2/Klf4/c-Myc/Lmo2/c-For/c-Myb for 3 days. The day after infection, the cells were reseeded at $1–2 \times 10^5$ cells per well in 0.1% gelatin-coated six-well plates containing Mitomycin C (Sigma-Aldrich)-treated MEF feeder cells. The cells were cultured in Myelocult long-term culture medium (ST05350, STEMCELL Technologies, Vancouver, British Columbia, Canada) supplemented with HSC cytokines (2 μg/ml indomethacin, 20 μg/ml gentamicin, 30 ng/ml SCF, 50 ng/ml Flt3L, 20 ng/ml TPO and 20 ng/ml IL-6). After 14-21 days, colonies were picked using a 100 μl pipette. To break the colonies into small mass, the colonies were treated with trypsin-EDTA (Hyclone SH30042.01) for 15min at 37°C. The colonies were reseeded in MEF feeder cells. The colonies were cultured in Myelocult long-term culture medium supplemented with HSC cytokines. To ensure the generation of HPCs, the colonies were stained for HSC markers (c-Kit$^+$, Sca-1$^+$, CD34$^{+/-}$, Flk2$^{+/-}$, CD150$^{+/-}$, CD48$^{+/-}$, and lack lineage markers) and were analyzed via FACS. To confirm HPCs generation, we performed PCR and western blot analysis. To validate the retrovirus-mediated over expression of the candidate genes, the mRNA levels of each candidate gene and the GAPDH gene were determined via RT-PCR analysis.

Flow cytometry

To analyze HSC marker expression, the cells were stained with antibodies against CD34, FLK2, hCD34, CD48, CD150, CD117 and Ly6a for 30 min at 4°C. Lineage staining was performed using a mixture of biotinylated anti-mouse antibodies against Mac-1 (CD11b), Gr-1 (Ly6C), Ter-119, NK1.1, CD2 and B220. To distinguish between donor and recipient cells, antibodies against CD45.1 and CD45.2 were used, the donor cells were stained using the CD45.2 antibody and the recipient cells were stained using the CD45.1 antibody. To analyze myeloid and erythroid marker expression, antibodies against Gr-1, Mac-1 and Ter-119 were used. These antibodies were purchased from BD Biosciences or eBiosciences. The data were generated using a FACSCanto flow cytometer (BD Biosciences) and were analyzed using FlowJo software (ThreeStar, Ashland, OR, USA).

Western blot analysis

Cells were lysed in lysis buffer containing 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, 10 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1 mM Na_3VO_4 and 1 mM PMSF. The lysates were resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to PVDF membranes (Millipore) and stained with 0.1% *Ponceau S* solution. After blocking with 5% nonfat milk or BSA, the membranes were incubated in anti-c-Kit (Abcam, Cambridge, MA, USA), anti-CD150 (Abcam, Cambridge, MA, USA) or anti-actin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) antibodies.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative RT-PCR (QPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen). For the RT-PCR analyses, each RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase and oligo (dT) 15 primers (Promega, Madison, WI, USA). Then, this cDNA was amplified using Emerald Amp PCR Master Mix (Takara), and qPCR was performed using SYBR Premix Ex Taq (Takara) according to the manufacturer's protocol. All RT-PCR and qPCR data were normalized to the GAPDH expression levels. The primer sequences used are listed in Supplementary table 1.

Colony-Forming cell (CFC) assays

To perform the CFC assays, Methocult M3434 medium (STEMCELL Technologies) was used. First, 0.4 ml of pMXs-7TF MEFs was added to 4 ml of methylcellulose-based medium containing the appropriate cytokines, and the tubes were vortexed to ensure the mixing of the cells with the medium. The cell mixtures were incubated for 5 min; then, 1.1 ml of the mixtures was inserted as droplets into a 30-mm dish. The cells were incubated at 37°C in 5% CO_2 at 95% humidity for 14 days. The colonies were morphologically evaluated and counted after 3, 7 or 14 days using an inverted microscope at 40X magnification.

Giemsa staining

To further analyze differentiation and proliferation, the cells were collected and resuspended in cold PBS. Then, the cells were transferred to glass slides using a Cytospin centrifuge for Giemsa staining. The cells were fixed with methanol for 2 min and stained with Giemsa staining solution (Sigma) for 4 min. The slides were transferred to phosphate buffer and rinsed with water. After the slides were completely dry, mounting solution was applied as droplets, and the slides were coverslipped. Photomicrographs were captured at 1000X magnification.

In vitro differentiation of NK cells from pMXs-7TF MEFs

NK cell differentiation from the HSCs was performed essentially as described previously [29]. In brief, HSCs or pMXs-7TF MEFs were seeded in 12-well plates and cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, indomethacin (2 μg/ml, Sigma), gentamicin (20 μg/ml), SCF (30 ng/ml, PeproTech), Flt3L (50 ng/ml, PeproTech) and IL-7 (0.5 ng/ml, PeproTech) for seven days to generate precursor natural killer (pNK) cells. The pNK cells were then harvested, reseeded and cultured in the presence of IL-15 (50 ng/ml, PeproTech) for six days to produce mature natural killer (mNK) cells. The purity of the mNK cells was determined via FACS analysis using anti-CD3e and anti-NK1.1 antibodies.

In vitro differentiation of the Myeloid lineage from pMXs-7TF MEFs

To differentiate cells of the myeloid lineage, pMXs-7TF MEFs and MEFs transfected with the pMXs control vector were seeded in 12-well plates and cultured in RPMI 1640 medium supplemented with 10 ng/ml IL6, 10 ng/ml GM-CSF, 10 ng/ml G-CSF, 50 ng/ml SCF, 20 ng/ml IL3 and 10 ng/ml BMP4. After two weeks, we observed that the pMXs-7TF MEFs were able to differentiate into the myeloid lineage. To analyze myeloid marker expression via FACS, the cells were stained with anti-Mac-1 and anti-Gr-1 antibodies.

Cytotoxicity analysis

NK cell death was evaluated using a calcein-AM release assay (Invitrogen) as previously described [29]. In brief, Yac-1 cells were incubated in 5 μg/ml calcein-AM for 1 hour at 37°C with occasional shaking. After washing, the cells were plated at the indicated effector:target cell (E:T) ratio in 96-well round-bottom plates and were incubated for four hours at 37°C. Following incubation, 100 μl of the supernatant was collected, and the plates were analyzed using a fluorescence plate reader (excitation filter 485 nm, emission filter 530 nm, BerThold, Germany).

Reconstitution assay

For the competitive reconstitution assays, MEF (CD 45.2$^+$, 5 × 10^4) or LK cells (CD45.2$^+$, 5 × 10^4) from pMXs-7TF MEFs or BM cells were mixed with whole BM cells (1 × 10^6) from C57BL/6 (CD 45.1$^+$) mice. Then, these mixtures were intravenously injected into eight-week-old C57BL/6 CD 45.1$^+$ congenic mice that had received lethal γ-irradiation (9 Gy). For serial BM transplantation (BMT), donor-derived BM cells (3 × 10^6) from recipients were injected into a second set of recipient mice (CD 45.1$^+$) at 16th week after the first BMT. Peripheral blood was collected and analyzed via FACS to determine the repopulation percentage of the donor-derived cells. Successful pMXs-7TF MEFs transplantation was confirmed based on the identification of donor-derived HSC-positive cells in the BM at 16th week after second transplantation. These transplanted recipients were maintained under specific pathogen-free conditions.

Statistical analysis

The data are presented as the mean ± SD values, unless stated otherwise. To compare two groups, we performed two-tailed paired t-tests using PRISM software (San Diego. CA, USA) and performed two-tailed paired Student t-tests using Microsoft Excel. A p value of less than 0.05 was considered to be significant.

Results

Reprogramming MEFs to generate HPCs

To generate HPCs, we selected candidate genes based on the results from an earlier serial analysis of gene expression (SAGE) dataset (Supplementary figure 1A) [30]. According to the SAGE dataset, c-Jun (J), Lmo2 (L), c-Fos (F), c-Myb (B), and c-Myc (M) were higher expressed in HSCs than natural killer (NK) cells. These TFs are known to play a role in the development of lymphoid lineages. To confirm the increased expression of these TFs in HSCs (LKS cells), we verified the mRNA levels of these candidate genes in HSCs via RT-PCR analysis. As shown in supplementary figure 1B, these genes displayed increased expression levels in HSCs compared with other cell types, such as MEFs and pNK, mNK cells and embryonic stem (ES) cells. Next, these candidate genes were applied to the MEFs in combination with pluripotency-related genes, including Oct4, Sox2, Klf4 and c-Myc (OSKM), using the pMXs-based retrovirus system. Flow cytometric analysis showed that RFP-positive MEF cells comprised more than 90% of the cells (Supplementary figure 1C) and that each gene was specifically overexpressed in the MEFs (Supplementary figure 1D).

For reprogramming, MEFs were transduced with the candidate genes and cultured on MEF feeder cells in long-term HSC culture medium (Myelocult, M5300) lacking leukemia inhibitory factor (LIF), a compound that plays a crucial role in the maintenance of self-renewal in mouse ES cells [31]. After 14-21 days in culture, colonies were picked and were seeded on MEF feeder cells in long-term HSC culture medium supplemented with indomethacin, gentamicin, and hematopoietic cytokines, such as SCF, Flt3L, TPO and IL-6. To ensure that we generated HPCs, the colonies were stained for HSC markers and were analyzed via FACS. The general scheme is depicted in figure 1A. The various combinations of candidate genes were tested to determine the optimal genes for transduction (Supplementary figure 2A). The number of colonies depended on which of the genes were combined with OSKM. The combinations, such as OSKML, OSKMF, OSKMB, OSKMLF, OSKMLB, OSKMFB, and OSKMLFB induced colony formation (Supplementary figure 2B). In particular, the OSKMLFB combination produced the largest number of colonies (Supplementary figure 2C) and generated a distinct population of LKS cells (Supplementary figure 2D). Therefore, we selected the OSKMLFB combination (pMXs-7TF) as the optimal reprogramming conditions. figure 1B shows the morphology of control MEFs (pMXs), pMXs-7TF MEFs, and primitive HSCs. The pMXs-7TF MEFs exhibited a round morphology that was similar to that of primitive HSCs. Transduction with pMXs-7TF increased the number of formed colonies compared to transduction with pMXs (Figure 1C). Next, to define the emergent hematopoietic cells, we examined the time-course of gene expression during pMXs-7TF MEFs generation. After transduction, the cells were harvested at the indicated times, and HSC marker expression was analyzed via qPCR. The expression of fibroblast-specific genes, such

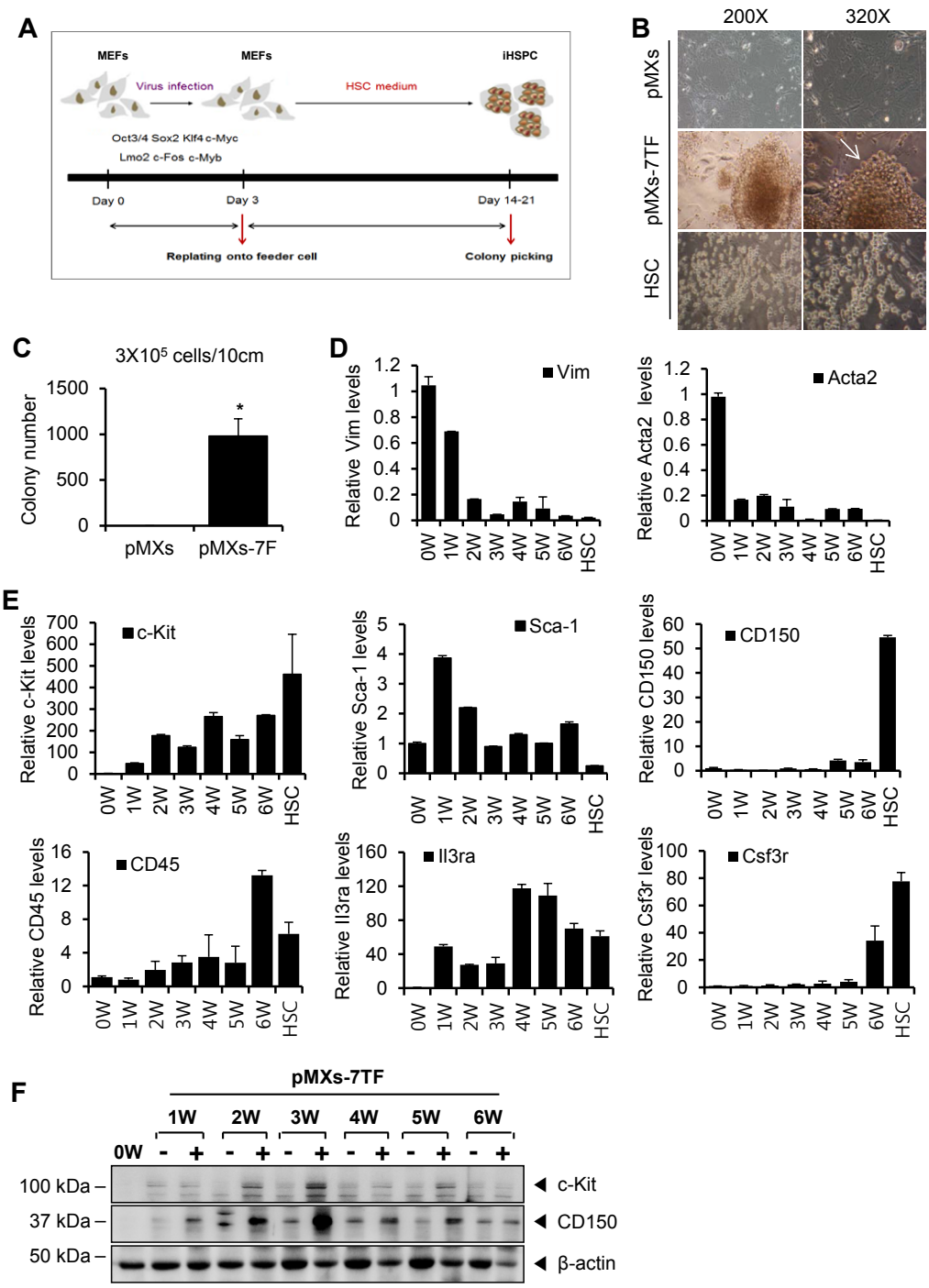

Figure 1: Reprogramming of MEFs to HPCSs using seven factors. **(A)** A schematic showing the pMXs-7TF MEFs reprogramming process. MEFs were infected with retrovirus for a total of three days. After the first day, the virus-infected MEFs were reseeded on Mitomycin C-treated MEF feeder cells. The cells were cultured in Myelocult long-term medium supplemented with several hematopoietic cytokines. After 14-21 days, colonies were picked and reseeded in 24-well plates. **(B)** A bright-field micrograph showing the morphology of the cells transduced with the pMXs control vector or the pMXs-7F vector and HSCs. Small round cells were detected among the pMXs-7F-treated cells (white arrow). Original magnifications: 200X and 320X. **(C)** The MEFs were transduced with the seven transcription factor-expressing (pMXs-7TF) vector or the pMXs control vector. The number of colonies was counted 14-21 days after infection. The error bars represent the s.d. (n=3); *p<0.05 compared with the pMXs-transduced MEFs. **(D)** The relative expression levels of fibroblast-associated genes (Vim and Acta2) in the pMXs-7TF-transduced MEFs. The transduced MEFs or colonies were harvested at 0-6 week (W) after reseeding on the MEF feeder cells. Target gene expression was evaluated via qPCR and was normalized to the GAPDH expression levels. HSCs were used as controls. **(E)** MEFs were transduced with the seven transcription factor-expressing vector (pMXs-7TF). After transduction, the cells were harvested at the indicated times, and HSC marker expression was analyzed. HSCs or ES cells were used as controls. Target gene expression was evaluated via qPCR and was normalized to the GAPDH levels. All experiments were independently repeated at least three times, and the error bars represent the s.d. **(F)** Time course analysis of HSC marker expression. The expression of c-Kit, CD150 and β-actin was determined via Western blot analysis. (–) indicates pMXs-control vector-transduced cells; (+) indicates pMXs-7TF-transduced cells.

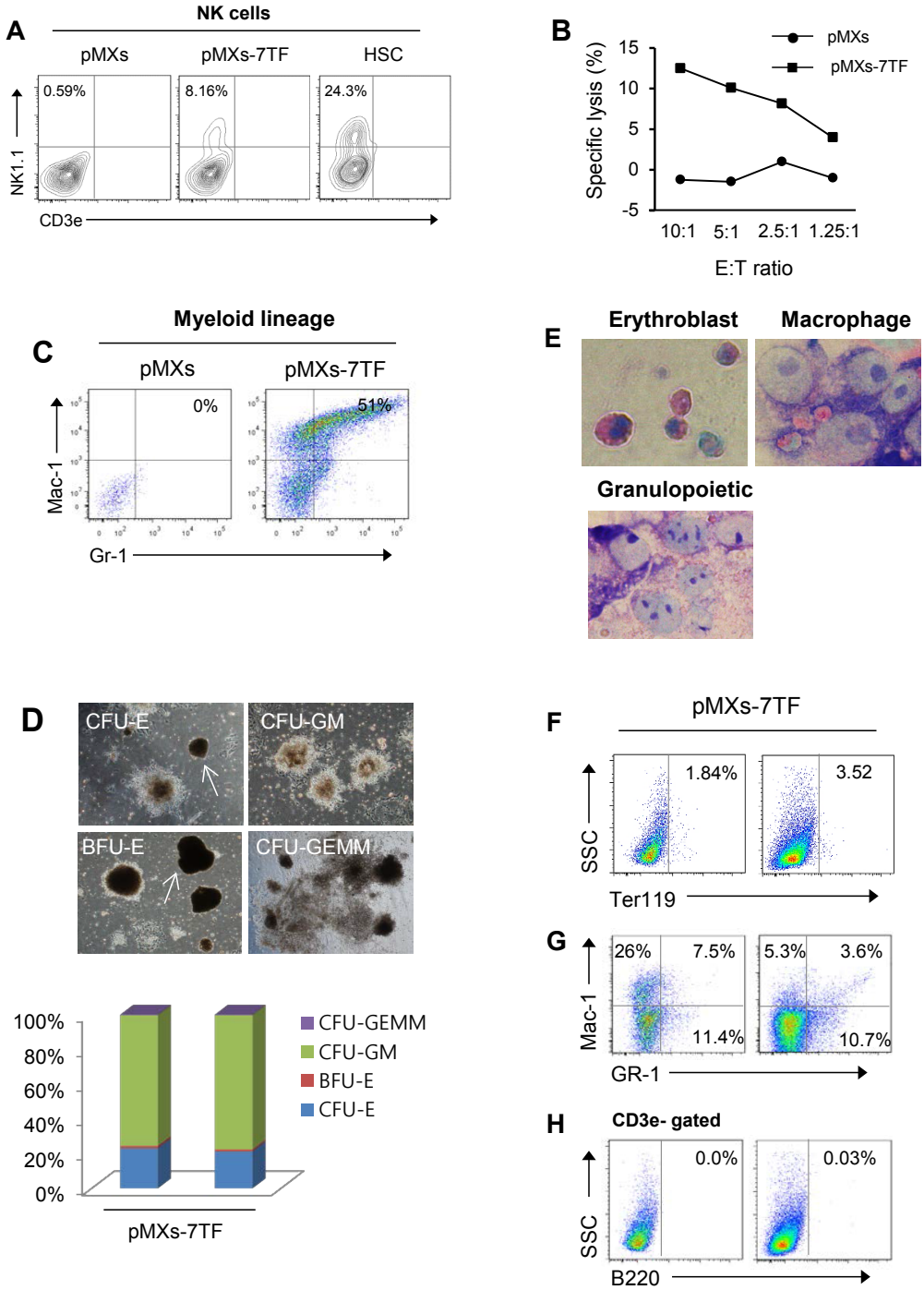

Figure 2: Multipotent capacity of pMXs-7TF MEFs *in vitro*. **(A)** To explore the capacity of the cells to differentiate into NK cells, the HSCs or the pMXs-7TF MEFs were co-cultured with Mitomycin C-treated OP9 cells in the presence of Flt3L, SCF and IL-7 for 6 days. After 6 days, to generate mNK cells, the cells were cultured in the presence of IL-15 for 6 additional days. Then, the cells were stained with anti-CD3 and anti-NK1.1 antibodies. The phenotypes of the CD3e-NK1.1+ subsets were determined via FACS analysis. The percentage of NK cells among the gated lymphocyte population was evaluated. HSCs were used as positive controls. **(B)** The cytotoxicity of mNK cells derived from pMXs-7TF MEFs or pMXs-transduced MEFs. The mNK cells were incubated in calcein-AM-labeled Yac-1 cells at the indicated E:T ratio (10:1, 5:1, 2.5:1 or 1.25:1). After four hours, the supernatant was collected, and the plate was examined using a fluorescence plate reader (485 nm excitation, 530 nm emission). All experiments were independently repeated at least three times. **(C)** To induce the myeloid differentiation of pMXs-7TF MEFs in vitro, the pMXs-7TF MEFs were maintained in myeloid culture medium containing several cytokines. After two weeks, the cells were stained with anti-Mac-1 and anti-Gr-1 antibodies and were analyzed via flow cytometry. **(D)** CFC images and the relative frequency of CFU formation from the pMXs-7TF-transduced cells. The left panel shows images of CFU-E, CFU-GM, BFU-E, and CFU-GEMM colonies. Original magnification: 40X. The right panel shows the relative frequency of colony formation. Representative results from three independent experiments are shown. **(E)** Giemsa staining of Methocult colonies derived from pMXs-7TF MEFs on day 14. Original magnification: 1000X. (F-H) Flow cytometry to determine the differentiation states of the cell populations. Erythroid cells **(F)**, myeloid cells **(G)** and B cells **(H)**. After 14 days, Methocult colonies were harvested and stained with lineage-specific antibodies.

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as Vim and Acta2, decreased gradually (Figure 1D and supplementary figure 3A), but the expression of HSC-specific markers, including c-Kit, Sca-1, CD150, CD45, Il3ra and Csf3r [20], increased in cells transduced with pMXs-7TF (Figure 1E and supplementary figure 3B). Western blot analysis confirmed that c-Kit and CD150 were highly expressed in the pMXs-7TF MEFs compared with the control pMXs-transduced cells (Figure 1F).

To validate the commitment to pluripotency, we examined the expression patterns of pluripotency markers at the indicated time points. The total levels of Oct4, Sox2 and Klf4 decreased in a time-dependent manner, and the expression level of c-Myc, which is highly expressed in HSCs, was elevated in the pMXs-7TF MEFs and HSCs (Supplementary figure 4A). The endogenous expression of Oct4 and Sox2 was lower in the pMXs-7TF MEFs than in the ES cells (Supplementary figure 4B). Nanog and E-Ras, which are markers for pluripotent cells, were expressed at basal levels in the pMXs-7TF MEFs (Supplementary figure 4C). Taken together; these findings indicated that the pMXs-7TF MEFs reprogramming process does not require the generation of iPS cells.

To further analyze the cellular phenotypes, we examined surface antigen expression in the transduced cells. LKS cell, long-term HSC, short-term HSC and MPP ($CD34^+Flk2^+CD150^-CD48^-$ LKS) cell populations were detected in the pMXs-7TF MEFs that were derived from individual colonies. As shown in supplementary figures 5A-5B, Sca-1, but not c-Kit, was expressed in the MEFs transduced with the pMXs control vector. Sca-1 is expressed in a variety of cells, including mesenchymal stem cells, cardiomyocytes and fibroblasts [32,33]; this broad expression explains the detection of Sca-1 in the pMXs-transduced cells. Although surface antigen expression in the pMXs-7TF MEFs was not exactly the same as that in the primitive HSCs, the pMXs-7TF MEFs clearly included an LKS cell population. Next, we performed qPCR to compare the gene expression profiles between pMXs-7TF MEFs, HSCs and ES cells (Supplementary figure 5C). The expression levels of these HSC markers in the pMXs-7TF MEFs were similar to those in the HSCs. Next, we performed RT-PCR analysis. As shown in supplementary figure 5D, HSC surface markers, such as c-Kit, Sca-1, CD150 and CD34, and TFs, such as c-Jun, Lmo2, c-Fos, c-Myb and Trim28, that are highly expressed in HSCs were strongly expressed in our pMXs-7TF MEFs and HSCs. Nanog was not expressed in the pMXs-7TF-transduced cells. In addition, the protein levels of these factors were verified via Western blot analysis. As expected, c-Kit and CD150 were expressed in the pMXs-7TF MEFs (Supplementary figure 5E), and these results were verified via immunocytochemistry using antibodies against SSEA (a pluripotent cell marker), c-Kit and Sca-1 (Supplementary figure 5F). The HSC surface markers c-Kit and Sca-1 were highly expressed in pMXs-7TF MEFs and HSCs, but SSEA was not expressed in pMXs-7TF MEFs. Taken together, these results demonstrated that pMXs-7TF MEFs display similar molecular characteristics to primitive HSCs. To determine whether these pMXs-7TF MEFs continually expand and maintain their HSC properties *in vitro*, pMXs-7TF MEFs were cultured in long-term HSC culture medium for two months. We found that the LKS cells population remained present after two months of culturing (Supplementary figure 5G).

Assessment of differentiation potential *in vitro*

Next, we examined the multipotency of the pMXs-7TF MEFs by measuring their capacity to differentiate into the lymphoid and myeloid lineages. To confirm lymphoid lineage differentiation, we generated NK cells from pMXs-7TF MEFs using a two-step *in vitro* differentiation protocol [34]. As shown in figure 2A, the pMXs-7TF MEFs could be differentiated into CD3e-NK 1.1^+ mNK cells, as has been observed for primitive HSCs, and these cells were positive for other NK cell markers, such as DX5 (Supplementary figure 6A) [35]. We then examined the cytotoxicity of mNK cells derived from pMXs-7TF MEFs. The mNK cells that were derived from pMXs-7TF MEFs (Supplementary figure 6B) displayed cytotoxic activity against Yac-1 tumor cells, whereas the pMXs-transduced cells did not display this activity (Figure 2B). Next, to verify the differentiation of pMXs-7TF MEFs into the myeloid lineage, the cells were cultured in differentiation medium containing several cytokines, including IL6, GM-CSF, G-CSF, SCF, IL3 and BMP4. After two weeks, we observed that the pMXs-7TF MEFs differentiated into the $Gr\text{-}1^+Mac\text{-}1^+$ myeloid lineage (Figure 2C). To determine the specific hematopoietic potential of the pMXs-7TF-transduced cells, we performed a CFC assay using methylcellulose-based medium supplemented with recombinant cytokines (M3434). Erythroid colony-forming unit (CFU-E), granulocyte-macrophage colony-forming unit (CFU-GM), erythroid burst-forming unit (BFU-E) and granulocyte-erythroid-macrophage-megakaryocyte colony-forming unit (CFU-GEMM) colonies were successfully derived from the pMXs-7TF MEFs (Figure 2D). The colonies derived based on the CFC assay were stained with Giemsa, and the majority of the colony-forming cells exhibited granulocyte and macrophage lineage characteristics (Figure 2E). To further verify the identity of these lineages, the colonies derived based on the CFC assay were stained with antibodies against Mac-1 and GR-1 (myeloid lineage, figure 2F), Ter119 (erythroid lineage, figure 2G) and B220 (lymphoid lineage, figure 2H). We found that these cells expressed myeloid and erythroid markers. These results confirmed the colony-forming potential and multilineage differentiation capacity of these pMXs-7TF-MEFs.

Functional properties of the pMXs-7TF MEFs *in vivo*

HSCs are crucial for the reconstitution of hematopoiesis upon transplantation into recipients who have undergone BM ablation [3,36,37]. To investigate the ability of these pMXs-7TF MEFs to regenerate the hematopoietic system, we cultured the reprogrammed cells on MEF feeder cells. After 2-3 weeks, LK cells from pMXs-7TF MEFs ($CD45.2^+$) or BM (HSC, $CD45.2^+$) were sorted and transplanted with congenic competitors (BM, $CD45.1^+$) into lethally γ-irradiated congenic mice ($CD45.1^+$). After 16 weeks, we analyzed the reconstituted cell population via FACS analysis (Figure 3A). To verify the engraftment of donor-derived cells in the blood, we collected peripheral blood at 5, 9 and 12 weeks after transplantation. Similar to BM-derived LK cells (HSC), pMXs-7TF MEFs -derived LK cells (pMXs-7TF) exhibited the capacity for reconstitution in the peripheral blood (Figures 3B-3C), and donor-derived $CD45.2^+$ LKS cell populations were detected in the BM of the transplant-recipient mice. In addition, donor-derived ($CD45.2^+$) immune cells, including NK cells, T cells and B cells were observed in the recipient mice (Figure 3D and supplementary figure 7A).

To confirm the reconstitutive properties of these cells, pMXs-7TF MEFs were transplanted into Rag2/Il2 g double-knockout mice, which contain no detectable $NK1.1^+$ NK cells or $Thy1^+$ T cells [38]. Nine weeks after transplantation, NK1.1+DX5+NK and $CD4^+CD8^+$ T cells were observed in the spleens of the pMXs-7TF MEFs- and HSC-transplant-recipient mice (Figure 3E and supplementary figure 7B). As previous reports indicated that Oct 4 can induce the direct conversion of blood progenitors from human fibroblasts and that these cells can differentiate into a myeloid lineage but not lymphoid cells [19], we examined the presence of myeloid-lineage cells in recipient mice (CD45.1) transplanted with LK cells (CD45.2) derived from pMXs-7TF MEFs or BM cells. We found that $CD45.2^+Gr\text{-}1^+Mac\text{-}1^+$ myeloid lineage cells were present in the recipient BM (Figure 3F and supplementary figure 7C). These data demonstrated that pMXs-7TF MEFs exhibit the potential to differentiate into both the lymphoid and myeloid lineages.

To confirm the ability of these pMXs-7TF MEFs to regenerate the hematopoietic system, we performed a serial BMT. LK cells from pMXs-7TF MEFs ($CD45.2^+$) or BM (HSC, $CD45.2^+$) were sorted and transplanted with congenic competitors (BM, $CD45.1^+$) into lethally

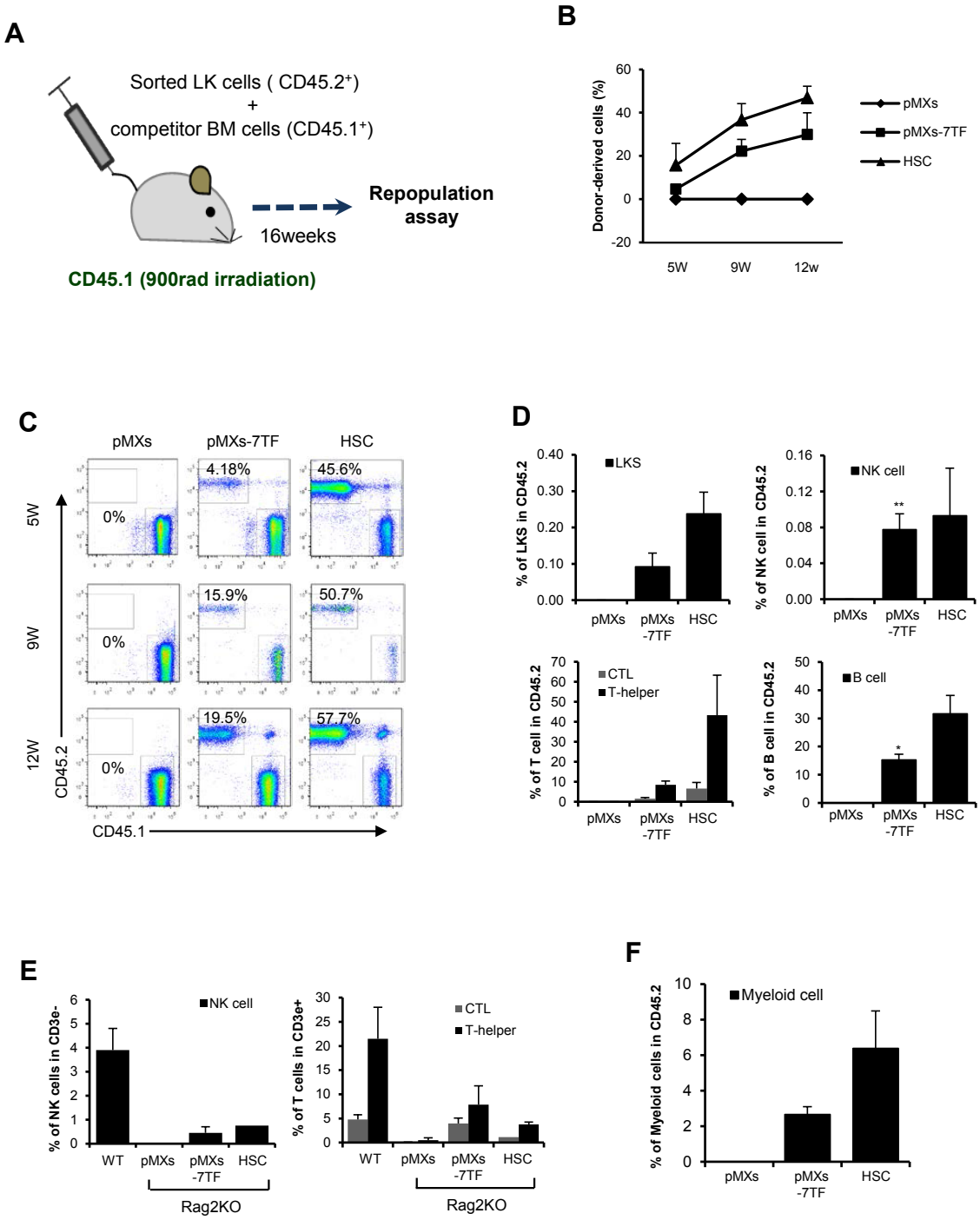

Figure 3: Multipotent capacity of pMXs-7TF MEFs *in vivo*. **(A)** The experimental design of the competitive reconstitution assay. Irradiated CD45.1 congenic mice were transplanted with 5 × 10^5 LK cells from donor-derived BM (CD45.2) or pMXs-7TF MEFs (CD45.2) with competitor cells (CD45.1, 1 × 10^6). After 16 weeks, the frequencies of donor-derived LKS, NK, T and B cells were determined via FACS analysis. **(B)** Peripheral blood was collected at 5, 9 and 12 weeks after BM transplantation to determine the percentage of donor-derived cells. The error bars represent the s.d. (n=4). **(C)** Donor-derived cells (CD45.2+) in peripheral blood were analyzed by flow cytometry. **(D)** Donor-derived (CD45.2+) LKS, NK, B or T cells in the recipient BM (CD45.1+) at 16 week after transplantation. The error bars represent the s.d. (n=4). *p<0.05 and **p<0.01 compared with pMXs control vector-transduced MEFs. ; see also Supplementary Figure 7. **(E)** pMXs-7TF MEFs were transplanted into Rag2 KO mice. After nine weeks, the wild type mice (C57BL/6J, WT), the Rag2 KO mice (pMXs), and the HSC-transplanted (HSC) or pMXs-7TF MEFs-transplanted (pMXs-7TF MEFs) Rag2 KO mice were euthanized and analyzed via FACS analysis. The left panel indicates NK cells, and the right panel indicates T cells. ; see also Supplementary Figure 7. The error bars represent the s.d. (n=3). Representative results from one of three repeated experiments are shown. **(F)** To confirm the generation of myeloid cell populations in vivo, we verified the presence of the CD45+GR-1+Mac-1+ cell populations in the donor-derived cells. LK cells (5 × 10^5) from iHSCs or BM cells were transplanted into lethally irradiated congenic mice (CD45.1). After nine weeks, donor-derived myeloid cells (CD45.2+) were analyzed in the peripheral blood of the recipients (CD45.1+). ; see also Supplementary Figure 7. The error bars represent the s.d. (n=3).

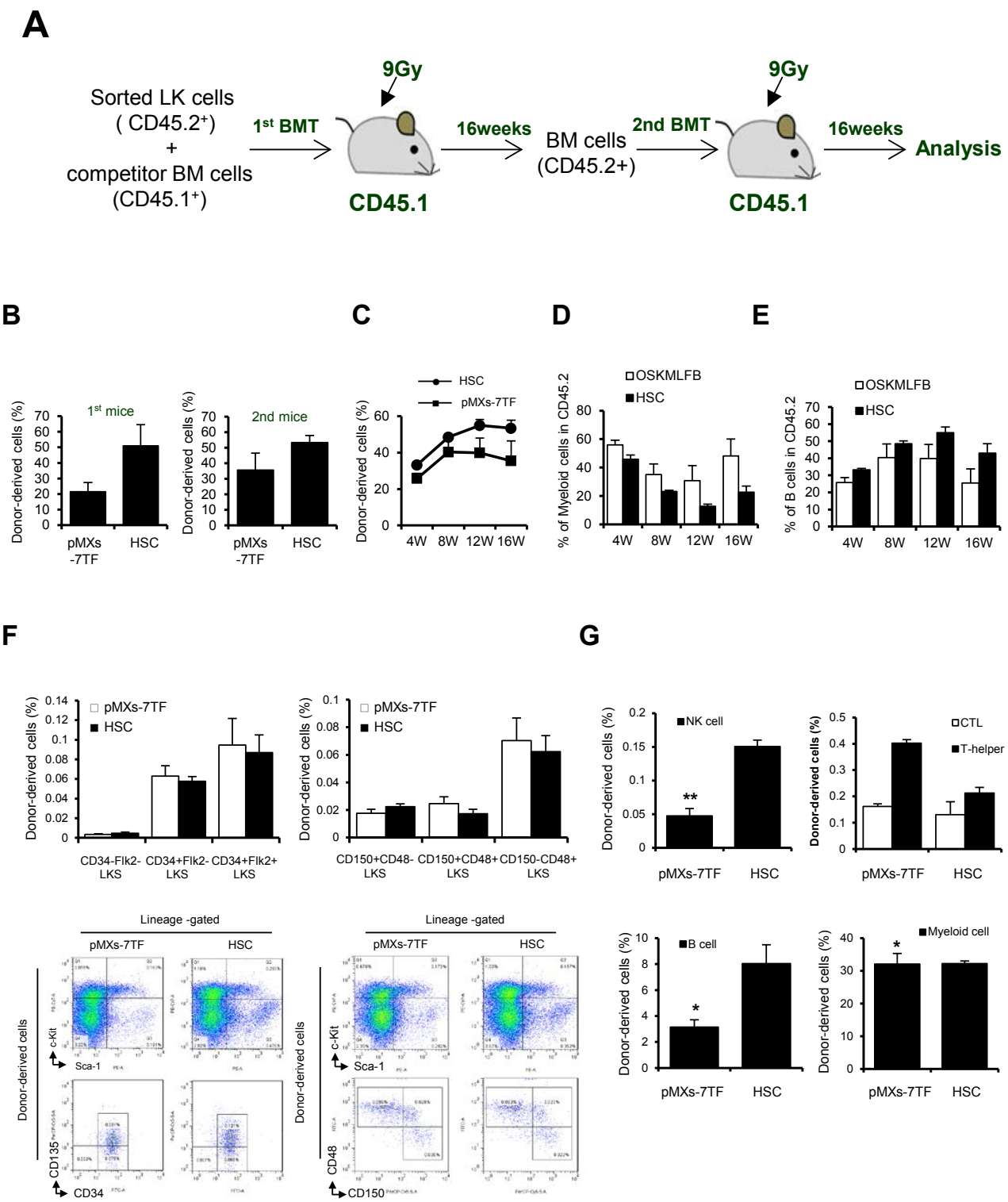

Figure 4: A serial BMT of pMXs-7TF MEFs. **(A)** The experimental design of serial BMT assay. Irradiated CD45.1 congenic mice were transplanted with 5 × 10^5 LK cells from donor-derived BM (CD45.2) or pMXs-7TF MEFs(CD45.2) with competitor cells (CD45.1, 1 × 10^6). At 16week after the first BMT, donor-derived BM cells (3×10^6) from recipients were injected into a second set of recipient mice (CD 45.1+). **(B)** PB was collected at 16 week after first BMT and secondary BMT to determine the percentage of donor-derived cells; see also Supplementary Figure 8. **(C)** To verify the engraftment of donor-derived cells in the blood, we collected PB at 4, 8, 12 and 16 week after secondary BMT. **(D-E)** Reconstitution of the indicated populations in PB at 16 week after secondary BMT. Donor chimerism presented as percentage of donor cells. **(F-G)** Donor-derived (CD45.2+) HSC populations, NK, T, B and myeloid cells in the recipient BM (CD 45.1+) at 16 week after second BMT assay; see also Supplementary Figure 9. The error bars represent the s.d. (n=3). *p<0.05 and **p<0.01 compared with HSC

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irradiated congenic mice (CD45.1$^+$) At 16 week after the first BMT, donor-derived BM cells from recipients were injected into secondary recipient mice (CD 45.1$^+$) (Figure 4A). At 16 week after each BMT, peripheral blood (PB) was collected and analyzed via FACS to determine the repopulation percentage of the donor-derived cells (Figure 4B and supplementary figure 8A). At 16 week after second BMT, we analyzed the reconstituted cell population via FACS analysis. First, to verify the engraftment of donor-derived cells in the blood, we collected PB at 4, 8, 12 and 16 weeks after transplantation (Figure 4C and supplementary figure 8B). Similar to BM-derived LK cells (HSCs), pMXs-7TF-MEFs-derived LK cells exhibited the multi-lineage reconstitution through the degree of B cells and myeloid cells in the PB (Figures 4D-4E and supplementary figure 8C). In addition, donor-derived CD45.2$^+$ HSC populations were detected in the BM of the transplant-recipient mice (Figure 4F and supplementary figure 9A). Donor-derived (CD45.2$^+$) immune cells, including NK cells, T cells, B cells and Myeloid cells were observed in the recipient mice (Figure 4G, supplementary figures 9B and 9C). These data demonstrated that pMXs-7TF MEFs exhibit the potential to differentiate into both the lymphoid and myeloid lineages.

hCD34 expression in mouse LT-HSCs

To further confirm the reprogramming capacity of pMXs-7TF-MEFs, we used transgenic (tg) mice carrying the hCD34 locus and 12.8 kb of 5' and 25.6 kb of 3' flanking regions, which are necessary to direct hCD34 expression exclusively in LT-HSCs [27], such that pMXs-7TF MEFs regeneration could be monitored based on hCD34 expression. MEFs obtained from hCD34 tg mice were transduced with a retrovirus carrying the candidate genes Oct 4, Sox2, Klf4, c-Myc, LMO2, c-Fos and c-Myb. After obtaining pMXs-7TF MEFs from the hCD34 tg-derived MEFs, the expression of HSC markers was analyzed. As shown in supplementary figure 10A, hCD34$^+$ cells were observed in the LT-HSC population. We then performed qPCR and RT-PCR to verify the expression of hCD34 and the HSC marker genes. c-Kit, Sca-1, CD150 and hCD34 were highly expressed in the pMXs-7TF-transduced cells, as expected; however, hCD34 was not expressed in the pMXs-transduced wild type or hCD34 tg MEFs, ES cells, or HSCs (Supplementary figures 10B and 10C). These results confirmed that pMXs-7TF MEFs are similar to primitive HSCs with respect to hCD34 gene expression.

Discussion

In this study, we demonstrated that MEFs can be converted to mouse HPCs via the expression of Lmo2, c-Fos, c-Myb, Oct4, Sox2, Klf4 and c-Myc. First, the reprogrammed pMXs-7TF MEFs expressed HSC surface markers, such as c-Kit and Sca-1, and they produced detectable long-term HSC, short-term HSC and MPP cell populations. Second, the pMXs-7TF MEFs were maintained *in vitro* for extended periods. To confirm whether pMXs-7TF MEFs can undergo long-term expansion, the pMXs-7TF MEFs were cultured in Myelocult long-term culture medium supplemented with hematopoietic cytokines. After two months, we observed that the LKS cell population remained present in the pMXs-7TF-transduced cells. Third, the gene expression profiles of the pMXs-7TF MEFs were nearly identical to those of primitive HSCs. HSC surface markers (c-Kit, Sca-1, CD34 and CD150) and transcription factors (c-Jun, Lmo2, c-Fos, c-Myb, c-Myc and Trim28) that are highly expressed in HSCs were strongly expressed in pMXs-7TF-transduced cells, although the pluripotent cell markers SSEA, Nanog and E-Ras were not detectable in these cells. Fourth, the pMXs-7TF MEFs displayed the potential to differentiate into multiple lineages *in vivo* and *in vitro*. When the pMXs-7TF MEFs were injected into lethally irradiated congenic or immunodeficient mice, they exhibited potent reconstitutive capacities. Moreover, the pMXs-7TF MEFs differentiated into both the lymphoid and myeloid lineages, which perform distinct functions. Taken together, these results demonstrated that pMXs-7TF MEFs can be reprogrammed using specific genes without the need to generate iPS cells and that these pMXs-7TF MEFs display phenotypic and functional characteristics of HPCs.

According to the SAGE dataset, the expression of c-Jun, Lmo2, c-Fos, c-Myb and c-Myc were selectively elevated in HSCs. These TFs are necessary for the regulation of HSC properties such as self-renewal and differentiation potential. c-Myc is one of the transcription factors important for HSC differentiation and survival [39,40] and regulates the balance between the self-renewal and differentiation of HSCs [41]. Lmo2 and c-Myb are critical for the development of HSCs [42-44], and Lmo2 in particular is necessary for promoting a hematopoietic fate in embryonic fibroblasts [23]. c-Fos, a member of the AP-1 transcription factor, in a complex with c-Jun protein regulates expression of AP-1 binding genes [45]. AP-1 transcription factor plays multiple roles in development of hematopoietic precursor cells into mature blood cells including the granulocyte monocyte and erythroid lineages [46,47]. c-Fos is highly expressed in HSCs [48] and is important for cell growth and the G0/G2 transition in HSCs. According to recent study, c-Fos promotes endothelial and hematopoietic gene expression [20]. In the present study, we identified a combination of HSC-specific transcription factors that induce the conversion of MEFs to HPCs in the presence of Oct4, Sox2 and Klf4.

In conclusion, the combination of HSC-specific transcription factors such as Lmo2, c-Fos and c-Myb and pluripotency-related genes such as Oct 3/4, Sox2, Klf4 and c-Myc can induce the direct conversion of multi-potent iHSCs from MEFs. The present study demonstrates the iHSCs have the potential to differentiate into immune cells and to repopulate hematopoiesis *in vivo*. The success of iHSC generation from the patient's own somatic cells will facilitate the treatment of several diseases and can be used instead of HSCs. This method will accelerate the clinical application of iHSC generation as a platform technology for regenerating patient-specific HSCs.

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Author Contributions

H.Y.S. designed the experiments and wrote the paper. H.Y.S., H.W.S., Y.K.K., W.K. and S.Y. performed the experiments. E.L. generated the hCD34 transgenic mice. E.L., H.J., S.R.Y., T.D.K., and Y.J.P. provided critical comments. I.C. supervised the project and wrote the paper.

Conflicts of Interest Disclosure

The authors declare no competing financial interests.

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