Role of SOX17 in hematopoietic development from human embryonic stem cells

Yaeko Nakajima-Takagi, Mitsujiro Osawa, Motohiko Oshima, Haruna Takagi, Satoru Miyagi, Mitsuhiro Endoh, Takaho A Endo, Naoya Takayama, Koji Eto, Tetsuro Toyoda, Haruhiko Koseki, Hiromitsu Nakauchi, and Atsushi Iwama

1Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
2RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan.
3RIKEN Genomic Sciences Center, Yokohama 230-0045, Japan.
4JST, CREST, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan.
5Center for iPS Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan.
6Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, the Institute of Medical Science, the University of Tokyo, Tokyo 113-0033, Japan.

Scientific category: hematopoiesis and stem cells
Short title: Role of Sox17 in hematopoietic development

Corresponding author: Mitsujiro Osawa, Ph.D. (mitsujiro.osawa@faculty.chiba-u.jp) and Atsushi Iwama, M.D. Ph.D. (aiwama@faculty.chiba-u.jp), 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Phone: +81-43-226-2187, Fax: +81-43-226-2191
Abstract

To search for genes that promote hematopoietic development from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), we overexpressed several known hematopoietic regulator genes in hESCs/iPSCs-derived CD34^+CD43^- endothelial cells (ECs) enriched in hemogenic endothelium. Among the genes tested, only Sox17, a gene encoding a transcription factor of the SOX family, promoted cell growth and supported expansion of CD34^+CD43^-CD45^-/low cells expressing the hemogenic endothelial marker VE-cadherin. SOX17 was expressed at high levels in CD34^+CD43^- ECs compared to low levels in CD34^+CD43^-CD45^- pre-hematopoietic progenitor cells (pre-HPCs) and CD34^+CD43^-CD45^- HPCs. Sox17-overexpressing cells formed semi-adherent cell aggregates and generated few hematopoietic progenies. However, they retained hemogenic potential and gave rise to hematopoietic progenies upon inactivation of Sox17. Global gene expression analyses revealed that the CD34^+CD43^-CD45^-/low cells expanded upon overexpression of Sox17 are hemogenic endothelium-like cells developmentally placed between ECs and pre-HPCs. Of interest, Sox17 overexpression also reprogrammed both pre-HPCs and HPCs into hemogenic endothelium-like cells. Genome-wide mapping of Sox17 binding sites revealed that Sox17 directly activates transcription of key regulator genes for vasculogenesis, hematopoiesis, and erythocyte differentiation. Depletion of SOX17 in CD34^+CD43^- ECs severely compromised their hemogenic activity. These findings suggest that SOX17 plays a key role in priming hemogenic potential in ECs, thereby regulating hematopoietic development from hESCs/iPSCs.
Introduction

During mammalian development, two waves of hematopoiesis occur in sequential stages: first, a transient wave of primitive hematopoiesis, and then definitive hematopoiesis. These stages are temporally and anatomically distinct, involving unique cellular and molecular regulators. The formation of primitive blood cells occurs early during fetal life, with coordinated progression from extraembryonic to intraembryonic sites of hematopoiesis. Within the embryo, definitive hematopoiesis undergoes developmentally stereotyped transitions; HSCs arising from the aorta-gonad-mesonephros (AGM) region migrate first to the placenta and fetal liver, and then to the spleen. Eventually, hematopoiesis shifts to the bone marrow (BM), where homeostatic blood formation is maintained postnatally.1

During definitive fetal hematopoiesis, hematopoietic stem cells (HSCs) emerge directly from a small population of ECs in the conceptus, referred to as ‘‘hemogenic endothelium’’.2-4 Hemogenic endothelium is located in all sites of HSC emergence including the ventral aspect of the dorsal aorta, vitelline and umbilical arteries, yolk sac, and placenta. The process by which blood forms from hemogenic endothelium involves an endothelial-to-hematopoietic cell transition during which individual cells bud out and detach from the endothelial layer.2-4 Hemogenic endothelium is distinguished from all other endothelial cells by the presence of a transcription factor called Runx1.5 Runx1 is expressed in hemogenic endothelial cells, in newly formed hematopoietic cell clusters, and in all functional HSCs.6,7 A similar process occurs during hemangioblast differentiation in primitive blood cell formation. The extraembryonic yolk sac is considered to be the first site of emergence of “hemangioblast”, which is a mesodermal precursor with both endothelial and hematopoietic potential. Hemangioblasts differentiate into a
hemogenic endothelium intermediate, which gives rise to primitive hematopoietic cells but also definitive hematopoietic cells upon activation of Runx1.8

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have been demonstrated to reproduce many aspects of embryonic hematopoiesis in stromal coculture or embryoid body (EB) culture. A recent study has provided evidence that hematopoietic differentiation of hESCs progresses through sequential stages: first hemogenic endothelium, then primitive hematopoiesis, and finally definitive hematopoiesis, resembling the development of physiological hematopoiesis.9 However, the induction of hematopoietic cells from hESCs/iPSCs is still inefficient. Significant innovations are required before it will be possible to obtain sufficient numbers of the specific types of hematopoietic cells needed for therapeutic uses.

Sry-related HMG (High-mobility group) box 17 (SOX17) is a member of the SOX family of DNA binding transcription factors. Sox17 participates in various developmental processes and biological activities, such as formation of definitive endoderm10 and vascular development.11 Moreover, recent studies have shown that Sox17 also plays an important role in fetal hematopoiesis in the yolk sac and fetal liver, especially in the maintenance of fetal and neonatal HSCs, but not adult HSCs.12 Overexpression of Sox17 has also been shown to confer fetal HSC characteristics onto adult hematopoietic progenitors.13 Among SOX family, Sox7, -17, and -18 are highly related and constitute the Sox subgroup F (SoxF). Of interest, Sox7 and Sox18 are transiently expressed in hemangioblasts and hematopoietic precursors, respectively, at the onset of blood specification. Sustained expression of Sox7 and Sox18, but not Sox17, in early hematopoietic precursors from mouse ES cells and embryos enhances their proliferation while blocking their maturation.14,15 However, the role of Sox17 in early hematopoietic development, particularly from hESCs, has not yet been clarified. In this study, we tested the effect of
overexpression of known hematopoietic regulator genes in hiPSC-derived CD34⁺CD43⁻ endothelial cells enriched in hemogenic endothelium in order to find genes that can be manipulated to efficiently produce hematopoietic cells from hESCs. We found that Sox17 promotes expansion of hemogenic endothelial-like cells. We demonstrate that SOX17 functions in hemogenic endothelium and plays a role in the development of hematopoietic cells from hESCs/iPSCs.
Materials and methods

Cell lines

H1 hESCs (WiCell Research Institute, Madison, WI) and TkCBV4-7 hiPSCs generated from human cord blood CD34+ cells were maintained on irradiated murine embryonic fibroblasts in Dulbecco’s Modified Eagle Medium (DMEM)-F12 (Sigma) supplemented with 1x MEM non-essential amino acids (Gibco-Invitrogen), 1x GlutaMAX-I (Gibco-Invitrogen), 20% knockout serum replacement (KSR) (Gibco-Invitrogen), 0.1 mM 2-mercaptoethanol (2-ME)(Sigma), 1% penicillin/streptomycin solution (Sigma) and 5 ng/mL human basic fibroblast growth factor (bFGF)(ReproCELL). Every 3 to 4 days, the cells were dissected into clumps of about 300–500 cells in a dissociation solution consisting of 0.25% trypsin, 20% KSR, and 1 mM CaCl2 in PBS and transferred to a new feeder layer to maintain them in an undifferentiated state. OP9 stromal cell line was kindly provided by Toru Nakano (Osaka University, Osaka, Japan). OP9 cells were maintained in α-Minimal essential medium (Gibco-Invitrogen) supplemented with 2.2 g/liter sodium bicarbonate, 20% fetal bovine serum (FBS), and 1% L-glutamine, penicillin/streptomycin solution (Sigma).

EB differentiation

H1 hESCs or TkCBV4-7 hiPSCs were dissociated into single cells with Accumax (Innovative Cell Technologies). The cells were washed with DMEM-F12 and recultured at 1x10^6 cells per 60 mm petri dish (FALCON) in 5 ml mTeSR1 (StemCell Technologies) supplemented with 10 μM LY27632 (Cayman), 2 ng/ml human Bone Morphogenetic Protein 4 (BMP4) (PeproTech), 2 ng/ml human Activin A (PeproTech). At day 2 of culture, EBs were split one 60 mm petri dish to
two 60 mm petri dishes, and cultured in EB medium consisting of Iscove modified Dulbecco medium (IMDM)(Sigma) containing 15% FBS, 1x GlutaMAX-I, 1% penicillin/streptomycin solution, 200 μg/ml bovine holo transferrin (BOVOGEN), 50 μg/ml ascorbic acid (Sigma) and 450 μM 1-thioglycerol (Sigma) supplemented with 2 ng/ml human BMP4, and 5 ng/ml human VEGF (PeproTech). At day 4 of culture, medium conditions were changed as described in Figure 1B. LY363947 (Cayman) was used as an inhibitor of TGFβ signaling. EBs were constantly cultured on a shaker at 70 rpm.

**Flow cytometric analysis and OP9 coculture**

EBs were dissociated with 0.25% trypsin-EDTA solution (Sigma) and filtered through a nylon screen to obtain a single cell suspension. Flow cytometric analysis and cell sorting were performed using FACSIAriaII (BD Biosciences) and the data was analyzed using FlowJo software (Tree Star). The following antibodies were used for the flow cytometric analysis: CD34 (clone 581, Alexa Fluor 647 or PE-Cy7), CD43 (clone CD43-10G7, PE), CD45 (clone HI30, PE-Cy7), CD11b (clone M1/70, Brilliant Violet 421), CD235a (clone HIR2, PE), CD144 (VE-Cad) (clone 16B1, PE), and CD309 (KDR) (clone HKDR-1, APC). Sorted cells were resuspended in HE medium [IMDM, 10% FBS, 1% L-glutamine and penicillin/streptomycin solution] supplemented with 20 ng/mL human stem cell factor (SCF) and 20 ng/ml human thrombopoietin (TPO)(PeproTech), and transferred onto semiconfluent irradiated OP9 cells. For mature hematopoietic cell differentiation, sorted cells were resuspended in HE medium supplemented with 20 ng/ml SCF, 20 ng/ml TPO, 10 ng/ml human interleukin-3 (IL-3) (PeproTech) and 3 unit/ml human EPO.
Retrovirus and lentivirus vectors, virus production, and transduction

Mouse Sox17 fused to ERT with a 1x or 3x Flag tag was subcloned into the MIG retrovirus vector, which contains the long-terminal repeats (LTRs) from the murine stem cell virus (MSCV) and an internal ribosomal entry site (IRES) upstream of the enhanced green fluorescent protein (GFP) as a marker gene. A recombinant vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped high-titer retrovirus was generated using a 293gpg packaging cell line.16

The virus containing media from the 293gpg cell cultures was concentrated by centrifugation at 6,000g for 16 hours. To knockdown SOX17, lentiviral vectors (CS-H1-shRNA-EF-1α-EGFP) expressing shRNA against human SOX17 and luciferase were prepared. Target sequences were as follows; Sh-SOX17#1143; GCATGACTCCGGTGTGAAT, and Sh-SOX17#1273; GCCCAGAAGCAGTGTTACA. The viruses were produced as previously described.17 EBs at day 5~12 of culture were dissociated and the indicated cell populations were sorted using a FACS AriaII. Sorted cells were seeded onto semiconfluent irradiated OP9 cells and transduced with a SOX17-ERT retrovirus or a SOX17 knockdown virus. Transduced cells were cocultured with OP9 cells in the presence of the indicated cytokines. To induce nuclear translocation of SOX17-ERT, 4-hydroxy tamoxifen (4-OHT) was added to the medium to a concentration of 200 nM on the following day.

Quantitative RT-PCR analysis

Total RNA was extracted using Trizol reagent according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized from total RNA using ThermoScript RT-PCR System (Invitrogen). Quantitative RT-PCR was carried out using FastStart Universal Probe Master (Roche Applied Science), Universal ProbeLibrary (Roche Applied Science), and the Applied
Biosystems 7300 Fast Real-Time PCR system (Applied Biosystems). Primer sequences and probe numbers used are listed in Supplemental methods.

**Colony-forming assay**

Colony assays were performed in methylcellulose (Stem Cell Technologies) containing IMDM supplemented with 20 ng/ml human SCF, 10 ng/ml human IL-3, 10 ng/ml human TPO, and 3 units/ml human EPO, and incubated at 37°C in a 5% CO₂ atmosphere. The number of colonies was evaluated by counting colonies at day 12 of culture.

**Gene expression microarray**

Total RNA was extracted using Trizol reagent according to the manufacturer’s instructions (Invitrogen). Purified total RNA was amplified and labeled using the WT expression kit (Ambion, USA) according to the manufacturer’s instructions. The labeled samples were hybridized to Human promoter Gene 1.0 ST GeneChip arrays (Affymetrix, Santa Clara, CA) to assess and compare overall gene expression profiles as described previously. Microarray data were submitted to Gene Expression Omnibus under accession number GSE38156. Expression profiles of the cells were clustered using hierarchical clustering. Distance between two samples was defined with Pearson's correlation using all or selected probes. Probes were selected using Gene Ontology (GO) database or using ChIP-chip data of Sox17.

**Chromatin-immunoprecipitation on microarray (ChIP-Chip) experiment**

CD34⁺CD43⁻ cells from EBs at day 6 of culture were seeded on irradiated OP9 cells and transduced with a 3x Flag SOX17-ERT retrovirus. The cells were further cultured on OP9 cells in
the presence of SCF and TPO (20 ng/ml) and 200 nM 4-OHT. CD34\(^+\) cells were collected at day 27 of culture by magnetic cell sorting using magnetic beads conjugated with anti-CD34 antibodies (Mylteni Biotech) and subjected to a chromatin immunoprecipitation (ChIP) using an anti-FLAG antibody (M2, Sigma). ChIP was carried out as previously described.\(^\text{18}\) ChIP on chip analysis was carried out using the SurePrint G3 Human Promoter Kit, 1x1M (G4873A, Agilent, Palo Alto, Calif., USA). Purified immunoprecipitated and input DNA was subjected to T7 RNA polymerase-based amplification as described previously.\(^\text{19}\) Labeling, hybridization and washing were carried out according to the Agilent mammalian ChIP-chip protocol (ver.9.0). Scanned images were quantified with Agilent Feature Extraction software under standard conditions. The assignment of regions bound by SOX17 around transcription start sites (TSSs) was carried out using direct sequence alignment on the human genome database (NCBI version 36). The location of SOX17-bound regions was compared with a set of transcripts derived from the MGI database. We assigned bound regions that were within -8.0 kb to +4.0 kb of the TSS. Alignments on the human genome and TSSs of genes were retrieved from Ensembl (http://www.ensembl.org). Intensity ratios (IP/input: fold enrichment) were calculated, and the maximum value for each promoter region of a gene was used to represent the fold enrichment of the gene. Fold enrichment was calculated only for probes whose signals both from IP and input DNA were significant ($P<10^{-3}$). ChIP-chip data were submitted to Gene Expression Omnibus under accession number GSE38156.

**Gene ontology (GO) analysis**

genes were collected from the database and enrichment of SOX17 binding genes was distributed to 2x2 contingency tables for all GO terms (having/not having GO and binding/not binding to SOX17). We calculated P-value of each contingency table using hypergeometric distribution. P-value reflects the likelihood that we would observe the distribution by chance and significant GO terms were selected when the P-value was less than 0.001.

**Immunostaining**

Sox17-ERT transduced cells were sorted by flow cytometry and cultured on MAS-coated glass slides (Matsunami Glass Industry, LTD) for four hours. The cells were then fixed with 2% PFA and immunostained with an anti-laminin antibody (ab11575; abcam) or an anti-FLAG antibody (M2, Sigma)

**Western blotting**

Total cell lysate was resolved by SDS-PAGE and transferred to a PVDF membrane. The blots were probed with an anti-Sox17 antibody (09-038; Millipore) or an anti-α-Tubulin (CP06; Calbiochem) and a horseradish peroxidase-conjugated secondary antibody. The protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo).
RESULTS

Screening of genes that promote hematopoietic development from hESCs/iPSCs

Hematopoietic development from hESCs and hiPSCs recapitulates physiological development, beginning in the conceptus and proceeding in a stepwise manner. CD34+CD43- endothelial cells (ECs) enriched in hemogenic endothelium give rise to the earliest hematopoietic progenitors, pre-hematopoietic progenitor cells (pre-HPCs) with an immunophenotype of CD34+CD43+CD45-. Pre-HPCs then mature into CD34+CD43+CD45+ HPCs that express CD45, a marker antigen specific to hematopoietic cells (Figure 1A).20,21 We improved the conventional culture system to efficiently induce HPCs in EB culture by modifying cytokine conditions and adding an inhibitor of TGF-β signaling (Figure 1B). In our culture system, expression of hematopoietic regulator genes, such as RUNX1 and SCL/TAL1, increased in EBs after day 4 of culture accompanied by the drop in expression of early mesodermal marker genes such as Brachyury (Figure 1C).

To identify genes that promote hematopoietic development from pluripotent stem cells, we transduced hiPSC-derived ECs purified from day 6 EBs with several known hematopoietic regulator genes. We selected 13 genes that are known to play an important role in the development and/or maintenance of HSCs, including RUNX1, Scl/Tal1, Gata2, and HOXB4. The growth of the transduced cells was monitored in the presence of SCF and TPO for 14 days. Unexpectedly, most of these known regulator genes did not promote cell growth, but Sox17 did. A similar effect was observed when we transduced CD34+CD43+ pre-HPCs/HPCs from day 8 EBs (Figure 1D). To confirm these findings, we overexpressed Sox17 in hESC-derived ECs and pre-HPCs/HPCs. Overexpression of Sox17 also promoted cell growth of hESCs as well (data not
shown). Based on these results, we decided to conduct a detailed analysis of the function of SOX17 using hESCs.

**Sox17 promotes expansion of hemogenic endothelium-like cells**

SOX17 mRNA was highly expressed in EBs between days 2 to 4 of culture (**Figure 1E**). SOX17 has been described as one of the master regulator genes for endodermal development.\(^{10,22}\) High expression of SOX17 in EBs at early time points supposedly reflects the development of endodermal cells. In contrast, ECs emerge around day 6 in our culture system at the same time as increased expression of hematopoietic regulator genes such as RUNX1 and SCL/TAL1 (**Figure 1C**). Thus, the expression of SOX17 after day 6 may indicate a role of SOX17 in hematopoietic development (**Figure 1E**). Indeed, SOX17 was expressed at high levels in ECs, but at significantly lower levels in pre-HPCs, HPCs, and human cord blood (CB) CD34\(^+\) cells. (**Figure 1F**). Of interest, other SOXF family genes, SOX7 and SOX18, showed a very similar pattern of expression profiles (**Figure 1F**).

To evaluate the effect of overexpression of Sox17 in hematopoietic development in detail, we produced a retrovirus containing Sox17 fused to ERT (Sox17-ERT). We transduced ECs from day 6 EBs with the Sox17-ERT retrovirus on OP9 stromal cells and cultured them in the presence of SCF and TPO. Addition of 4-OHT, which induces nuclear translocation of ERT fusion protein, considerably stimulated cells growth (**Figure 2A**). Overexpression of Sox17-ERT promoted cell growth moderately even without 4-OHT, suggesting leaky translocation of Sox17-ERT (data not shown). Indeed, Sox17-ERT was detected in both the nucleus and cytoplasm without 4-OHT, while addition of 4-OHT induced efficient nuclear translocation of Sox17-ERT (**Figure S1A**). Of interest, Sox17-overexpressing cells formed semi-adherent cell aggregates on OP9 cells (**Figure**...
Morphological analysis revealed that they showed a monotonous morphology intermediate between ECs and pre-HPCs (Figure 2C). We further performed immunostaining with an anti-laminin antibody. After incubation in slide chambers for four hours, ECs attached to the slide glasses and stretched their cytoplasm out. In contrast, Sox17-overexpressing cells behaved like pre-HPCs and maintained a round shape, suggesting that Sox17-overexpressing cells do not retain strong adhesive properties of ECs, although they form semi-adherent cell aggregates on OP9 cells (Figure S1B). Flow cytometric analysis demonstrated that Sox17-overexpressing cells expanded on OP9 cells were mostly CD34+CD43+ and did not express or expressed a low level of CD45 (CD45−/low). Of note, they co-expressed the hemogenic endothelial marker VE-cadherin (Figure 2D). Interestingly, overexpression of Sox17 in pre-HPCs and HPCs from day 8 EBs similarly expanded CD34+CD43+CD45−/lowVE-cadherin+ cells (Figure 2D). Although the endothelial-specific marker KDR/FLK1 was expressed in the majority of ECs from day 6 and 8 EBs (data not shown), its expression was immediately downregulated during differentiation into pre-HPCs and HPCs, and also upon activation of Sox17 (Figure 2D).

To understand the developmental stage of the cells expanded upon overexpression of Sox17, we performed comprehensive gene expression analyses using microarrays. ECs from day 6 and 8 EBs, pre-HPCs from day 8 EBs, and HPCs from day 8 and 12 EBs were transduced with Sox17-ERT and cultured on OP9 cells. These cells were then treated with 4-OHT, and the resulting CD34+CD43+CD45−/low cells were subjected to microarray analysis. Freshly isolated ECs from day 6, 8, and 12 EBs, pre-HSCs from day 8 EBs, and HPCs from day 8 and 12 EBs served as control samples. The CD34+CD43+CD45−/low cells overexpressing Sox17 appeared to express both endothelial cell-related genes, such as VE-cadherin/CDH5 and ESAM, as well as hematopoietic-related genes, such as RUNX1 and SCL/TAL1 (Table S1). Hierarchical clustering
of the cell populations based on the microarray data of total genes revealed that Sox17-overexpressing cells show very similar profiles of gene expression irrespective of the cell sources (ECs, pre-HPCs, and HPCs) (Figure 3A). We next performed clustering using probes corresponding to genes identified as “Transcription factor” and “Hemopoiesis” from the Gene Ontology (GO) database. Of note, Sox17-overexpressing cells were developmentally placed between ECs and pre-HPCs/HPCs (Figure 3B and C). These findings together with the intermediate morphology between ECs and pre-HPCs suggest that CD34⁺CD43⁺CD45⁻/low cells expanded upon the overexpression of Sox17 are at a developmental stage between hemogenic endothelium and early hematopoietic precursor cells.

To confirm this possibility, we then tested whether the CD34⁺CD43⁺CD45⁻/low cells overexpressing Sox17 give rise to mature hematopoietic cells upon inactivation of Sox17 (Figure 4A). As expected, after depletion of 4-OHT, CD34⁺CD43⁺CD45⁻/low cells lost expression of CD34 and VE-cadherin but gained a higher level of CD45 expression, and gave rise to CD235a⁺ erythroblasts and CD11b⁺ myeloid cells more efficiently than they did in the presence of 4-OHT (Figure 4B and C). This trend was confirmed in colony-forming assays. We seeded CD34⁺CD43⁺CD45⁻/low cells overexpressing Sox17 in methylcellulose media in the presence and absence of 4-OHT. Sox17-overexpressing cells in the presence of 4-OHT mainly formed compact colonies consisting of non-hemoglobinized cells with the morphology similar to endothelial cells as in Figure 2B, while they generated hemoglobinized erythroid colonies and myeloid colonies in the absence of 4-OHT (Figure 4D). These findings clearly indicate that the CD34⁺CD43⁺CD45⁻/low cells overexpressing Sox17 still retain hemogenic potential, which becomes apparent upon the removal of 4-OHT.

We next compared the expression of globin genes in Sox17-overexpressing
CD34+CD43+CD45-\textit{low} hemogenic endothelium-like cells and their hematopoietic progeny to the globin gene expression in CB CD34+ cells. CD34+CD43+CD45-\textit{low} cells expanded upon overexpression of \textit{Sox17} were further cultured in the presence and absence of 4-OHT for 7 days and then GFP+ cells expressing \textit{Sox17-ERT} were collected by cell-sorting. RT-PCR analysis revealed that embryonic type globin (ε) and fetal type globin (γ) but not adult type globin (β) were highly expressed in \textit{Sox17}-overexpressing cells and/or their hematopoietic progeny (Figure \textbf{S2}). These results raise the possibility that the CD34+CD43+CD45-\textit{low} hemogenic endothelium-like cells expanded upon overexpression of \textit{Sox17} are a hemogenic intermediate differentiated from hemangioblasts that primarily give rise to yolk sac-type blood cells.8

\textbf{SOX17 is essential for the hemogenic activity of hemogenic endothelial cells}

Our results so far indicate that the overexpression of \textit{Sox17} promotes the expansion of hemogenic endothelium-like cells, but inhibits their hematopoietic differentiation into pre-HPCs. Because \textit{SOX17} is highly expressed in ECs enriched in hemogenic endothelium, we examined the role of SOX17 by knockdown analysis. We transduced ECs from day 5 EBs with lentiviruses expressing shRNA against \textit{SOX17} on OP9 cells and allowed them to differentiate into hematopoietic cells for 9 days. The most effective shRNA, sh-\textit{SOX17}#1273 (Figure \textbf{5A}), significantly suppressed development and differentiation of hematopoietic cells including both erythroblasts and myeloid cells, whereas it only moderately diminished the growth of CD235a+CD11b- non-hematopoietic cells, the majority of which do not express SOX17, though 25~30\% of these cells are SOX17+ ECs (Figure \textbf{5B and C}). sh-\textit{SOX17}#1143 similarly, albeit modestly, suppressed the production of hematopoietic cells. Similar results were obtained when we knocked down \textit{SOX17} in ECs from day 6 EBs (data not shown). Of interest, however,
hematopoietic differentiation was not affected upon SOX17 knockdown in pre-HPCs from day 8 EBs (Figure 5D). These findings indicate that SOX17 plays a key role in the acquisition of hematopoietic potential in hemogenic endothelial cells.

**SOX17 directly regulates transcription of key regulator genes for hemogenic endothelial cells**

To identify the direct target genes of SOX17 in hemogenic endothelium cells, we conducted a ChIP-chip analysis. We transduced ECs cells from day 6 EBs with a 3xFlag-Sox17-ERT retrovirus and expanded CD34+CD43+CD45-/-low hemogenic endothelium-like cells on OP9 cells. At day 27 of culture, 94.2% of the expanded cells were positive for CD34. CD34+ cells were further enriched (99.8%) by magnetic cell sorting using magnetic beads conjugated with anti-CD34 antibodies, and these purified cells were then subjected to ChIP-chip analysis.

The ChIP-chip analysis was performed with human promoter microarrays containing about 21,000 probe sets covering from -8.0kb upstream to +4.0kb downstream of the TSS of RefSeq genes. 3xFlag-Sox17 was cross-linked to DNA and precipitated using the anti-FLAG M2 antibody. Gene promoters bound by Sox17 were ranked according to fold enrichments calculated in comparison with signals obtained with the input DNA. Of the 19,457 gene promoter regions analyzed, 182 and 98 regions showed Sox17-binding with a fold enrichment greater than 2 and 3-fold, respectively (Full data are listed in Table S2). The functional annotation of the genes bound by Sox17 with a fold enrichment greater than 3-fold was performed based on gene ontology (GO) and showed significant enrichment for genes which fell into categories such as “vasculogenesis”, “hemopoiesis”, and “positive regulation of erythrocyte differentiation” (Figure 6A, Table 1, and Table S2). The genes bound by Sox17 include genes well
characterized as regulators of hematopoietic development from hemogenic endothelial cells, such as VE-cadherin/CDH5, RUNX1, SCL/TAL1, and HHEX (Table 1 and Table S2). VE-cadherin, an endothelial marker antigen, is expressed by hemogenic endothelial cells and by early HSCs, which appear in the YS and the AGM, as well as by a transient HSC population of the fetal liver. RUNX1, SCL/TAL1, and HHEX encode transcription factors essential for the development of HSCs from hemogenic endothelial cells or hemangioblasts. The distribution of SOX17 signals at these genes varied greatly across the promoter region (Figure 6B). SOX17 is reported to bind to the consensus motif of ‘ATTGT’. The VE-cadherin/CDH5 promoter contains the consensus motif between -107bp and -103bp from the TSS. It was recently reported that this site is conserved in mouse and Sox7 directly binds to it to activate transcription. Our ChIP-chip data showed that Sox17 binds to this site as well (Figure 6B, arrowhead). These findings clearly indicate that SOX17 directly regulates expression of a set of key genes for hematopoietic development in hemogenic endothelial cells.

We next performed hierarchical clustering of the cell populations based on the microarray data using “SOX17 targets” which we arbitrarily selected as genes with Sox17 binding greater than 3 fold over the input levels in the ChIP-chip analysis (Table S2). As expected, Sox17-overexpressing cells were again developmentally placed between ECs and pre-HPCs/HPCs (Figure 6C).

Comparison of gene lists between the ChIP-chip and microarray assays

We next examined the changes in expression of the 98 genes bound by Sox17 (>3-fold enrichment in the ChIP-chip analysis) upon Sox17 overexpression. The microarray data of Sox17-overexpressing CD34+CD43+CD45low cells in Figure 3 were compared to those of
respective fresh controls. Sox17 is thought to activate transcription of target genes.\textsuperscript{22} As expected, 36 of the 98 genes showed up-regulation in expression more than 2 fold in at least in one cell type (\textbf{Figure 6D, Table 1 and Table S2}). This tendency was evident in the top 15 genes with Sox17 binding (\textbf{Table 1 and Table S2}), though in 4 genes out of 15, the effects of overexpression of Sox17 were obvious only in pre-HPCs and HPCs, but not in ECs which possess a high level of endogenous SOX17 (\textbf{Table S2}). Similarly, negative correlation was detected between the levels of Sox17 binding in ChIP-chip analysis and fold changes in expression during differentiation of ECs (SOX17\(^+\)) to HPCs (SOX17\(^-\)) (\textbf{Figure 6D}). Among genes bound by Sox17, \textit{EGFL7} as well as \textit{VE-cadherin/CDH5} have been shown to be up-regulated in BM hematopoietic progenitor cells transduced with Sox17.\textsuperscript{13} In our ChIP-chip analysis, however, Sox17 did not show any binding to the genes directly regulated by Sox17 during differentiation of ES cells into extraembryonic endoderm.\textsuperscript{22} Taken together, these data suggest that SOX17 regulates different targets in hematopoietic development and endodermal development.
DISCUSSION

In this study, we found that all of the SOXF subfamily genes, SOX7, -17, and -18, are highly expressed in hESC-derived ECs enriched in hemogenic endothelium and markedly downregulated in pre-HPCs and HPCs to the levels comparable to that in CB CD34+ cells. Overexpression of Sox17 in ECs resulted in expansion of monotonous cells with a CD34+CD43+CD45-/low immunophenotype. These cells co-expressed hematopoietic marker antigens, such as CD43 and a low level of CD45, as well as the hemogenic endothelial marker, VE-cadherin. These unique characteristics of Sox17-overexpressing ECs are reminiscent of hemogenic endothelial cells. Of interest, overexpression of Sox17 inhibited the hematopoietic differentiation of both pre-HPCs and HPCs and reprogrammed them into hemogenic endothelium-like cells. In contrast, depletion of SOX17 in pre-HPCs did not affect their hematopoietic differentiation. These findings suggest that SOX17 is one of the master regulators that define hemogenic endothelium but must be downregulated during the development of pre-HPCs to allow hematopoietic differentiation.

The effects of overexpression of SOX17 in hESC-derived ECs and hematopoietic precursors are very similar to that of overexpression of Sox7 and Sox18 in early hematopoietic precursors from mouse embryo and mouse ESCs. However, in mice, it has been reported that Sox17 remains marginally expressed during blood specification and overexpression of Sox17 in early hematopoietic precursors induces massive apoptosis. The contrasting effects of Sox17 between human and mouse is somewhat surprising but could be partially attributed to the difference in expression during early hematopoiesis as described above. The expression of all the SOXF subfamily genes in ECs evokes the possibility that they have redundant function in the
development of hematopoiesis from hESCs as they do in postnatal angiogenesis in mice.\textsuperscript{11} Nonetheless, the effects of knockdown of Sox7 in mice and SOX17 in this study are different. Sox7 knockdown in Brachyury\textsuperscript{+}Flk1\textsuperscript{−} mesodermal precursors, which give rise upon further differentiation to Flk1\textsuperscript{+} cells containing hemangioblast precursors, profoundly inhibited the production of both hematopoietic progenitors as well as endothelial progenitors, leaving open the possibility that Sox7 inhibits the production of hematopoietic progenitors through inhibiting the formation of hemogenic endothelium or hemangioblasts. In contrast, SOX17 knockdown in ECs enriched in hemogenic endothelium in this study mainly compromised the development of mature hematopoietic cells but only mildly affected the proliferation of non-hematopoietic cells. Furthermore, depletion of SOX17 in pre-HPCs did not significantly affect their hematopoietic differentiation. Therefore, the role of SOX17 at the developmental stage of blood specification could be more specific to the establishment of a hemogenic program in mesodermal or endothelial precursors compared to that of Sox7. Although we did not detect the effects of SOX17 knockdown in pre-HPCs, recent studies have shown that Sox17 also plays an important role in the maintenance of fetal and neonatal HSCs, but not adult HSCs.\textsuperscript{12} Sox17 has also been demonstrated to confer fetal HSC characteristics to adult hematopoietic progenitors.\textsuperscript{13} SOX17 may again exert its critical function at a stage later than the pre-HPC stage, when pre-HPCs/HPCs differentiate into embryonic HSCs.

Of interest, very similar results to ours have been demonstrated in the murine system with the transcription factor HoxA3. HoxA3 is a gene uniquely expressed in the embryonic vasculature, but not in the yolk sac vasculature. HoxA3 restrains hematopoietic differentiation of the earliest endothelial progenitors, and can induce reversion of the earliest hematopoietic progenitors into CD41-negative endothelial cells.\textsuperscript{29} This reversible modulation of endothelial–hematopoietic state
is accomplished by downregulation of key hematopoietic transcription factors. Among these factors, Runx1 is able to erase the endothelial program set up by HoxA3 and promote hematopoietic differentiation. Of note, Sox17 was listed as one of the targets regulated by HoxA3. Given that SOX17 appears to directly regulates the expression of RUNX1 in this study, it could be assumed that HoxA3 functions as an apical regulator of hemogenic endothelium eventually activating the transcription of Runx1 via upregulation of Sox17 in order to initiate hematopoietic differentiation. It would be intriguing to address this question.

The direct targets for Sox17 have been characterized during endodermal differentiation of mouse ESCs using ChIP-chip analysis. The Sox17-binding consensus motif has also been identified using de novo motif analysis from the ChIP-chip data. As expected, the genes bound by Sox17 in Sox17-overexpressing hemogenic endothelium-like cells were quite different from those detected during endodermal differentiation, and were related to the GO terms, “vasculogenesis”, “hemopoiesis”, or “positive regulation of erythrocyte differentiation”. Among them, VE-cadherin/CDH5 encodes one of the well-known marker antigens of hemogenic endothelium and is also expressed by embryonic HSCs. Of note, Sox17 appears to directly bind to the promoters of RUNX1, SCL/TAL1, and HHEX, which encode key transcription factors essential to the development of HSCs from hemogenic endothelial cells or hemangioblasts. Other target genes included BAZF/BCL6B, JUNB, and EGFL7, which encodes a POZ/BTB zinc finger protein, a basic HLH transcription factor, and a secreted angiogenic factor, respectively. These genes have been implicated in vasculogenesis and/or angiogenesis, and BAZF/BCL6B and JUNB have also been implicated in hematopoiesis. The profiles of these Sox17 targets during early hematopoietic development further support the critical role of SOX17 in the regulation of hemogenic endothelium.
Our findings in this study unveiled a novel function of SOX17 in hematopoietic development. Because overexpression of Sox17 expands hemogenic endothelium-like cells, it is possible that conditional expression of SOX17 in hESC-derived endothelial progenitors facilitates hematopoietic development. Therefore, SOX17 could be a novel target for manipulation to improve the yield of hematopoietic progenies from hESCs for regenerative cell therapies.
ACKNOWLEDGEMENTS

We thank Toru Nakano for providing us with OP9 cells, Makiko Yui and Atsunori Saraya for technical assistance, George Wendt for critical reading of the manuscript, and Mieko Tanemura and Akemi Matsumura for laboratory assistance. This work was supported in part by Grants-in-aid for Scientific Research (#21390289 and 23659483) and for the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT, Japan, a Grant-in-aid for Core Research for Evoulutional Science and Technology (CREST) from the Japan Science and Technology Corporation (JST), and grants from the Astellas Foundation for Research on Metabolic Disorders, and the Tokyo Biochemical Research Foundation.

Authorship Contribution: Y. N-T. performed the experiments, analyzed results, made the figures, and actively wrote the manuscript; M. Osawa, M. Oshima, H.T., and S.M. assisted with the experiments including the hematopoietic analyses; M.E., T-E., T.T, and H.K. performed microarray and ChIP-chip analyses; N.T., K.E., and H.N. generated iPSCs, M. Osawa and A.I. conceived of and directed the project, and A.I. secured funding and actively wrote the manuscript.

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


**FIGURE LEGENDS**

Figure 1. Screening of genes that promote expansion of hematopoietic cells from hESCs/hiPSCs.

(A) The hematopoietic fractions derived from hESCs in EB culture used in this study. (B) Schematic representation of the protocol modified for efficient induction of pre-HPCs/HPCs from hESCs/hiPSCs in EB culture. (C) Expression of *BRACHYURY*, *RUNX1* and *TAL1/SCL* expression during differentiation of hESCs in EBs determined by quantitative RT-PCR analysis. mRNA levels were normalized to *GAPDH* expression. Expression levels relative to that in hESCs (day 0 of EB culture) are shown as the mean ± S.D. for triplicate analyses. (D) Cell growth of CD34^+CD43^- cells from day 6 EBs and CD34^+CD43^+ cells from day 8 EBs. EBs were formed by suspension culture of hiPSCs. Sorted cells (2x10^4) were transduced with the indicated hematopoietic regulator genes and cultured on OP9 cells in the presence of 20 ng/ml of SCF and TPO. At day 14 of culture, the absolute numbers of cells were determined and are indicated in bars. Representative data from repeated experiments are shown. (E) Expression of *SOX17* during differentiation of hESCs in EBs determined by quantitative RT-PCR analysis. mRNA levels were normalized to *GAPDH* expression. Expression levels relative to that in hESCs (day 0 of EB culture) are shown as the mean ± S.D. for triplicate analyses. (F) Expression of *SOX17, SOX7* and *SOX18* in bulk EB cells, CD34^+CD43^- cells (ECs), CD34^+CD43^-CD45^- cells (pre-HPCs), and CD34^+CD43^-CD45^+ cells (HPCs) from day 8 EBs determined by quantitative RT-PCR analysis. mRNA levels were normalized to *GAPDH* expression. Expression levels relative to those in CB CD34^+ cells are shown as the mean ± S.D. for triplicate analyses.
Figure 2. Sox17 promotes the expansion of CD34^+CD43^+CD45^{low} cells.

(A) Growth curve of ECs from day 6 EBs that were transduced with a Sox17-ERT or a control retrovirus. ECs (2x10^4) were transduced with the indicated retrovirus on OP9 cells and cultured in the presence of 20 ng/ml of SCF and TPO and 200 nM 4-OHT. Absolute numbers of cells were determined and plotted. Representative data from repeated experiments are shown. (B) Appearance of a representative colony generated by Sox17-overexpressing cells in (A) observed under an inverted microscope. (C) Typical cell morphology of Sox17-overexpressing cells in (A). Sorted cells were cytospun onto glass slides and observed after Wright-Giemsa staining. ECs, pre-HPCs, and HPCs from day 8 EBs served as controls. (D) Flow cytometric analysis of expanded cells on overexpression of Sox17. ECs from day 6 EBs and pre-HPCs and HPCs from day 8 EBs were transduced with a Sox17-ERT or a control retrovirus, cultured on OP9 in the presence of 20 ng/ml of SCF and TPO and 200 nM 4-OHT for 10-15 days, and then analyzed for their immunophenotypes.

Figure 3. CD34^+CD43^+CD45^{low} cells expanded upon overexpression of Sox17 developmentally place between ECs and pre-HPCs/HPCs.

Gene expression patterns of wild type and Sox17-overexpressing cells obtained in microarray analyses were clustered using hierarchical clustering. Distance between two samples was defined with Pearson's correlation using total genes (A) or certain probes selected from the Gene Ontology (GO) database (B-C). "Transcription factor" represents genes which are located in the nucleus and have at least one of the GO terms "regulation of transcription, DNA-dependent", "transcription factor activity" or "transcription factor complex" (B). "Hemopoiesis" represents genes that are annotated with the GO terms "hemopoiesis", "vasculogenesis", "erythrocyte
differentiation", "erythrocyte maturation" and/or "erythrocyte development" (C). The color of each cell represents the value of correlation indicated on the right side of the matrix.

Figure 4. CD34⁺CD43⁺CD45⁻/low cells expanded upon overexpression of Sox17 retain hemogenic potential.

(A) Experimental design to evaluate effects of withdrawal of 4-OHT on Sox17-overexpressing cells. ECs from day 6 EBs transduced with a Sox17-ERT retrovirus were cultured in the presence of 20 ng/ml of SCF and TPO and 200 nM 4-OHT for 15 days. Then, the cells were subjected to co-culture with OP9 cells and colony-forming assays. For co-culture with OP9 cells, the cells were replated onto OP9 cells and cultured in the presence of 20 ng/ml of SCF and TPO, 10 ng/ml IL-3, and 3 unit/ml EPO with and without 4-OHT. At day 7 of culture, the cells were analyzed for their immunophenotypes by Flow cytometry. For colony-forming assays, the cells were replated in methylcellulose in the presence of 20 ng/ml SCF, 10 ng/ml of TPO and IL-3, and 3 units/ml EPO with and without 4-OHT. At day 12 of culture, the number of colonies was evaluated. (B) Representative flow cytometric profiles of cells overexpressing Sox17-ERT before and after depletion of 4-OHT. (C) The absolute numbers of CD235⁺ erythroblasts and CD11b⁺ myeloid cells in culture at 7 days after depletion of 4-OHT. Data are shown as the mean ± S.D. for triplicate cultures. (D) Ability of Sox17-ERT-overexpressing cells to form hematopoietic colonies in methylcellulose cultures with or without 4-OHT. The numbers of colony-forming units (CFUs) in culture are presented (left panel). CFU-GM, CFU-M, CFU-G, BFU-E, and CFU-E denote colony-forming unit (CFU)-granulocyte-macrophage, CFU-macrophage, CFU-granulocyte, burst-forming unit-erythroid, and colony-forming unit-erythroid, respectively. Compact colonies indicate colonies composed by hemogenic endothelial cell-like cells.
Appearance of a representative compact colony and an erythroid colony observed under an inverted microscope is depicted (right panel).

**Figure 5. Hematopoietic differentiation from hemogenic endothelium is inhibited by depletion of SOX17.**

(A) Knockdown efficiencies of shRNAs against *SOX17*. Western blot analysis of SOX17 in 293T cells transduced with shRNAs against *SOX17* (upper panel). α-Tubulin was used as the loading control. Pre-HPCs from day 8 EBs were transduced with shRNAs against *SOX17* on OP9 cells and were cultured in the presence of 20 ng/ml of SCF and TPO, 10 ng/ml IL-3, and 3 unit/ml EPO for 7 days. Levels of endogenous *SOX17* were analyzed by quantitative RT-PCR analysis (lower panel). mRNA levels were normalized to *GAPDH* expression. Expression levels relative to that in the control cells transduced with an shRNA against *Luciferase* are shown as the mean ± S.D. for triplicate analyses. (B) Effects of depletion of SOX17 on hematopoietic development from hemogenic endothelial cells. ECs from day 5 EBs were transduced with shRNAs against *SOX17* on OP9 cells and were cultured in the presence of 20 ng/ml of SCF and TPO, 10 ng/ml IL-3, and 3 unit/ml EPO for 9 days. Representative flow cytometric profiles of cells at day 9 of culture are depicted. (C) Absolute numbers and proportion of CD235a⁺ erythroblasts and CD11b⁺ myeloid cells in (B) at day 9 of culture. Data are shown as the mean ± S.D. for three independent cultures. (D) Effects of depletion of SOX17 on pre-HPCs. pre-HPCs from day 8 EBs were transduced with shRNAs against *SOX17* on OP9 cells and were cultured in the presence of 20 ng/ml of SCF and TPO, 10 ng/ml IL-3, and 3 unit/ml EPO for 7 days. Absolute numbers and proportion of CD235a⁺ erythroblasts and CD11b⁺ myeloid cells at day 7 of culture are presented. Data are shown as the mean ± S.D. for triplicate cultures of one
experiment from the two independent experiments that gave similar results.

**Figure 6. Targets of SOX17 detected by Chip-chip analysis**

(A) GO analysis of the Sox17 targets detected by Chip-chip analysis. CD34⁺CD43⁻ cells from day 6 EBs were transduced with a 3x Flag \(SOX17\)-ERT retrovirus. The cells were further cultured on OP9 cells in the presence of 20 ng/ml of SCF and TPO and 200 nM 4-OHT. CD34⁺ cells were collected at day 27 of culture and subjected to ChIP-chip analysis. The \(P\)-value of each GO term is indicated. (B) Chip-chip profile of SOX17 occupancy at genes related hematopoietic development from hemogenic endothelial cells. Plot under the x-axis show the position of probe sets. Arrowhead at \(VE\)-cadherin/\(CDH5\) promoter indicate consensus motif of Sox17-binding site. (C) Gene expression patterns of wild-type and engineered cells obtained in microarray analyses clustered using hierarchical clustering. Distance between two samples was defined with Pearson's correlation of Sox17 target genes with Sox17 binding more than 3 fold in the ChIP-chip analysis presented in Table S2. Color of each cell represents the value of correlation indicated on the right side of the matrix. (D) Comparative analysis of ChIP-chip and microarray data. Venn diagrams showing the number of genes bound by Sox17 (>3.0 fold enrichment) and the number of genes up-regulated in expression more than 2 fold in at least in one cell type among ECs, pre-HPCs, and HPCs upon overexpression of Sox17 (Sox17-overexpressing CD34⁺CD43⁺CD45⁻/low cells compared to those of respective fresh controls)(left panel). The percentages of overlapping and non-overlapping bound genes are indicated in parentheses. Correlation of Sox17 binding (fold enrichment) in ChIP-chip analysis and fold changes in expression during differentiation of ECs to HPCs (right panel). The fold enrichments and fold changes in expression were plotted for 84 genes out of 98 showing fold...
enrichments greater than 3.0 fold (the microarray data was not available for the remaining 14 genes). Correlation coefficients (R) are indicated for genes with fold enrichment greater than 3.0 and 6.0 fold, respectively.
<table>
<thead>
<tr>
<th>Rank</th>
<th>Symbol</th>
<th>Gene name</th>
<th>Fold enrichment</th>
<th>GO term</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vasculo-vasculogenesis</td>
<td>positive regulation of erythrocyte differentiation</td>
<td>Hemo-poiesis</td>
</tr>
<tr>
<td>1</td>
<td>GPSM3</td>
<td>G-protein signaling modulator 3</td>
<td>48.50</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>MAST4</td>
<td>microtubule associated serine/threonine kinase family member 4</td>
<td>41.62</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>TXLNB</td>
<td>taxilin beta, muscle-derived protein 77</td>
<td>38.05</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>EGOT</td>
<td>eosinophil granule ontogeny transcript</td>
<td>37.53</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>PBPPL1</td>
<td>pro-platelet basic protein-like 1</td>
<td>35.26</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>MFSD6</td>
<td>major facilitator superfamily domain containing 6</td>
<td>30.27</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>7</td>
<td>CDH5</td>
<td>cadherin 5, type 2</td>
<td>29.86</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>BCL6B</td>
<td>B-cell CLL/lymphoma 6, member B</td>
<td>28.05</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>9</td>
<td>C1orf55</td>
<td>chromosome 1 open reading frame 55</td>
<td>25.81</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>10</td>
<td>PTTG1IP</td>
<td>pituitary tumor-transforming 1 interacting protein</td>
<td>25.28</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>11</td>
<td>TRIM67</td>
<td>tripartite motif containing 67</td>
<td>24.08</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>12</td>
<td>MYCT1</td>
<td>myc target 1/myc target 1</td>
<td>21.26</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>13</td>
<td>CD40LG</td>
<td>CD40 ligand</td>
<td>14.12</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>14</td>
<td>SCOC</td>
<td>short coiled-coil protein</td>
<td>13.45</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>15</td>
<td>PPP1R16B</td>
<td>protein phosphatase 1, regulatory subunit 16B</td>
<td>13.18</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>19</td>
<td>ACVR2A</td>
<td>activin A receptor, type II A</td>
<td>9.45</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>26</td>
<td>HHEX</td>
<td>hematopoietically expressed homeobox</td>
<td>6.87</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>37</td>
<td>RUNX1</td>
<td>runt-related transcription factor 1</td>
<td>5.46</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>41</td>
<td>TAL1/SCL</td>
<td>T-cell acute lymphocytic leukemia 1</td>
<td>5.28</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>50</td>
<td>EGFL7</td>
<td>EGF-like-domain, multiple 7</td>
<td>4.69</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>69</td>
<td>JUNB</td>
<td>jun B proto-oncogene</td>
<td>3.78</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

ND indicates no data.
Figure 1

(A) EB day 6

(B) Cytokine

(C) BRACHYURY, RUNX1, SCL/TAL1 expression

(D) Cell number / sorted 20,000 cells

(E) SOX17 expression

(F) SOX17, SOX7, SOX18 expression
Figure 2

A. Graph showing cell number over culture days for control and Sox17-ERT (+ 4-OHT) conditions.

B. Image showing cell morphology on EB day 8.

C. Images of Sox17-ERT cells at EB day 8, showing ECs, pre-HPCs, and HPCs.

D. Flow cytometry plots for CD34, CD43, CD45, and VE-cadherin expression in control and Sox17 conditions.
Figure 4

A

SOX17-overexpressing CD34^+CD43^+CD45^{low} cells → Co-culture with OP9 cells → Colony-forming assay

B

4-OHT (+) vs 4-OHT (-) effects on CD34, CD45, and VE-cadherin expression.

C

P<0.01 for CD235a+ cell number and P=0.0118 for CD11b+ cell number.

D

Number of CFUs (CFU-GM, CFU-M, CFU-G, BFU-E, CFC-E) affected by 4-OHT. Compact colony formation is shown with images.
Figure 5

A

- **α-SOX17**
- **α-Tubulin**

**SOX17 relative expression**

- **sh-Luc**
- **sh-SOX17#1143**
- **sh-SOX17#1273**

B

**CD235a+ cells**

**CD11b+ cells**

**Others**

(CD235a-CD11b- cells)

C

- **Absolute cell numbers**
- **Proportion of cells (%)**

D

- **Absolute cell numbers**
- **Proportion of cells (%)**
Figure 6

A

<table>
<thead>
<tr>
<th>Category</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA binding</td>
<td></td>
</tr>
<tr>
<td>Vasculogenesis</td>
<td></td>
</tr>
<tr>
<td>Positive regulation of erythrocyte differentiation</td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Hemopoiesis</td>
<td></td>
</tr>
<tr>
<td>Transcription factor activity</td>
<td></td>
</tr>
<tr>
<td>Protein N-terminus binding</td>
<td></td>
</tr>
<tr>
<td>Histone deacetylase complex</td>
<td></td>
</tr>
<tr>
<td>Growth factor binding</td>
<td></td>
</tr>
<tr>
<td>RNA metabolism</td>
<td></td>
</tr>
<tr>
<td>Protein processing</td>
<td></td>
</tr>
</tbody>
</table>

B

- **VE-cadherin/CDH5**
  - Distance from TSS (bp)
  - log2

- **RUNX1**
  - Distance from TSS (bp)
  - log2

- **HHEX**
  - Distance from TSS (bp)
  - log2

C

**Sox17 targets**

D

<table>
<thead>
<tr>
<th>Bound genes (&gt;3.0 fold)</th>
<th>Up-regulated genes (&gt;2.0 fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>36</td>
</tr>
<tr>
<td>63.3%</td>
<td>36.7%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fold expression changes (HPCs/ECs)</th>
<th>Fold enrichments of SOX17</th>
</tr>
</thead>
<tbody>
<tr>
<td>R= -0.182 (&gt;3.0 fold)</td>
<td>R= -0.308 (&gt;6.0 fold)</td>
</tr>
</tbody>
</table>
Role of SOX17 in hematopoietic development from human embryonic stem cells

Yaeko Nakajima-Takagi, Mitsuiro Osawa, Motohiko Oshima, Haruna Takagi, Satoru Miyagi, Mitsuhiro Endoh, Takaho A. Endo, Naoya Takayama, Koji Eto, Tetsuro Toyoda, Haruhiko Koseki, Hiromitsu Yaeko Nakajima-Takagi, Mitsujiro Osawa, Motohiko Oshima, Haruna Takagi, Satoru Miyagi, Mitsuhiro Endoh, Takaho A. Endo, Naoya Takayama, Koji Eto, Tetsuro Toyoda, Haruhiko Koseki, Hiromitsu Nakauchi and Atsushi Iwama