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OP9-Lhx2 stromal cells facilitate derivation of hematopoietic progenitors both in vitro and in vivo☆

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ABSTRACT

Generating engraftable hematopoietic stem cells (HSCs) from pluripotent stem cells (PSCs) is an ideal approach for obtaining induced HSCs for cell therapy. However, the path from PSCs to robustly induced HSCs (iHSCs) in vitro remains elusive. We hypothesize that the modification of hematopoietic niche cells by transcription factors facilitates the derivation of induced HSCs from PSCs. The Lhx2 transcription factor is expressed in fetal liver stromal cells but not in fetal blood cells. Knocking out Lhx2 leads to a fetal hematopoietic defect in a cell non-autonomous role. In this study, we demonstrate that the ectopic expression of Lhx2 in OP9 cells (OP9-Lhx2) accelerates the hematopoietic differentiation of PSCs. OP9-Lhx2 significantly increased the yields of hematopoietic progenitor cells via co-culture with PSCs in vitro. Interestingly, the co-injection of OP9-Lhx2 and PSCs into immune deficient mice also increased the proportion of hematopoietic progenitors via the formation of teratomas. The transplantation of phenotypic HSCs from OP9-Lhx2 teratomas but not from the OP9 control supported a transient repopulating capability. The upregulation of *Apln* gene by Lhx2 is correlated to the hematopoietic commitment property of OP9-Lhx2. Furthermore, the enforced expression of *Apln* in OP9 cells significantly increased the hematopoietic differentiation of PSCs. These results indicate that OP9-Lhx2 is a good cell line for regeneration of hematopoietic progenitors both in vitro and in vivo.

1. Introduction

Induced hematopoietic stem cells (iHSCs) derived from either pluripotent stem cells (PSCs) or somatic cells are essential to cell therapy based on HSC transplantation. However, the derivation of iHSCs with physiologic functions after engraftment has remained a significant challenge (Slukvin, 2013). Several bottlenecks, including the key conditions of hematopoietic specification, maturation, maintenance, and expansion, must be addressed (Shepard and Talib, 2014). The ectopic expression of intrinsic regulatory factors has been hypothesized to directly convert somatic cell types to hematopoietic stem cells. As a result, HSC-like cells have been obtained via the conversion of pro-B (Riddell et al., 2014) and PSC-derived hematopoietic progenitors (Doulatov et al., 2013). In addition to some defects, these iHSCs may constitute a tumorigenic risk due to the enforced expression of factors via lenti- or retro-viral transduction approaches.

To date, the in vitro niche modeling of hematopoietic development has failed to yield functional HSCs from PSCs (Kennedy et al., 2012), indicating that current extrinsic niche factors are not sufficient for the derivation of iHSCs from PSCs in vitro without the direct combination of manipulating intrinsic factors. Recently, iHSCs were identified to result from intra-teratoma hematopoiesis with low efficiency (Amabile et al., 2013; Suzuki et al., 2013). The efficiency of iHSCs derived from teratomas was further increased by combining the OP9- and OP9-derived-cell lines as well as hematopoietic cytokines. Thus, teratoma hematopoiesis constitutes a new 3-D in vivo model for studying niche factors for the derivation of iHSCs from PSCs.

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Lhx2 is a LIM homeobox gene that plays important roles in eye, forebrain, and definitive erythrocyte development. Knocking out Lhx2 leads to severe anemia in a cell non-autonomous manner (Porter et al., 1997). The enforced expression of Lhx2 in hematopoietic progenitors from either PSCs (Pinto do et al., 1998) or adult mouse bone marrow (Pinto do et al., 2002) results in SCF-dependent cell lines via a cell non-autonomous mechanism (Pinto do et al., 2001). PSCs that overexpress Lhx2 can produce HSC-like cells via OP9/PSCs co-culture; however, the related mechanism remains elusive (Kitajima et al., 2011). In addition to Lhx2, multiple transcription factors, including Fos, Tcfec, Hmgb1, and Sfpi1, can enhance the repopulating activity of HSCs in a cell non-autonomous manner (Deneault et al., 2009). We hypothesize that certain transcription factors can enhance the hematopoietic specification of PSCs in a cell non-autonomous manner. We report for the first time that the enforced expression of Lhx2 in OP9 stromal cells promotes the production of hematopoietic progenitors both in vitro and in vivo. OP9-Lhx2 facilitates hematopoietic specification partially via the upregulation of the *Apln* gene. Thus, the OP9-Lhx2 cell line can be widely used for hematopoietic derivation from PSCs, both in vitro and in vivo.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice and NOD/SCID mice were purchased from Vital River Laboratories (Beijing, China) and housed in the specific-pathogen-free (SPF)-grade experimental animal center of Guangzhou Institutes of Biomedicine and Health (GIBH). All experiments were conducted with the ethical approval of the Animal Ethics Committee of GIBH.

2.2. Cell lines and culture condition

OP9 cells were maintained in α-MEM medium (Gibco) supplemented with 20% fetal bovine serum (Gibco). hPSCs (National Institutes of Health code: WA01) were maintained in the feeder-free culture medium mTeSR™1 (Stemcell Technologies). OP9 cells and hPSCs were co-cultured in differentiation medium consisting of α-MEM medium supplemented with 10% defined fetal bovine serum (Hyclone) and monothioglycerol (100 μmol/L).

2.3. In vitro hematopoietic differentiation of hPSCs through stromal cell co-culture

OP9-GFP/OP9-Lhx2/OP9-*Apln* cells ($8–10 \times 10^5$) were plated in 100 mm dishes. When the OP9 cells reached confluence, hPSCs were plated onto the related stromal cell layer ($4–6 \times 10^6$ ES cells/dish) and cultured with differentiation medium. On day 9, $CD34^+$ cells were enriched using immunomagnetic beads and re-plated to a new dish containing confluent OP9 cells. BMP4 (20 ng/ml), SCF (50 ng/ml), TPO (20 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), and Flt3-L cytokines (50 ng/ml) were added to the culture. Half of the differentiation medium was replaced every other day. On day 18, the cells were collected and analyzed by flow cytometry.

2.4. Teratoma formation and single cell preparation

For mouse teratoma formation, 1×10^6 mPSCs and 1×10^6 OP9-GFP/OP9-Lhx2 cells were mixed with Matrigel and co-injected into the inguen of NOD/SCID mice (6–8 weeks old). For human teratoma formation, 4×10^6 hPSCs and 1×10^6 OP9-GFP/OP9-Lhx2 cells were mixed with Matrigel and co-injected into the inguen of NOD/SCID mice (6–8 weeks old). Single teratoma cells were prepared as described with minor modification (Amabile et al., 2013). Briefly, teratomas were minced into small pieces and dissociated with the gentleMACS™ system (Miltenyi Biotec). The dissociated cells were further digested in 0.2% collagenase IV (Gibco) and 60 U/ml DNAse (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for 40 min. The cells were then successively filtered through 70 μm and 30 μm strainers. Dead cells were depleted via low-speed centrifugation (100 g, 10 min, 4 °C) in ammonium-chloride-potassium lysing buffer. The single live cells were then resuspended in 2% FBS for further phenotypic and functional analyses.

2.5. Flow cytometry analysis and cell sorting

Human antibodies were purchased from BD Biosciences, and mouse antibodies were purchased from eBioscience. The following antibodies were added for human blood cell analysis: FITC-conjugated anti-mCD45 (30-F11), FITC-conjugated anti-mTER-119 (TER-119), PE-Cy7-conjugated anti-humanCD45, APC-conjugated anti-humanCD34, and APC-Cy7-conjugated anti-humanCD38. The following antibodies were added for mouse blood cell analysis: APC-Cy7-conjugated anti-mCD45.1 and anti-mTER-119, PE-conjugated anti-mCD45.2 (104), APC-conjugated anti-mCD150 (TC15-12F12.2), and PE-Cy7-conjugated anti-mCD48 (HM48-1). The following antibodies were added to analyze the lineage of cells engrafted in the recipients: FITC-conjugated anti-mCD45.1, PE-conjugated anti-mCD45.2 (104), APC-conjugated anti-mB220, APC-cy7-conjugated mCD11b (M1/70), and PE-Cy7-conjugated anti-mCD3e (500A2). After washing, the cells were stained with 2 μg/ml DAPI in 10% FBS. The cells were analyzed or sorted on a BD LSRFortessa™ MoFlo Astrios (Beckman Coulter) and BD FACSAria™ II sorter (BD Biosciences) at the flow cytometry core facility of the instrument center of GIBH.

2.6. Colony-forming assay

Hematopoietic progenitors derived from either in vitro co-culture or in vivo teratoma formation were suspended in 3.3 ml of methylcellulose medium (MethoCult H4434, Stem Cell Technologies) and plated in triplicate in 35 mm dishes at 37 °C in a humidified atmosphere of 5% CO_2 for 10–14 days. Colonies of more than 50 cells were scored as positive clones.

2.7. RNA-seq

Two micrograms of RNA from the OP9-Lhx2 or OP9-GFP control sample was used to create libraries for deep sequencing according to the manual of the TruSeq RNA Sample Preparation Kit (Illumia). The samples were sequenced using the Illumina Myseq sequencer. Sequence reads were processed and analyzed by the bioinformatics core of GIBH.

2.8. Statistical analysis

All quantitative analyses were based on a minimum of three replicates. Data are presented as means ± SDs. Student's t tests were performed using the statistic software GraphPad Prism (GraphPad Software).

3. Results

3.1. OP9-Lhx2/PSCs co-culture enhances the production of hematopoietic progenitor cells

To generate a OP9-Lhx2 cell line that stably overexpressed mouse Lhx2, Lhx2 cDNA (OriGene) was subcloned into pMYs-IRES-EGFP (RTV-021, Cell Biolabs, INC) to generate the pMYs-Lhx2-IRES-EGFP vector. OP9 cells were transduced with Lhx2 or GFP retrovirus packaged using plat-E cells via calcium phosphate-mediated transfection as described previously (Yang et al., 2015). As shown in Fig. S1A, the GFP+ OP9 cells reached 100% after two rounds of viral transduction. To confirm the ectopic expression of Lhx2, we first carried out quantitative

RT-PCR. The mRNA level of Lhx2 was more than ten thousand times higher in OP9-Lhx2 than in OP9 cells transduced with the GFP control (Fig. S1B). We conducted a western blot assay using anti-Lhx2 monoclonal antibody to further confirm the expression level of Lhx2 at the protein level (N-20, Santa Cruz Biotech). A 55 kDa band was specifically detected in the OP9-Lhx2 sample but was absent in the OP9 control sample (Fig. S1C). The overexpression of Lhx2 in mouse bone marrow cells produced SCF-dependent cell lines (Pinto do et al., 2002), which is a feature of Lhx2 function. To functionally confirm Lhx2 expression, we also cultured Lhx2-transduced mouse bone marrow cells in medium containing mSCF. As expected, the ectopic expression of Lhx2 in mouse bone marrow allowed the cells to replicate indefinitely in the presence of the cytokine mSCF (Fig. S1D). Thus, we successfully overexpressed functional Lhx2 in OP9 cells.

To investigate whether the OP9-Lhx2 line could promote the hematopoietic differentiation of PSCs, we first carried out an in vitro OP9-Lhx2/PSCs co-culture assay (Fig. 1A). Five million PSCs (H1) were seeded onto an OP9 monolayer in a 100 mm culture dish. The mixed cells were cultured in differentiation medium consisting of α-MEM medium supplemented with 10% defined fetal bovine serum (Hyclone) and monothioglycerol (100 μmol). After 9 days of co-culture, $CD34^+$ cells were enriched with immunomagnetic beads and re-plated onto an OP9 monolayer for a second round of co-culture in differentiation medium supplemented with 20 ng/ml BMP4, 50 ng/ml SCF, 20 ng/ml TPO,

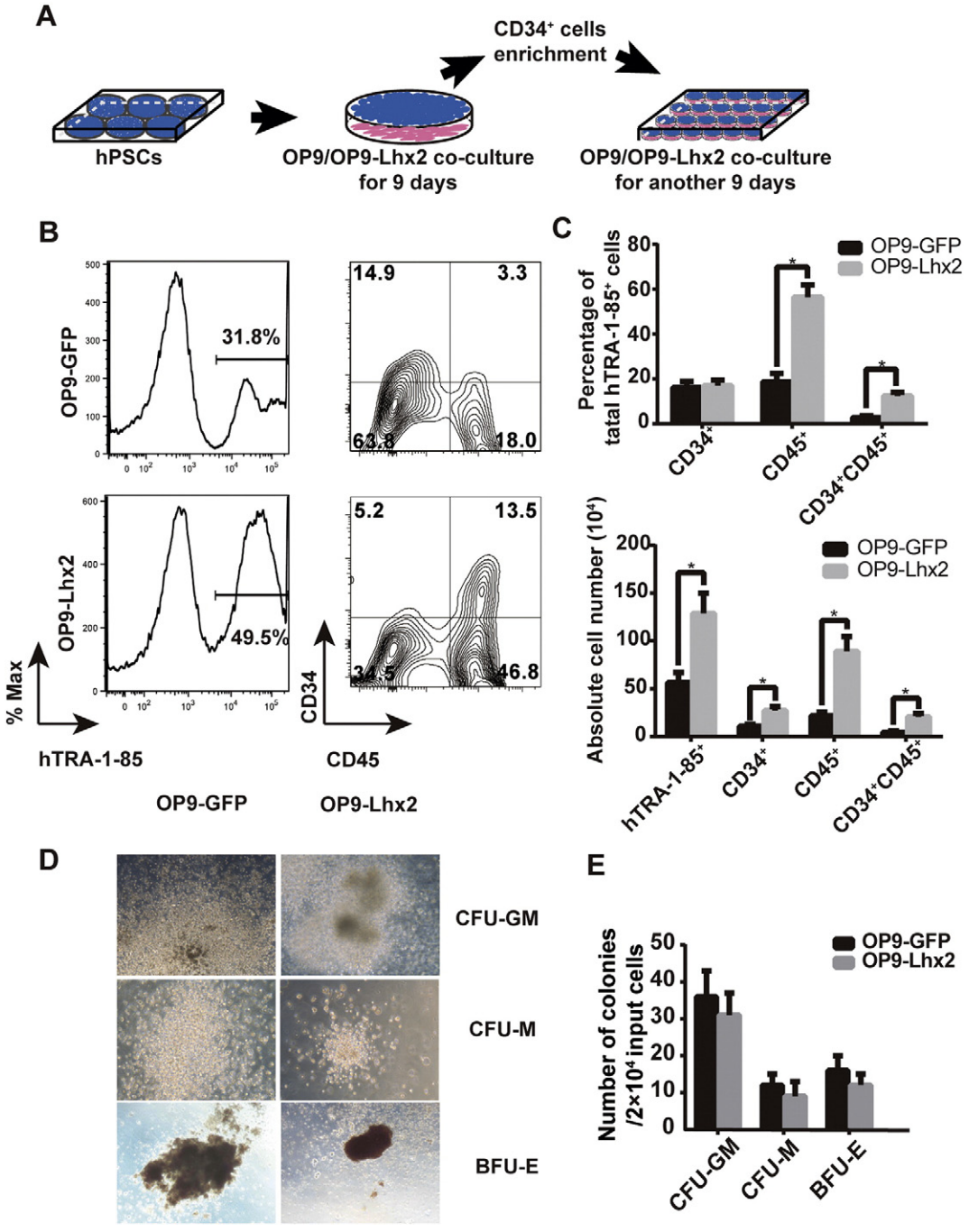

Fig. 1. OP9-Lhx2 enhances generation of ESCs-derived hematopoietic cells. (A) Strategy for hematopoietic differentiation by ESC (H1 line)/stromal cell co-culture. (B) Phenotypic identification of hematopoietic cells by FACS analysis. Dead cells and debris were excluded. (C) Statistical analysis of hematopoietic cells derived from co-culture system. Each column represents the mean of triplicate wells. Data are shown as means + SDs (*$p < 0.05$). (D), (E) Colony-forming assay of $CD34^+$ cells derived from co-culture. The hematopoietic progenitors from OP9-Lhx2/ESCs and OP9-GFP/ESCs co-culture formed CFU-GM, CFU-M and BFU-E colonies.

20 ng/ml IL-3, 20 ng/ml IL-6, and 50 ng/ml Flt3-L. Round and bright blood cells began to become evident at day 15, and they were robust by day 18. To identify the hematopoietic cells, we analyzed the phenotypes of hematopoietic cells using flow cytometry. Consistent with our hypothesis, OP9-Lhx2/PSCs co-culture produced significantly more hematopoietic progenitors ($CD45^+CD34^+$) and mature blood cells ($CD45^+CD34^-$) than the counterparts derived from OP9/PSCs co-culture (Fig. 1B, C). We further performed a colony-forming-unit assay to evaluate the function of hematopoietic progenitors derived from our co-culture system. Both hematopoietic progenitors derived from OP9-Lhx2/PSCs and OP9/PSCs yielded multiple colony types, including CFU-GM, CFU-M and BFU-E (Fig. 1D, E). Thus, OP9-Lhx2/PSCs co-culture efficiently produced multipotent hematopoietic progenitors.

3.2. OP9-Lhx2 facilitates the derivation of murine and human hematopoietic progenitors in vivo via teratoma formation

To investigate the ability of the OP9-Lhx2 line to facilitate the derivation of murine hematopoietic progenitors with engraftment potential, we used a teratoma formation model to produce hematopoietic cells in a 3-D in vivo microenvironment (Fig. 2A). We first co-injected one million OP9-Lhx2 cells with one million mouse PSCs ($tdTomato^+CD45.2^+$)

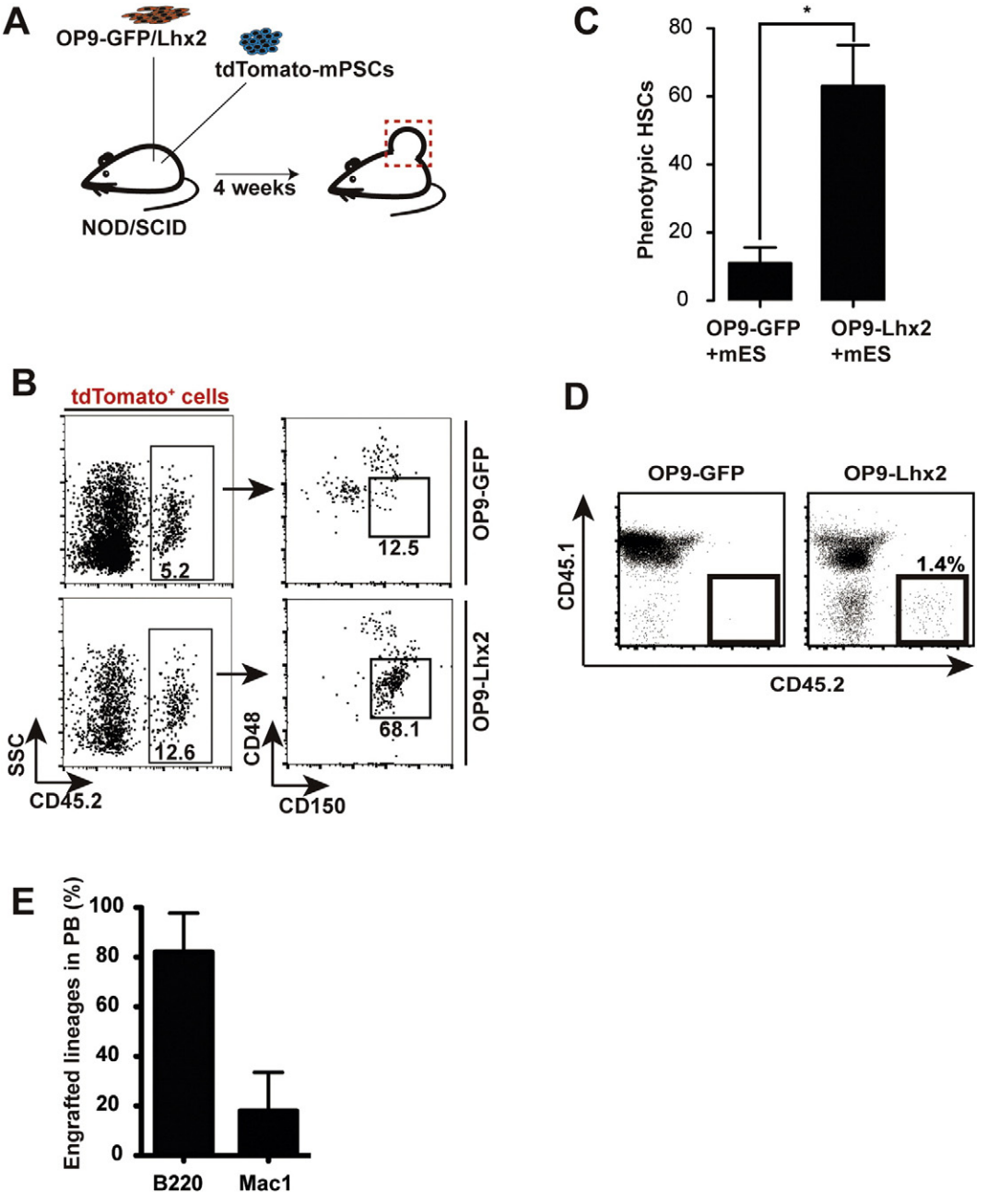

Fig. 2. OP9-Lhx2 facilitates derivation of murine hematopoietic progenitors in vivo via teratoma formation. (A) Strategy for producing hematopoietic cells in vivo via teratoma formation. (B) OP9-Lhx2/ESCs (C57/B6 background, $CD45.2^+$)-formed teratomas produced a higher proportion of hematopoietic progenitors compared with OP9-GFP/ESCs teratoma controls. Teratoma-derived blood cells were defined as $tdTomato^+CD45.2^+$ cells; teratoma derived hematopoietic progenitors were defined as $tdTomato^+CD45.2^+CD150^+CD48^-$ cells. Plots show the analysis of representative teratomas of two groups. (C) Statistic analysis of phenotypic HSCs. Teratomas from three teratoma-bearing mice from each group were analyzed. Data are shown as mean + SD (*$p < 0.01$). (D) Hematopoietic progenitors derived from OP9-Lhx2/mESCs teratoma are transiently transplantable. Five hundred hematopoietic progenitors ($tdTomato^+CD45.2^+CD48^-CD150^+$) were transplanted into each lethally irradiated recipient ($CD45.1^+$) via an injection into the femur cavity. A flow cytometric analysis of peripheral blood (PB) detected a low engraftment of OP9-Lhx2/mESCs teratoma-derived hematopoietic cells ($CD45.2^+CD45.1^-$) four weeks after transplantation. (E) Lineages analysis of engrafted cells in peripheral blood in recipients ($n = 3$) one month after transplantation.

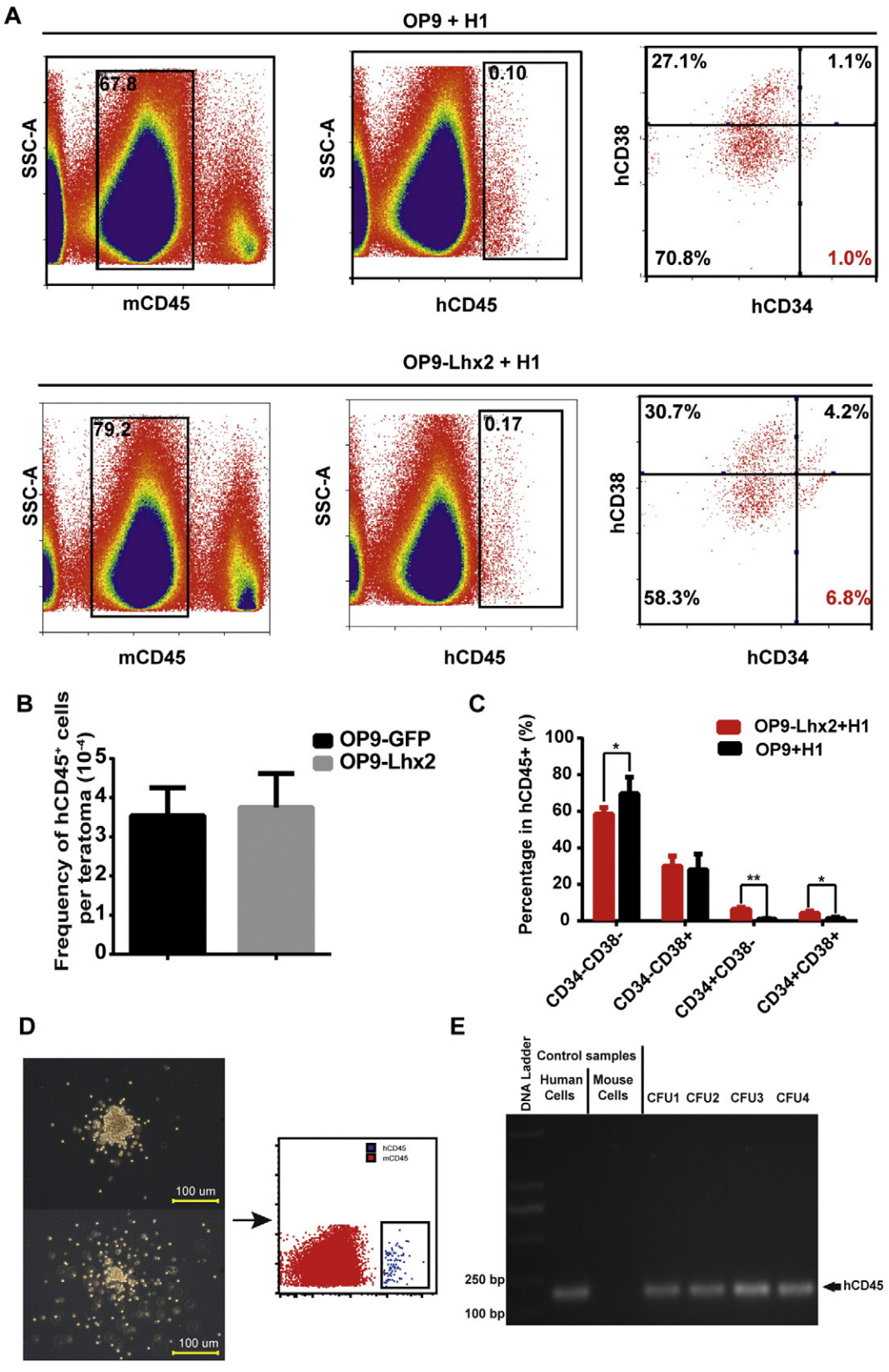

Fig. 3. OP9-Lhx2 facilitates the derivation of human hematopoietic progenitors in vivo via teratoma formation. (A) Flow cytometry analysis of human hematopoietic progenitors derived from OP9/hESCs and OP9-Lhx2/hESCs teratomas. An analysis was conducted 8–10 weeks after ESC injection. Human hematopoietic progenitors were phenotypically defined as $mCD45^-$ $hCD45^+hCD34^+hCD38^-$ cells. Representative plots show the gating strategy of teratoma-derived live blood cells. (B) Frequencies of $hCD45^+$ cells in teratomas. Single live cells prepared from teratomas ($n = 3$) were analyzed. (C) Statistic analysis of hematopoietic cells derived from three representative teratomas of each group in the same batch experiments. Data are shown as means + SDs ($^*p < 0.05$). (D) Human teratoma-derived hematopoietic progenitors gave rise to colonies in the CFU assay. Flow cytometry analysis confirmed that the cells from CFU colonies were human blood cells. Colonies were selected and mixed with 0.25 million mouse bone marrow cells ($mCD45^+$) to protect the few human cells ($hCD45^+$). (E) PCR confirmed the human origin of CFU colonies. Four representative colonies from the CFU assay were picked up. Then cDNAs of individual colonies were used for PCR to detect human CD45 gene.

into NOD-SCID recipients ($CD45.1^+$). Four weeks later, the teratomas were separated from the teratoma-bearing mice. To assess the production of hematopoietic progenitors, we analyzed the single live cells derived from teratomas by flow cytometry (Fig. 2B). To deplete mouse blood contaminants from teratoma-bearing mice, we first gated out tdTomato-negative cells. Teratoma derived hematopoietic cells were further defined as $tdTomato^+CD45.2^+$ cells. We further combined CD150 and CD48 surface markers to define the HSC-enriched population ($CD150^+CD48^-$). Interestingly, teratomas from OP9-Lhx2/PSCs produced much more hematopoietic progenitors than teratomas from OP9/PSCs (Fig. 2C). To verify the engraftment potential of hematopoietic cells derived from teratoma formation, $CD150^+CD48^-$ progenitor cells were sorted from teratomas and transplanted into lethally irradiated mice. One month after transplantation, the peripheral blood of each recipient was analyzed for engraftment. Hematopoietic progenitors from some OP9-Lhx2/PSCs teratomas showed low levels of engraftment in the peripheral blood (Fig. 2D). However, hematopoietic progenitors from OP9/PSCs teratomas (0/20) showed no engraftment. On average, hematopoietic progenitors from 15% OP9-Lhx2/PSCs teratomas showed transient engraftment potential, but none produced long-term engraftable blood cells. A further phenotypic analysis indicated that hematopoietic progenitors from OP9-Lhx2/PSCs teratomas reconstituted myeloid and B lymphoid lineages but lacked T cells (Fig. 2E).

To investigate whether OP9-Lhx2 also enhances human hematopoietic differentiation in vivo, we analyzed hematopoiesis in OP9-Lhx2/hPSCs teratoma formation. Four million human PSCs and one million OP9-Lhx2 or OP9 control cells were co-injected into the inguen of NOD-SCID mice. Seven to nine weeks after co-injection, single cells were prepared from the teratomas for flow cytometry analysis. To exclude mouse blood cell contaminants, we first gated out $mCD45.1^+$ cells. We then used cord blood white cells as a positive control to gate the human $CD45^+$ cells in teratoma single cells. Human blood cells from teratoma were further defined as $hCD45^+mCD45^-mTER\text{-}119^-$ cells. To assess whether OP9-Lhx2/hPSCs teratomas produce a higher proportion of human hematopoietic progenitors, we combined the lineage marker CD38 and hematopoietic stem/progenitor marker CD34 in subsequent analyses. Significantly more hematopoietic progenitors ($CD38^-CD34^+$) were derived from OP9-Lhx2/hPSCs teratomas compared with the counterparts derived from the OP9/hPSCs control (Fig. 3A-C). To further evaluate the function of human hematopoietic progenitors derived from teratoma hematopoiesis, we sorted the $CD34^+CD38^-$ cells and carried out a CFU assay. CFU colonies were evident in the culture dish ten to fourteen days after plating (Fig. 3D). Because human progenitors were rare in teratoma hematopoiesis (~10,000 $CD34^+$ cells per teratoma), we attempted to exclude the possibility that these CFU colonies are contaminants of murine blood cells that arise during the long-term sorting of billions of cells for each teratoma. We selected the cells from each colony and combined them with 0.25 million mouse CD45.1 protector cells for flow cytometry analysis. A population of human blood cells ($mCD45.1^-hCD45^+$) was clearly evident (Fig. 3D). To further confirm the human origin of these colonies, we performed PCR and successfully detected the human CD45 gene using the cDNAs from the picked colonies (Fig. 3E). To investigate the niche of teratoma hematopoiesis, we also performed morphologic examination of teratomas sections. Hematoxylin/Eosin staining demonstrated that blood cells were identified in a bone-marrow-like niche surrounded by cartilage (Fig. S2). However, we failed to detect engrafted human blood cells when we transplanted the $CD34^+CD38^-$

Table 1
Differentially expressed genes in OP9-Lhx2 versus OP9-GFP control cells.

Gene symbol	GenBank accession	Log2 fold change	*p*-value	Biological process
Tnn	NM_177839	Inf	2E-75	Cell-matrix adhesion/cell growth and migration
Megf10	NM_001001979	4.76	5E-61	Cell proliferation/cell differentiation
Nrp2	NM_002077403	4.04	6E-80	Angiogenesis/negative chemotaxis
Sorcs2	NM_030889	3.00	5E-36	Transport
Cdh2	NM_007664	1.92	7E-36	Signal transduction/cell adhesion and migration
Adam8	NM_001291066	1.88	6E-11	T cell differentiation in thymus/cell adhesion
Tfrc	NM_011638	1.66	5E-39	Osteoclast differentiation/bone resorption
Apln	NM_013912	1.50	8E-17	Signal transduction/cell proliferation
Procr	NM_011171	1.44	2E-12	Antigen processing and presentation/blood Coagulation
Sema5a	NM_009154	1.40	9E-09	Cell chemotaxis/endothelial cell proliferation
Met	NM_008591	1.34	4E-26	Phosphorylation/cell migration
Adnp	NM_009628	1.27	0.007	Activation of protein kinase/transcription
Emp2	NM_007929	1.17	2E-11	Signaling pathway/cell-matrix adhesion
Ctsl	NM_009984	1.11	4E-29	Cell communication/protein processing
Mrc2	NM_008626	−1.15	2E-25	Collagen catabolic/osteoblast differentiation
Bmp1	NM_009755	−1.18	7E-16	Cell differentiation/proteolysis
Fgfr2	NM_010207	−1.24	7E-08	Angiogenesis/bone development
Thbs1	NM_011580	−1.27	2E-23	Cell adhesion/part of the HSC niche
Itgfb3	NM_016780	−1.33	4E-10	Cell adhesion/signaling pathway
Vcam1	NM_011693	−1.35	4E-15	Cell adhesion/T cell proliferation
Lrp6	NM_008514	−1.59	2E-12	Wnt-activated receptor activity/protein binding
Thbs2	NM_011581	−1.68	3E-70	Cell adhesion/angiogenesis
Jag1	NM_013822	−1.81	7E-25	Notch signaling/T cell mediated immunity
Notch3	NM_008716	−2.34	4E-41	Notch signaling pathway/cell differentiation
Ctsh	NM_007801	−2.60	2E-22	Cell proliferation/apoptotic signaling pathway
Slc1a3	NM_148938	−2.82	3E-16	Metabolic process/neuron differentiation
Sned1	NM_172463	−2.86	5E-109	Cell-matrix adhesion
Hs6st2	NM_001077202	−2.89	5E-137	Transferase activity
Npr3	NM_001039181	−3.03	4E-25	Signaling pathway/osteoclast proliferation
Chst15	NM_029935	−3.61	8E-27	Hexose biosynthetic process
Clec2d	NM_053109	−3.64	2E-24	Osteoclast differentiation/signal transduction
Mest	NM_001252292	−4.54	6E-86	Regulation of lipid storage
Itga11	NM_176922	−5.12	2E-72	Cell adhesion/signaling pathway
Epha4	NM_007936	−6.09	3E-54	Cell adhesion/signaling pathway
Aoc3	NM_009675	−6.21	5E-59	Leukocyte migration/cell adhesion

cells into immune-deficient mice (data not shown). Thus, OP9-Lhx2 facilitated the derivation of murine/human hematopoietic progenitors in vivo via teratoma hematopoiesis.

3.3. OP9-Lhx2 enhances hematopoietic fate partially via the upregulation of *Apln*

To investigate the underlying molecular effectors that facilitate hematopoietic differentiation by OP9-Lhx2, we carried out RNA-seq to obtain the gene expression profile of OP9-Lhx2 and OP9-GFP control cells. Considering the niche cell effect of OP9-Lhx2 on hematopoietic differentiation, we focused on secreted proteins and membrane proteins (SP/MP) that were upregulated or downregulated by Lhx2 in OP9 cells. The GO analysis of the RNA-seq data demonstrated that the ectopic expression of Lhx2 in OP9 upregulated 14 SP/MP and downregulated 21 SP/MP genes (Table 1). A quantitative RT-PCR assay further confirmed the differential expression of these genes (Fig. S3).

To identify the key effectors of Lhx2 that improve the efficiency of hematopoietic induction, we first searched the literature for the 35 differentially expressed SP/MP genes. Notably, *Apln* has been reported to promote the hematopoietic differentiation of human embryonic stem cells (Yu et al., 2012). We then evaluated whether the enforced expression of *Apln* in OP9 cells could enhance hematopoietic differentiation. *Apln* cDNA was inserted into the same vector to overexpress the Lhx2 gene. An OP9-*Apln* cell line was established via the retroviral transduction of the *Apln* gene into OP9 cells (Fig. 4A). We then used a OP9-*Apln*/PSCs co-culture to assess the production of hematopoietic cells. As expected, OP9-*Apln* increased the ratio of CD34 positive hematopoietic progenitors compared with the effect observed for the OP9/PSCs co-culture control (Fig. 4B). To confirm the functions of $CD34^+$ cells derived from OP9-*Apln*/ESC co-culture, we performed CFU assays. Our results confirmed that $CD34^+$ cells formed CFU-GM, CFU-G and BFU-E colonies (Fig. 4C, D). Thus, OP9-Lhx2 enhanced hematopoietic fate in part via the upregulation of *Apln*.

4. Discussion

The generation of induced HSCs from PSCs is an ideal approach for overcoming the lack of suitable HSC resources and graft rejection. Attempts to derive robustly induced HSCs from PSCs in recent decades failed due to multiple bottlenecks, including limited approaches for identifying and manipulating the stemness of HSCs in vitro (Shepard and Talib, 2014). In this study, we established a niche cell line, OP9-Lhx2 that can promote hematopoietic derivation using in vitro co-culture and in vivo teratoma formation methods. Interestingly, the ability of OP9-Lhx2 to facilitate the production of hematopoietic progenitors from PSCs is conserved from murine to human PSCs. Previous modifications of OP9 cells directly focused on extrinsic factors that play roles in hematopoietic development (Holmes and Zuniga-Pflucker, 2009; Ichii et al., 2012). In this study, we developed a new approach and showed that the ectopic expression of transcription factors in OP9 cells can also improve the niche to produce hematopoietic progenitors from PSCs. A mechanistic analysis demonstrated that OP9-Lhx2 promotes hematopoietic derivation partially via the upregulation of *Apln*, which has been reported to enhance the hematopoietic specification of PSCs in vitro (Yu et al., 2012). Because Lhx2 regulated the expression level of

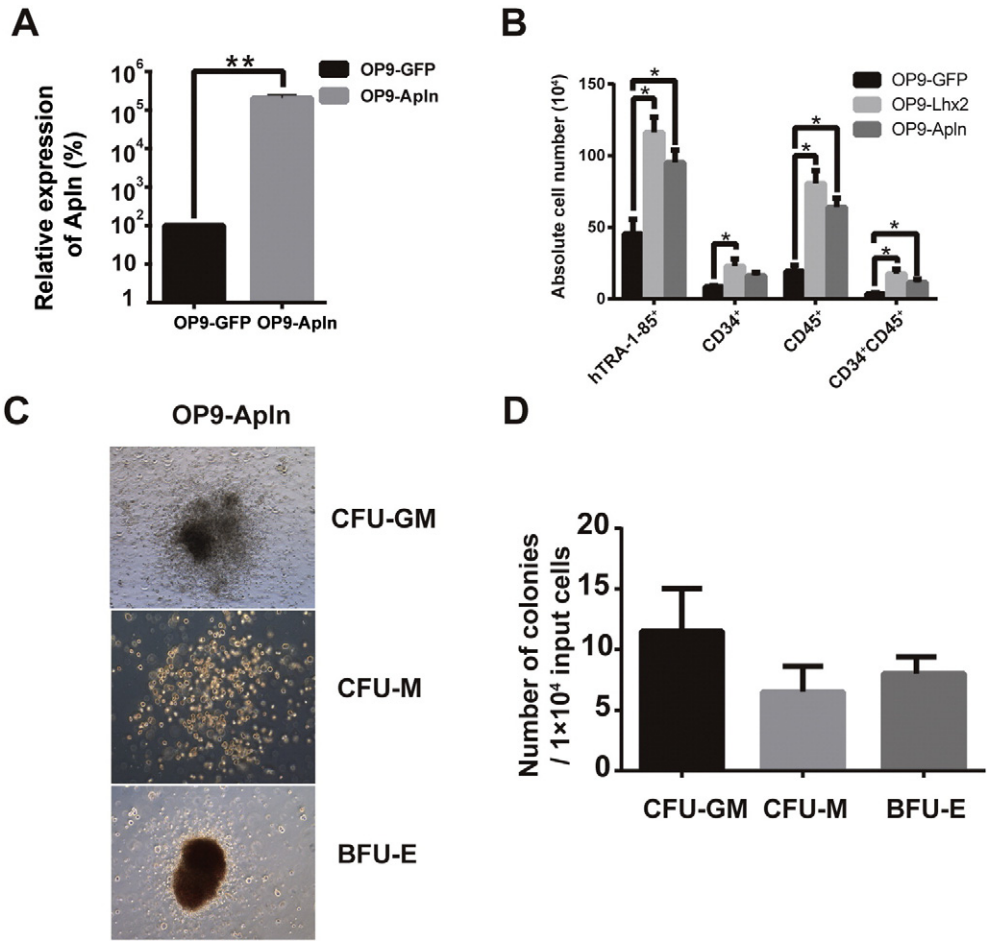

Fig. 4. Enforced expression of *Apln* in OP9 cells promotes generation of ESCs-derived hematopoietic cells. (A) Quantitative PCR of *Apln* expression level in OP9-GFP/OP9-*Apln* stromal cells. (B) Statistical analysis of hematopoietic cells derived from OP9-GFP/ESCs and OP9-*Apln*/ESCs co-culture. Data are shown as means + SDs (*$p < 0.05$, **$p < 0.01$). (C), (D) Colony-forming assay of $CD34^+$ cells derived from OP9-*Apln*/ESC co-culture. The hematopoietic progenitors from OP9-*Apln*/ESCs formed CFU-GM, CFU-M and BFU-E colonies.

several genes involved in cell adhesion/signaling pathways, additional effectors might also enhance the hematopoietic specification by OP9-Lhx2.

Induced HSCs were derived from both mouse and human PSCs via intra-teratoma hematopoiesis, which provides a new tool for investigating the extrinsic and intrinsic factors of hematopoietic development (Amabile et al., 2013; Suzuki et al., 2013). We used this approach to confirm that OP9-Lhx2 promotes the production of both mouse and human hematopoietic progenitors in vivo. The derived hematopoietic progenitors engrafted and reconstituted myeloid and B lymphoid lineages in recipients, but they lacked the potential for T lymphopoiesis. We tested multiple ESC and iPSC lines and excluded the possibility that the defect in the T potential was due to defects in the individual PSC line. Hematopoietic progenitors derived from PSCs have been reported to possess a homing defect. This possibility was excluded in our experiment because engraftments via the intra-medullary tibia injection of teratoma-derived hematopoietic progenitors failed. Hematopoietic progenitors were also detected in the bone marrow of teratoma-bearing mice, but the transplantation of these cells into myeloablated mice again failed to show engraftments. Thus, the hematopoietic progenitors derived from teratoma hematopoiesis in our experiment possessed intrinsic defects and could not self-renew in the long term. In our experimental settings, even the hematopoietic progenitors from individual teratomas showed transient engraftment only in a low ratio of the recipients, which indicated functional variations of hematopoetic cells derived from teratoma hematopoiesis. We detected human blood cells from bulk teratoma cells using FACS analysis and PCR of CFU cells, however, blood cells surrounded by bone-marrow-like niche in teratomas in our experiments are extremely rare, and the identity of human blood cells origin must be further evaluated by situ staining using human CD45 antibodies. In fact, the very limited 0.1%–2% hematopoietic chimerism in the bone marrow with predominant myeloid engraftment in as few as 4% recipients demonstrated that the teratoma hematopoiesis tool must be optimized further (Slukvin, 2013; Amabile et al., 2013; Suzuki et al., 2013).

In conclusion, OP9-Lhx2 cells provide a better niche for the production of hematopoietic progenitors from PSCs, partially via the upregulation of *Apln*. OP9-Lhx2 might be used as a new tool for hematopoietic derivation both in vitro and in vivo. Our strategy might also be used to build additional niche cell lines by screening additional transcription factors that play important roles in hematopoietic development in a cell non-autonomous manner.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.08.009.

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