**Maintenance of hematopoietic stem and progenitor cells in fetal intra-aortic hematopoietic clusters by the *Sox17*-*Notch1*-*Hes1* axis**

Kiyoka Saitoa, Ikuo Nobuhisaa,\*, Kaho Haradaa, Satomi Takahashia, Maha Anania,b, Heiko Lickertc, Masami Kanai-Azumad, Yoshiakira Kanaie, Tetsuya Tagaa,\*

a Department of Stem Cell Regulation, Medical Research Institute, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113 - 8510, Japan.

b Department of Clinical Pathology, Suez Canal University, 4.5 Km the Ring Road, Ismailia, 41522, Egypt.

c Institute of Stem Cell Research, Ingolstädter Landstraße 1 D-85764, Neuherberg, Germany.

d Department of Experimental Animal Model for Human Disease, Center for Experimental Animals, TMDU, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113 - 8510, Japan.

e Department of Veterinary Anatomy, Graduate School of Agricultural and Life Science, the University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan

\*Address correspondence to:

*Ikuo Nobuhisa, PhD and Prof. Tetsuya Taga*

*Department of Stem Cell Regulation, Medical Research Institute,*

*Tokyo Medical and Dental University (TMDU)*

*1-5-45, Yushima, Bunkyo-ku,*

*Tokyo, 113-8510*

*Japan*

*E-mail*: nobuhisa.scr@mri.tmd.ac.jp (I. Nobuhisa), taga.scr@mri.tmd.ac.jp (T. Taga)

**Abstract**

The aorta-gonad-mesonephros region, from which definitive hematopoiesis first arises in midgestation mouse embryos, has intra-aortic hematopoietic clusters (IAHCs) containing hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). We previously reported expression of the transcription factor *Sox17* in IAHCs, and overexpression of *Sox17* in CD45lowc-KIThigh cells comprising IAHCs maintains the formation of cell clusters and their multipotency in vitro over multiple passages. Here, we demonstrate the importance of NOTCH1 in IAHC formation and maintenance of the HSC/HPC phenotype. We further show that *Notch1* expression is positively regulated by SOX17 via direct binding to its gene promoter. SOX17 and NOTCH1 were both found to be expressed in vivo in cells of IAHCs by whole mount immunostaining. We found that cells transduced with the active form of NOTCH1 or its downstream target, *Hes1*, maintained their multipotent colony-forming capacity in semisolid medium. Moreover, cells stimulated by NOTCH1 ligand, Jagged1, or Delta-like protein 1, had the capacity to form multilineage colonies. Conversely, knockdown of *Notch1* and *Hes1* led to a reduction of their multipotent colony-forming capacity. These results suggest that the *Sox17*-*Notch1*-*Hes1* pathway is critical for maintaining the undifferentiated state of IAHCs.

**Abbreviations**

AGM, aorta-gonad-mesonephros; HSCs, hematopoietic stem cells; HPCs, hematopoietic progenitor cells; IAHCs, intra-aortic hematopoietic clusters; Sox, Sry-related high mobility group box; NICD, Notch intra-cellular domain; SCF, stem cell factor; IL, interleukin; TPO, thrombopoietin

**Keywords**

AGM, IAHCs, Hematopoietic stem cells, Sox17, Notch1

**Highlights**

・IAHCs of E10.5 AGM co-expressed SOX17 and NOTCH1.

・SOX17 directly binds to the *Notch1* promoter and induces *Notch1* expression.

・*Notch1*- and *Hes1*-transduced HSCs and HPCs maintain their undifferentiated states.

**1. Introduction**

In mouse ontogeny, definitive hematopoiesis, which generates long-term repopulating hematopoietic stem cells (HSCs), first arises in the aorta-gonad-mesonephros (AGM) region at midgestation [1]. During AGM hematopoiesis, haemogenic endothelial cells, a common progenitor of hematopoietic and endothelial cells, lie in the haemogenic endothelium lining the aortic lumen [2]. In general, intra-aortic hematopoietic clusters (IAHCs) arise from the haemogenic endothelium [3–6]. IAHCs express various marker proteins [3,5,7,8], among which CD31, a marker of endothelial cells, is specifically expressed in cells located in the vessel wall side of the cluster. Moreover, CD45, a marker of hematopoietic cells, is highly expressed in lumen-side cells of IAHCs, while c-KIT, a marker of HSCs, is expressed throughout IAHCs. IAHCs are not found in mouse embryos deficient for *Runx1*, which is one of the essential transcription factors for definitive hematopoiesis [7–9]. In addition, transplantation analyses have demonstrated that IAHCs contain HSCs and hematopoietic progenitor cells (HPCs) [7,8]. These data indicate that hematopoietic cluster cells play an important role in AGM hematopoiesis.

We have previously reported that CD45lowc-KIThigh cells, which are minor population in the embryonic day (E)10.5 AGM region, display the highest hematopoietic ability to maintain the undifferentiated state [10]. CD45lowc-KIThigh cells transduced with the transcription factor *Sox17* are maintained in the undifferentiated state for at least eight passages [11]. Moreover, as demonstrated by transplantation analysis of *Sox17*-transduced cells in lethally irradiated mice, *Sox17*-transduced cells have a long-term repopulating activity [11]. *Sox17* belongs to the SoxF family and contains a high mobility group box. It plays critical roles in several developmental events including not only hematopoiesis, but also cardiovascular development [12,13], endoderm formation [14], and implantation [15]. During hematopoiesis, as demonstrated by analysis of conditional knockout mice, SOX17 is important for fetal hematopoiesis, but not adult hematopoiesis [16]. However, ectopic expression of *Sox17* induces HSC self-renewal and redirection of adult HSCs to fetal-like HSCs [17]. A previous study has reported expression of *Notch1* in IAHC cells and endothelial cells, while *Notch4* is mainly expressed in endothelial cells [18,19]. *Sox17* induces expression of *Notch1* [20,21], and NOTCH1 signaling is important for E10.5 AGM hematopoiesis [22]. In this study, we investigated the molecular mechanisms of *Sox17* and *Notch1*. The NOTCH signaling pathway regulates cell fate determination and differentiation in various cell systems. NOTCH family members are single-pass transmembrane proteins containing an extracellular domain, EGF-like repeats, and an intracellular domain that serves as an active transcription factor. After interacting with its ligand, the Notch intracellular domain (NICD) is released by γ-secretase-mediated cleavage and moves into the nucleus where it regulates transcription [23]. In principle, the NOTCH signaling pathway is governed by direct cell-cell interactions [24]. In analyses of the functional role of NOTCH1 in hematopoiesis, the AGM region in NOTCH1-knockout mouse embryos was found to be defective for definitive hematopoiesis [25]. In addition, NOTCH1-knockout mouse embryos exhibit abnormal vascular formation [26]. These data suggest a critical role of NOTCH1 in the specification of both endothelial and hematopoietic cells in mouse embryos at midgestation. Moreover, SOX17 functions during the specification stage of the haemogenic endothelium via NOTCH signaling in mouse embryonic stem (ES) cells [21]. However, the mechanisms through which HSCs and HPCs in the AGM region maintain the undifferentiated state and whether SOX17 directly binds to the *Notch1* promoter are unclear. In this study, to clarify these points, we focus on IAHCs containing HSCs and HPCs, and elucidate the maintenance mechanism of the undifferentiated state of IAHC cells by SOX17 and NOTCH1 during AGM hematopoiesis.

**2. Material and Methods**

*2.1. Isolation of hematopoietic cluster cells from the AGM region*

AGM regions were excised from ICR mice at E10.5 and incubated in 1 mg/ml Dispase II (Roche, Basel, Switzerland) for 20 min at 37°C. After washing with stop solution [Hank’s balanced salt solution containing 10% (v/v) fetal calf serum (FCS) and 250 µg/ml DNase I (Roche)], cells were treated with Cell Dissociation Buffer (Invitrogen, Carlsbad, CA) for 20 min at 37°C. Dissociated cells were washed with stop solution, resuspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 2% (v/v) FCS, and then subjected to immunostaining with phycoerythrin (PE)-conjugated anti-mouse CD45 (30-F11) and allophycocyanin (APC)-conjugated anti-mouse c-Kit (2B8) antibodies (TONBO Biosciences, San Diego, CA). Stained cells were resuspended in DMEM supplemented with 2% (v/v) FCS and 1 µg/ml propidium iodide (Calbiochem, San Diego, CA), and analyzed by fluorescence-activated cell sorting (FACS, FACSAria™ II; BD Biosciences, San Diego, CA). Animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care Committee of Tokyo Medical and Dental University (approval number: A2017-128C).

*2.2. Gene transduction of CD45lowc-KIThigh cells*

Sorted CD45lowc-KIThigh cells were infected with retroviruses carrying pMY vectors [27] encoding internal ribosome entry site (*IRES*)*-*green fluorescent protein (*GFP*) (Mock) or *NICD-IRES-GFP* (NICD) in the presence of 10 µg/ml polybrene for 2.5 hours at 37°C. The infected cells were co-cultured with OP9 stromal cells [28] in α-minimal essential medium (MEM) supplemented with 10% (v/v) FCS, 50 ng/ml stem cell factor (SCF; PeproTech, Rocky Hill, NJ, 250-03), 10 ng/ml interleukin (IL)-3 (PeproTech, 213-13), and 10 ng/ml thrombopoietin (TPO; PeproTech, 315-14). We have previously described packaging [11].

*2.3. Reverse transcription-polymerase chain reaction (RT-PCR)*

Sorted GFP+ cells (gene-transduced cells) were co-cultured with OP9 cells in α-MEM supplemented with 10% (v/v) FCS, 50 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml TPO for 11 days and then sorted again by FACS. RNA of sorted cells was extracted by ISOGEN (WAKO, Osaka, Japan), and then cDNA was synthesized by Superscript III (Invitrogen). The same amounts of cDNA were subjected to PCR using rTaq (TaKaRa, Kyoto, Japan). Primer sequences were as follows: *Notch1*, 5′-AGGTGCAGCCACAGAACTTA-3′ and 5′-TCGGACCAATCAGAGATGTT-3′; *Notch4*, 5′-CCCTTAAACTCGGTTGT-3′ and 5′-GGTGCTTAATAAATAGTTGCC-3′; *Sox17*, 5′-TTTATGGTGTGGGCCAAAG-3′ and 5′-GCCCATGTGCGGAGACAT-3′; *Hes1*, 5′-CAGCCAGTGTCAACACGACAC-3′ and 5′-TCGTTCATGCACTCGCTGAG-3′; *Hes5*, 5′-GCGTCGGGACCGCATCAACA-3′ and 5′-GCGGCGAAGGCTTTGCTGTG-3′; *Gata2*, 5′-ACCACCCGATACCCACCTAT-3′ and 5′-GCCATGGCAGTCACCATGCT-3′; *c-myb*, 5′-GAGAGGTGGCACAACCATTT-3′ and 5′-GGGAACGTGACTGGAGATGT-3′; *Runx1*, 5′-CCAGCAAGCTGAGGAGCGGCG-3′ and 5′-CCGACAAACCTGAGGTCGTTG-3′; *c-Mpl*, 5′-CGGTATGCCTACCGAGGAGAGAAG-3′ and 5′-CGCGGGACACATTCTTCACCCAG-3′; *EpoR*, 5′-GGACACCTACTTGGTATTGG-3′ and 5′-GACGTTGTAGGCTGGAGTCC-3′; *β-major globin*, 5′-CTGACAGATGCTCTCTTGGG-3′ and 5′-CACAACCCCAGAAACAGACA-3′; *β-actin*, 5′-CCAGGGTGTGATGGTGGGAA-3′ and 5′-CAGCCTGGATGGCTACGTACA-3′; *Hey1*, 5′-AGATCCTGCAGATGACCGTG-3′ and 5′-CAAACTCCGATAGTCCATAGC-3′; *Hey2*, 5′-ATGAGCATAGGATTCCGAGAGTG-3′ and 5′-GGCAGGAGGCACTTCTGAAG -3′; *G3PDH*, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

*2.4. Colony-forming assay*

Sorted cells were embedded and cultured in Methocult™ (M3434; StemCell Technologies, Vancouver, Canada) at 37°C for 7 days. Individual colonies were scored by their morphology.

*2.5. Luciferase assay*

Plat-E cells [29] (2×106) were transduced with a pGL3-Basic vector (Promega, Fitchburg, WI) carrying the *Notch1* promoter region (-735 to +119, Fig. 1A) together with pEF-BOS vectors [30] encoding *Sox17* or *Sox17-MA*, in which Met76 is replaced by Ala [11], by TransIT®293 reagent (Mirus Bio, Madison, WI). Luciferase activity was measured by the Pikka-gene dual luciferase assay system (Toyo Ink, Tokyo, Japan), and a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany) was used for quantitation. The pRL-TK vector (Promega, Fitchburg, WI) was used as a control.

*2.6. Chromatin immunoprecipitation (ChIP) assay*

ChIP assays were performed using a ChIP Assay Kit (Merck Millipore, Darmstadt, Germany). Sorted CD45lowc-KIThigh cells from AGM regions were infected with a retrovirus carrying a pMY vector encoding *3×FLAG-Sox17-IRES-GFP* in the presence of 10 µg/ml polybrene for 2.5 hours at 37°C and then co-cultured with OP9 stromal cells in αMEM supplemented with 10% (v/v) FCS, 50 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml TPO. After three passages of the infected cells, GFP+ cells were sorted (≥2×106) and treated with 1% formaldehyde for 10 minutes. The following ChIP assay was performed according to a previously described procedure [31]. The lysates were mixed with 1 µg anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) or 1 µg normal rabbit IgG (Merck Millipore). DNA fragments were amplified by PCR with the following primers: *Sox17-binding* sense, 5′-CTGCGCCTGTCCGGGCTAGG-3′ and antisense, 5′-ACCTAACTCCAGGATGGAGC-3′; *Non Sox17-binding* sense, 5′-ATCTGCATCGCATCATGAAT-3′ and antisense, 5′-TCTCATTCTACCTGCACCCT-3′.

*2.7. Intracellular staining of Notch1 in CD45lowc-KIThigh cells*

Dissociated AGM cells were immunostained with fluorescein isothiocyanate-conjugated anti-mouse CD45 (30-F11; BioLegend, San Diego, CA) and APC-conjugated anti-mouse c-Kit (2B8; TONBO Biosciences) antibodies. Cells were centrifuged at 1,300 rpm for 3 minutes and resuspended in DMEM containing 2% (v/v) FCS. The cells were then fixed in 4% paraformaldehyde (PFA) for 15 minutes at 4°C. After permeabilization with 1× Perm/Wash buffer (Transcription Factor Buffer Set; BD Biosciences), the cells were immunostained with PE-conjugated anti-mouse IgG (MOPC-21; BioLegend) or a PE-conjugated anti-mouse Notch1 antibody (mN1A; BioLegend) and then analyzed by FACS.

*2.8. AGM and endothelial cell cultures in the presence of NOTCH ligands*

Mouse anti-human IgG (R&D Systems, Minneapolis, MN) was mixed with either recombinant rat Jagged1 human IgG1 Fc chimera protein (R&D Systems) or recombinant human IgG1 (R&D Systems) at a ratio of 1:2 (final concentration: 10 µg/ml) to form the respective complex. CD45lowc-KIThigh cells were co-cultured with OP9 cells in the presence of pre-formed Jagged1 or control IgG complexes. After 4 days, sorted CD45lowc-KIThigh cells (1×103) were subjected to the colony-forming assay. Moreover, CD45lowc-KIThigh cells were co-cultured with OP9/G-DLL1 cells overexpressing NOTCH ligand DLL1 and GFP [32]. OP9/G-DLL1 and OP9/G cells were provided by the RIKEN Bioresource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. After 4 days, sorted CD45lowc-KIThigh cells (1×103) were subjected to the colony-forming assay. Sorted CD45-c-KIT-CD31+ endothelial cells were cultured in Myelocult M-5300 medium (StemCell Technologies) containing TPO (10 ng/ml), IL-6 (10 ng/ml, R&D Systems, 206-IL), DMSO [1/1000 (v/v)], bFGF (10 ng/ml, Pepro Tech, 100-18C), SCF (50 ng/ml), IL-3 (10 ng/ml), and VEGF (1 ng/ml, R&D Systems, 293-VE).

*2.9. Whole mount immunostaining*

Whole mount immunostaining was performed according to a previously described protocol [33]. E10.5 mouse embryos of SOX17-mCherry fusion transgenic mice [34] were fixed in 2% PFA for 20 minutes on ice. After washing with PBS, the embryos were blocked by streptavidin and biotin (BLOCKING KIT; Vector Laboratories, Burlingame, CA). Blocked embryos were treated with PBS-MT/BSA [1% (w/v) skim milk powder, and 0.4% (v/v) Triton X-100 containing 2% (w/v) bovine serum albumin] for 1 hour on ice and then immunostained with a rat anti-mouse CD117 (c-Kit) antibody (2B8; eBioscience, San Diego, CA), goat anti-mCherry antibody (SICGEN, Cantanhede, Portugal) and rabbit anti-mouse-Notch1 XP monoclonal antibody (Cell Signaling Technology, Beverly, MA) in Can Get Signal® Immunostain solution A (TOYOBO, Osaka, Japan) overnight at 4°C. Immunostained embryos were washed three times with PBS-MT for 1 hour each and then stained with Alexa Fluor® 647-conjugated donkey anti-rat IgG (Jackson Immuno Research, West Grove, PA), Alexa Fluor® 488-conjugated donkey anti-goat IgG (Life Technologies, Carlsbad, CA), and Alexa Fluor® 546-conjugated donkey anti-rabbit IgG (Jackson Immuno Research) in PBS-MT overnight at 4°C. After washing three times with PBS-MT for 1 hour each, embryos were stained with Hoechst 33258 (Nacalai Tesque, Kyoto, Japan) in PBS-T (0.4% [v/v] Triton X-100 in PBS) for 20 minutes at 4°C, followed by washing twice with PBS-T for 20 minutes each at 4°C. Moreover, embryos were treated with 50% (v/v) methanol/PBS for 10 minutes at 4°C and then treated twice with 100% methanol for 10 minutes each at 4°C. The embryos were washed in 50% (v/v) methanol/a mixture of benzyl alcohol and benzyl benzoate (1:2, BABB) on a CoverWell chamber (0.5 mm, Thermo Fisher Scientific, Waltham, MA) three times. Finally, the embryos were mounted in BABB. Observations were performed by confocal laser-scanning microscopy (LSM 510; Carl Zeiss, Oberkochen, Germany).

*2.10. Design of short hairpin RNAs (shRNAs) and cell transfection*

shRNA sequences were as follow: *shNotch1#1*, GCGTGTGTAATGTCAGCAT; *shNotch1#2*,GCAATCTGCGGTGTAGTAA; *shHes1#1*, GCCGATTTGTCTTTCTCGT; *shHes1#2*, GTGGAGAGTTGTATTGAGT; *shHey1#1*, GCCGACGAGACCGAATCAATA; *shHey1#2*, GCCTTTGAGAAGCAGGGATCT. The shRNA sequences and hairpin oligonucleotides designed with the 9-mer nucleotide spacer TTCAAGAGA [35] were annealed downstream of the U6 promoter in the pMKO.1 GFP retrovirus vector that expresses GFP under the control of the SV40 promoter [11]. shRNA constructs were transfected into *Sox17*-transduced cells, followed by co-culture with OP9 cells for 4 days. After two passages, gene expression levels in the cells were assessed by RT-PCR or colony-forming assays. Primer sequences were as follows: *Notch1* (for shNotch1#1), 5′-GCGAAGTGGACATTGACGAG-3′ and 5′-ACTGTTGCACTCGTTGACCT-3′; *Notch1* (for shNotch1#2), 5′-ACAGTGCAACCCCCTGTATG-3′ and 5′-GAAGCACTGCGAGGATGACT-3′; *Hes1* (for shHes1#1), 5′-GCTTCCTGTCCACGTGTGAG-3′ and 5′-TCCAAGTTCGTTTTTAGTGT-3′; and *Hes1* (for shHes1#2), 5’-AGTGTCACCTTCCAGTGGCT-3′ and 5′-TCTCGTGTAAAAAACGAAAT-3′; *Hey1* (for shHey1#1 and #2), 5′-GCGGACGAGAATGGAAACTTG-3′ and 5′-GGAGGCATCGAGTCCTTCAA-3′. We had previously prepared shSox17 constructs and used them in this study [11].

*2.11. Statistical analysis*

All data of luciferase activities and colony counts are represented as the mean ± standard deviation. Comparisons between two samples were performed using Student’s t-tests.

**3. Results**

*3.1. NICD is present in CD45lowc-KIThigh cells of the E10.5 AGM where SOX17 induces Notch1 expression*

We have previously demonstrated expression of *Sox17* in basal cells of E10.5 AGM IAHCs by whole mount in situ hybridization, and that E10.5 AGM CD45lowc-KIThigh cells transduced with *Sox17* maintain their undifferentiated state [11]. However, it is unclear how IAHCs containing HSCs maintain their undifferentiated state by *Sox17*. Studies have reported expression of *Notch1* and *Notch4* in the E10.5 AGM region [18] and activation of the *Notch1* promoter by *Sox17* in U2OS osteosarcoma cells [21]. However, these studies did not show that SOX17 and NOTCH1 co-localize in IAHCs or whether SOX17 directly binds to putative SOX17-binding sites of the *Notch1* promoter region in AGM cells. Thus, we first examined the role of *Sox17* in *Notch1* expression by luciferase and ChIP assays. As shown in Fig. 1B, introduction of *Sox17* dose-dependently increased *Notch1* promoter activity as reported previously [21]. In contrast, SOX17-MA, in which methionine 76 was substituted with alanine to disrupt its DNA-binding ability, did not induce *Notch1* promoter activation (Fig. 1B; right three columns). Next, we investigated whether SOX17 directly binds to putative SOX17-binding sites in the *Notch1* promoter region by a ChIP assay. A *Notch1* promoter fragment (from -429 to -139) including the two putative SOX17-binding sites [21] was found to interact with FLAG-tagged SOX17, whereas SOX17-binding was not found in another fragment (from -2471 to -2184) without the putative SOX17-binding sites (Fig. 1C). Next, we examined the expression level of *Notch* family genes in *Mock*- and *Sox17*-transduced cells by RT-PCR. As shown in Fig. 1D, *Sox17*-transduced cells highly expressed *Notch1*, *Notch4*, *Hes1*, and *Hes5* genes compared with *Mock*-transduced cells. Conversely, *Sox17* knockdown cells had decreased expression of *Notch* family genes (Fig. 1E). Moreover, we analyzed expression of NOTCH1 and NOTCH4 at the single cell level in *Sox17*-transduced IAHC cells of the E10.5 AGM by flow cytometry. As shown in Fig. 1F, NOTCH1 was highly expressed in these cells, whereas NOTCH4 expression was not significantly different compared with *Mock*-transduced cells. These data suggest that SOX17 directly binds to the *Notch1* promoter region and induces expression of *Notch1*.

As shown in Figure 1G, NICD (active form of NOTCH1) was observed in most CD45lowc-KIThigh cells, which are a minor population in hematopoietic clusters of the AGM region, but the main population responsible for hematopoietic activity. E10.5 SOX17-mCherry fusion transgenic mouse embryos [34] were stained with anti-NOTCH1, -mCherry (Sox17), and -c-KIT antibodies as well as Hoechst 33258 using the whole mount immunostaining method. We used the anti-mCherry antibody because mCherry fluorescence is quenched by PFA treatment. As shown in Fig. 2A, NOTCH1 immunoreactivity was detected in the membrane of IAHC cells, and SOX17 expression (mCherry immunoreactivity) was observed in the nuclei of IAHC cells. In wild-type mice, NOTCH1 and SOX17 immunoreactivity was also detected in IAHC cells (Fig. 2B). Among the endothelial cells, SOX17-expressing and non-expressing cells were observed (Fig. 2C). Moreover, we found that the high expression of SOX17 in endothelial cells of the dorsal aorta corresponded with that of NOTCH1 (Fig. 2C). As shown in Fig. 2, the expression levels of NOTCH1 and SOX17 in IAHC cells were generally lower than those in endothelial cells. However, it is of note that some IAHC cells expressed NOTCH1 and SOX17 simultaneously. Taken together, the results suggest that NOTCH1 is induced by SOX17, and the NOTCH1 signaling pathway is activated in portions of IAHCs in E10.5 AGM regions.

*3.2. NICD-transduced cells maintain their undifferentiated state in vitro*

To examine the function of NOTCH1 in IAHCs, we infected CD45lowc-KIThigh cells with retroviruses encoding *IRES-GFP* (Mock) or *NICD-IRES-GFP* (NICD) and co-cultured them with OP9 stromal cells. GFP+ cells transduced with each gene were sorted by FACS and subjected to RT-PCR. *NICD*-transduced cells exhibited increased expression levels of *Hes*1 and *Hes5*, which are downstream targets of NICD, transcription factors *c-Myb* and *Runx1*, which are essential transcription factors for definitive hematopoiesis [9,36], and TPO receptor *c-Mpl* that contributes to maintaining the undifferentiated state of hematopoietic cells [37] (Fig. 3A).

Moreover, to investigate the hematopoietic ability of *NICD*-overexpressing cells in vitro, CD45lowc-KIThigh cells in a 4-day culture of AGM-derived CD45lowc-KIThigh cells, which had been infected with retroviruses encoding *IRES-GFP* (Mock) or *NICD-IRES-GFP* (NICD), were sorted by FACS, as depicted in Fig. 3B (red squares), and embedded in semisolid medium for colony-forming assays. *NICD* transduction increased the number of total colonies (CFU-C), mixed colonies including granulocytes, macrophages, and erythrocytes (CFU-Mix), and erythrocyte colonies (CFU-E) (Fig. 3C, passage 1). Next, we investigated whether the undifferentiated state was maintained during subculture of *NICD*-transduced cells in the co-culture with OP9 stromal cells. Cells transduced with *NICD* maintained their multipotent colony-forming activity in semisolid medium over three passages (Fig. 3C, passage 3). In contrast, transduction of *Mock* dramatically impeded or abolished mixed colony formation. Similar results were observed for the number of total colonies and erythroid colonies. Taken together, these results suggest that activation of the NOTCH signal pathway contributes to maintenance of the undifferentiated state in CD45lowc-KIThigh cells of the AGM region.

*3.3. Stimulation of CD45lowc-KIThigh cells by NOTCH1 ligands increases their hematopoietic ability*

NOTCH ligands include two Jagged ligands (JAG1 and JAG2) and three Delta-like ligands (DLL1, DLL3, and DLL4). In particular, JAG1, JAG2, and DLL4 are expressed in both endothelial cells and hematopoietic cluster cells of the E10.5 AGM [18]. We examined whether CD45lowc-KIThigh cells could be maintained in the undifferentiated state by JAG1 stimulation. CD45lowc-KIThigh cells sorted from the E10.5 AGM were treated with a recombinant rat Jagged1 Fc chimera protein or recombinant human IgG1, which were pre-complexed with anti-human IgG [38], and then co-cultured with OP9 stromal cells. After 4 days, the colony-forming assay was performed with sorted CD45lowc-KIThigh cells. The number of mixed colonies was increased by JAG1 stimulation compared with the control (Fig. 4A, passage 1). Moreover, we cultured CD45lowc-KIThigh cells from the E10.5 AGM with OP9 cells that had been manipulated to express DLL1 and GFP (OP9/G-DLL1) [32]. CD45lowc-KIThigh cells co-cultured with OP9/G-DLL1 showed an increase in the number of mixed colonies (Fig. 4B, passage 1). However, the effect of NOTCH1 ligands was brief (Fig. 4A and B, passage 2).

To examine whether JAG1 stimulation of CD45-c-KIT-CD31+ haemogenic endothelial cells from the E10.5 AGM induced production of CD45+ hematopoietic cells, we treated CD45-c-KIT-CD31+ cells with JAG1 (Fig. 4C). The number of CD45+ hematopoietic cells was dramatically decreased by addition of JAG1 to CD45-c-KIT-CD31+ endothelial cell cultures (Fig. 4D). These data suggest that NOTCH signaling activated by their ligands maintains the undifferentiated state for at least a certain period of time in IAHCs but does not induce production of CD45+ hematopoietic cells from endothelial cells.

*3.4. Maintenance of hematopoietic cluster cells through the Sox17-Notch1-Hes1 axis*

Based on the above findings, we focused on *Hes1* and *Hes5* as target genes of NOTCH. In IAHCs of E10.5 mouse embryos, *Hes1*, but not *Hes5*, is expressed [39]. However, because HES1-null embryos exhibited an increase in the *Hes5* expression level, probably due to compensatory mechanisms, when analyzed in the explant culture of the E10.5 AGM region, both HES1 and HES5 were deleted in mice. As a result, cells with double knockout of HES1 and HES5 formed dramatically decreased numbers of mixed colonies in the colony-forming assay [39]. We thus infected CD45lowc-KIThigh cells with retroviruses encoding *IRES-GFP* (Mock), *Hes1-IRES-GFP* (Hes1), or *Hes5-IRES-GFP* (Hes5) and co-cultured them with OP9 stromal cells, which were then subjected to RT-PCR (Fig. 5A). The results indicated that *Hes1*-transduced cells displayed increased expression levels of *Runx1*, which is expressed in undifferentiated hematopoietic cells, whereas *Hes5*-transduced cells displayed increased expression levels of *EpoR* and *β-globin* that are erythroid markers. Similarly, *Hes1*-transduced cells had a capacity to form multilineage colonies, whereas *Hes5*-transduced cells showed a high ability to form erythroid colonies (Fig. 5B). Taken together, NOTCH1 activated by SOX17 induces *Hes1* and *Hes5* expression, and HES1 and HES5 play different roles in AGM hematopoiesis.

We previously showed that knockdown of *Sox17* in CD45lowc-KIThigh cells reduces the ability for multilineage colony formation [11]. Therefore, to examine whether the multilineage colony-forming ability was decreased by knockdown of *Notch1* or *Hes1* expression in *Sox17*-transduced cells, *Sox17*-transduced cells were infected with a retrovirus encoding shRNA against *Notch1*. As a result of the reduced *Notch1* expression level in *Sox17*-transduced cells, the expression level of *Hes1* was decreased significantly (Fig. 6A). Moreover, the expression levels of other target genes were decreased, such as *Hey1* and *Hey2*. In addition, we noted that *Sox17*-transduced cells with downregulated *Notch1* expression displayed a decrease in their multilineage colony-forming ability (Fig. 6B). Furthermore, *Sox17*-transduced cells with downregulated *Hes1* expression showed a moderate but significant decrease in the multilineage colony-forming ability (Fig. 6C and D). Next, to investigate the role of other NOTCH1 target genes, we transduced *Hey1* or *Hey2* into CD45lowc-KIThigh cells and knocked down *Hey1* expression in *Sox17*-transduced cells. As shown in Fig. 7A and B, *Sox17*-transduced cells expressed *Hey1* and *Hey2*, and *Hey1*- and *Hey2*-transduced cells had increased abilities for multilineage colony formation. However, *Sox17*-transduced cells with downregulated *Hey1* expression by introduction of shRNA did not have a clearly different ability for multilineage colony formation compared with the control. Therefore, these results suggest that CD45lowc-KIThigh cells of IAHCs are maintained by the *Sox17*-*Notch1*-*Hes1* axis.

**4. Discussion**

As shown in the schema of Fig. 8, in this study, we first showed that SOX17 directly binds to the *Notch1* promoter and induces expression of *Notch1*. Although another study has reported that NOTCH1 is important for the proliferation of endothelial cells and hematopoietic cluster cells that arise from the haemogenic endothelium [40], little was known about the molecular involvement of NOTCH signaling in the maintenance of HSCs in IAHCs. Here, we found that CD45lowc-KIThigh cells, which are a minor population but responsible for hematopoietic activity, can be maintained in the undifferentiated state by transduction of *NICD*. Moreover, we showed that forced expression of *Hes1*, a downstream target of NOTCH signaling, conferred CD45lowc-KIThigh cells with a high ability to maintain the undifferentiated state. In support of this observation, knockdown of *Notch1* expression by shRNA decreased the multilineage colony-forming ability of the cells, which was accompanied by a decrease in the expression level of *Hes1*. Similarly, knockdown of *Hes1* expression also decreased the capacity of the cells to form multilineage colonies. Somewhat inconsistent with our observations, Lizama et al. previously reported that SOX17 represses the expression of *Runx1* and *Gata2* in endothelial cells, which is necessary for hematopoiesis [20]. Conversely, our data support the idea that SOX17 positively regulates hematopoiesis through *Notch1* and *Hes1* expression in IAHCs, which arise from endothelial cells and contain HSCs and HPCs, as depicted in Fig. 8.

The AGM region of HES1 and HES5 double knockout mouse embryos displays an increased number of cells in each IAHC and a decreased number of functional HSCs [39]. In the present study, we found that *Hes5*-transduced cells formed an increased number of erythroid colonies, while HES1 is important to maintain the undifferentiated state of cells in IAHCs (Fig. 5B). These data suggest that HES1 and HES5 play different roles in AGM hematopoiesis. A previous study reported a functional difference between HES1 and HES5 in hematopoietic cell differentiation, in which *Hes1* transduction into the erythroid/megakaryocytic cell line K562 inhibited erythroid/megakaryocytic differentiation, whereas *Hes5* transduction induced megakaryocytic differentiation [41]. The detailed physiological role of HES5 remains to be elucidated in erythroid differentiation.

NOTCH1-deficient neonatal mice exhibit failure of T cell development, although other hematopoietic cells are normal [42]. These data imply that the NOTCH signaling pathway is not necessary for maintenance of HSCs in adults. In the embryonic stage, expression of *Gata2*, which is critical for the development and proliferation of HSCs and HPCs, is not detected in AGM regions of JAG1-deficient mice [43]. We found that a reduction of *Notch1* expression in *Sox17*-transduced cells decreased their ability to maintain the undifferentiated state. Taken together, these data indicate that activation of *Notch1* is necessary for definitive hematopoiesis in the AGM region.

In this study, we found that activation of NOTCH signaling in CD45lowc-KIThigh cells by JAG1 or DLL1 maintained their undifferentiated state for at least a certain period of time. Recently, Gama-Norton et al. reported that JAG1 is important for the specification of HSCs derived from endothelial cells, whereas DLL4 is pivotal for maintenance of the endothelial program [40]. We found that stimulation of hematopoietic cluster cells containing HSCs with Jagged1 ligand or Delta-like ligand maintained their undifferentiated state at least for a short period, but not long term (Fig. 4A and B). Conversely, introduction of *Notch1*- or *Hes1*-shRNAs into *Sox17*-transduced cells led to a moderately reduced number of the mixed colonies (Fig. 6B and C). These data suggest the presence of other unknown pathways facilitating maintenance of the undifferentiated state in IAHCs.

Ex vivo expansion of HSCs from bone marrow and cord blood has been investigated for transplantation by many studies. For example, *HoxB4*-transduced cells derived from mouse ES cells have a long-term repopulation ability in lethally irradiated recipient mice, but these cells have a low capacity to differentiate into lymphoid and erythroid lineages. [44,45]. Similarly, *HoxB4*-transduced human ES cells acquire the capacity for hematopoietic repopulation, but these HSCs have limited proliferative and migratory capacities compared with somatic HSCs [46]. Recently, induced pluripotent stem cells simultaneously transplanted with OP9 cells into immunodeficient mice with co-administration of hematopoietic cytokines were found to form teratomas containing functional HSCs [47]. This system will undoubtedly require careful modification to ensure its safety for use in therapy. Thus, for long-term ex vivo culture of HSCs, we need to determine the whole process of the maintenance mechanism of definitive HSCs. Here, we revealed one aspect to elucidate this process.

**5. Conclusion**

We suggest that the *Sox17*-*Notch1*-*Hes1* pathway is critical for maintaining the undifferentiated state of HSCs in the AGM region, which requires interactions with some other molecular pathways for complete hematopoiesis at the embryonic stage.

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**Conflict of interest**

No competing financial interests exist.

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**Figure Legends**

**FIG. 1.** Direct binding of SOX17 to the *Notch1* gene promoter and the presence of the active form of NOTCH1 in CD45lowc-KIThigh cells of IAHCs. **(A)** Promoter region of the *Notch1* gene. **(B)** Luciferase assays were performed with the pGL3 control vector or *Notch1* promoter constructs containing SOX17-binding sites. Expression plasmids for *Sox17* or *Sox17-MA* (a point mutant with substitution of Ala76 for Met76) were also transfected into Plat-E cells. Data represent averages and standard deviations of three independent sets of triplicate experiments. \**P*<0.05, \*\**P*<0.01. **(C)** CD45lowc-KIThigh cells were infected with a retrovirus encoding *3×FLAG-Sox17-IRES-GFP*, and cell lysates containing DNA fragments of 500-1000 bp were obtained. Lysates were treated with either an anti-FLAG antibody or normal mouse IgG. Immunoprecipitates were subjected to PCR. “*Sox17-binding*” represents a DNA fragment (from -693 to -406) containing two putative SOX17-binding sites. “*Non-Sox17-binding*” represents a DNA fragment (from -2471 to -2184) that did not contain the putative SOX17-binding sites. **(D)** RT-PCR of *Notch*-related genes in *Mock*- or *Sox17*-transduced cells. **(E)** RT-PCR of *Notch*-related genes in *Vector* (Vec), *Sox17#1* shRNA (shSox17#1)-, or *Sox17#2* shRNA (shSox17#2)-transduced CD45lowc-KIThigh cells. **(F)** CD45lowc-KIThigh cells from E10.5 AGM regions were infected with *IRES-GFP* (Mock) or *Sox17-IRES-GFP* (Sox17)-encoding retroviruses. *Mock*- or *Sox17*-transduced cells (GFP+ cells) were stained with anti-NOTCH1-APC or -NOTCH4-APC antibodies and analyzed by FACS. **(G)** Dissociated CD45lowc-KIThigh cells from E10.5 AGM regions were stained with an anti-NOTCH1-PE antibody and analyzed by FACS.

**FIG. 2.** Co-expression of SOX17 and NOTCH1 in IAHCs of E10.5 mouse embryos. **(A)** Confocal images of IAHCs in E10.5 SOX17-mCherry fusion mouse embryos at high magnification. ECs, endothelial cells; white arrows, IAHCs; dashed line, a border of endothelial cells and IAHCs; scale bar, 20 µm. **(B)** Confocal images of whole mount immunostaining of E10.5 mouse embryos. ECs, endothelial cells; white arrows, IAHCs; dashed line, a border of endothelial cells and IAHCs; scale bar, 20 µm. **(C)** Confocal images of an E10.5 SOX17-mCherry fusion mouse embryo stained for SOX17, NOTCH1, and c-KIT at high magnification. White arrowheads indicate immunoreactivities of SOX17 and NOTCH1 in the same endothelial cells. Scale bar, 50 µm.

**FIG. 3.** Maintenance of the undifferentiated state and elevated expression of undifferentiated cell marker genes in *NICD*-transduced cells. **(A)** RT-PCR of hematopoiesis-related genes in *Mock*- or *NICD*-transduced GFP+ cells. **(B)** Expression profile of CD45 and c-KIT in *Mock*- or *NICD*-transduced GFP+ cells. Red boxes indicate CD45lowc-KIThigh cells. **(C)** Numbers of total colonies (CFU-C), mixed colonies (CFU-Mix), and erythroid colonies (CFU-E) formed in cultures of *Mock*- or *NICD*-transduced CD45lowc-KIThigh cells (250 cells each) at passages 1-3 (passage 1, n=9; passages 2 and 3, n=4). \**P*<0.05, \*\*\**P*<0.005. Significant differences in NICD effects were observed in comparison with Mock effects.

**FIG. 4.** Enhancement of mixed colony formation by Jagged1 and Delta-like 1 stimulation. **(A)** Numbers of total colonies (CFU-C) and mixed colonies (CFU-Mix) formed with or without Jagged1 (n=3). Jagged1 and control IgG-Fc were pre-treated with anti-human IgG because the Jagged1-Fc/anti-Fc complex has a high *Notch*-activating ability. \**P*<0.05, \*\**P*<0.01. **(B)** Numbers of total colonies (CFU-C) and mixed colonies (CFU-Mix) formed in co-cultures with OP9/G or OP9/G-DLL1 cells (n=3). \**P*<0.05. **(C)** Expression profile of CD31 in CD45-c-KIT- cells. Green box shows CD45-c-KIT-CD31+ endothelial cells. **(D)** Percentage of CD45+ hematopoietic cells among cultured cells with or without Jagged 1 stimulation (n=4).

**FIG. 5.** Different potentials of HES1 and HES5 in hematopoietic differentiation. **(A)** RT-PCR of marker genes in hematopoietic *Mock*-, *Hes1*-, or *Hes5*-transduced CD45lowc-KIThigh cells. **(B)** Numbers of total colonies (CFU-C), mixed colonies (CFU-Mix), and erythroid colonies (CFU-E) formed by *Mock*-, *Hes1*-, or *Hes5*-transduced cells (n=5). \**P*<0.05, \*\*\**P*<0.005.

**FIG. 6.** Reduction of the colony-forming ability by knocking down *Notch1* or *Hes1* genes in *Sox17*-transduced cells. **(A)** RT-PCR of *Notch1* and NOTCH1 target genes in *Sox17*-transduced CD45lowc-KIThigh cellsthat were further transduced with *Luc*-shRNA (shLuc), *Notch1#1* shRNA (shNotch1#1), or *Notch1#2* shRNA (shNotch1#2). Expression levels of *Notch1* and NOTCH1 target genes were reduced by introduction of *shNotch1#1* and *shNotch1#2*. **(B)** Numbers of mixed colonies (CFU-Mix) formed by *shLuc*-, *shNotch1#1*-, and *shNotch1#2*-transduced cells (n=6). \**P*<0.05. **(C)** RT-PCR of *Hes1* in *Sox17*-transduced cells that were further transduced with *shHes1#1* shRNA (shHes1#1) or *shHes1#2* shRNA (shHes1#2). Expression levels of *Hes1* were reduced by introduction of *shHes1#1* and *shHes1#2*. **(D)** Numbers of mixed colonies (CFU-Mix) in *shLuc*-, *shHes1#1*-, and *shHes1#2*-transduced cell cultures (n=11). \**P*<0.05.

**FIG. 7.** Potentials of HEY1 and HEY2 in AGM hematopoiesis. **(A)** RT-PCR of *Hey1* and *Hey2* genes in *Mock*- or *Sox17*-transduced cells. **(B)** Numbers of total colonies (CFU-C), mixed colonies (CFU-Mix), and erythroid colonies (CFU-E) formed in cultures of *Mock*- or *NICD*-transduced cells (1×103 cells each, n=10). **(C)** RT-PCR of *Hey1* in *Sox17*-transduced cells that were further transduced with *shHey1#1* shRNA (shHey1#1) or *shHey1#2* shRNA (shHey1#2). Expression levels of *Hey1* were reduced by introduction of *shHey1#1* and *shHey1#2*. **(D)** Numbers of mixed colonies (CFU-Mix) in *shLuc*-, *shHey1#1*-, and *shHey1#2*-transduced cell cultures (n=7).

**FIG. 8.** Model of the function of the *Sox17*-*Notch1*-*Hes1* axis in IAHCs. SOX17 induces expression of *Notch1*, which eventually leads to maintenance of hematopoietic stem and progenitor cell phenotypes of IAHC cells. See text for details.