Eya2 is critical for the *E2A-HLF*-mediated immortalization of mouse hematopoietic stem/progenitor cells

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Abstract. The immunoglobulin enhancer-binding factor/hepatic leukemia factor (*E2A-HLF*) oncogenic fusion gene, generated by t(17;19)(q22;p13) translocation in childhood B-cell acute lymphoblastic leukemia with a very poor prognosis, encodes a chimeric transcription factor in which the transactivation domains of E2A are fused to the DNA-binding and dimerization domain of HLF. E2A-HLF has been demonstrated to have an anti-apoptotic effect. However, the molecular mechanism underlying *E2A-HLF*-mediated leukemogenesis remains unclear. The present study identified EYA transcriptional coactivator and phosphatase 2 (*Eya2*), the forced expression of which is known to immortalize mouse hematopoietic stem/progenitor cells (HSPCs), as a direct target molecule downstream of E2A-HLF. *E2A-HLF*-immortalized mouse HSPCs expressed *Eya2* at a high level in the aberrant self-renewal program. Chromatin immunoprecipitation-quantitative polymerase chain reaction and a reporter assay revealed that E2A-HLF enhanced the *Eya2* expression by binding to the promoter region containing the E2A-HLF-binding consensus sequence. *Eya2* knockdown in *E2A-HLF*-immortalized cells resulted in reduced colony-forming efficiency. These results suggest a critical role of Eya2 in *E2A-HLF*-mediated leukemogenesis.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy (1). Chromosomal translocations are the hallmark of pediatric ALL, and generate fusion genes encoding chimeric transcription factors. The translocation t(17;19)(q22;p13) that results in the fusion gene *E2A-HLF* (2,3) defines a rare subtype of ALL that accounts for ~1% of pediatric B-cell precursor ALL cases and is associated with a very poor prognosis (4,5).

The immunoglobulin enhancer-binding factor (*E2A*) gene encodes two proteins, E12 and E47, which are members of the basic helix-loop-helix (bHLH) family of transcriptions factors, and it is required for proper B-cell development (6-8). Hepatic leukemia factor (*HLF*) encodes a transcription factor of the basic leucine zipper (bZIP) family containing a proline- and acidic amino acid-rich (PAR) domain, which enables it to form either homodimers or heterodimers with other PAR protein family members (2,9,10). In the E2A-HLF fusion protein, the two transactivation domains, AD1 and AD2, of E2A are fused to the bZIP DNA-binding and dimerization domain of HLF (3). The expression of the *E2A-HLF* fusion gene results in transcriptional reprogramming with dedifferentiation in pre-leukemic cells (11). E2A-HLF promotes the anchorage-independent growth of murine fibroblasts (12). Human leukemic cells expressing E2A-HLF rapidly undergo apoptosis when programmed to express a dominant-negative mutant of E2A-HLF. In addition, the conditional expression of E2A-HLF prevents apoptosis induced by cytokine withdrawal in interleukin (IL)-3-dependent mouse Ba/F3 cells (13). However, B-cell progenitor-specific conditional *E2A-HLF* knock-in mice exhibit hyposplenia and lymphopenia, whereas hematopoietic stem/progenitor cell (HSPC)-specific ones are embryonically lethal (14). The E2A-HLF fusion likely requires additional events to cause leukemia, since immunoglobulin enhancer and promoter-driven E2A-HLF transgenic and knock-in mice exhibit maturation arrest and apoptosis in cells expressing *E2A-HLF* (14-16). Numerous molecules downstream of E2A-HLF or cooperative with E2A-HLF have since been identified, including transcription factor Lim domain only 2 (LMO2), which is involved in T-cell ALL (17,18); snail family transcriptional repressor 2 (19); nuclear factor, interleukin 3 regulated (20); Groucho-related genes (21); Annexin VIII and sushi-repeat protein upregulated in leukemia, which have paraneoplastic roles in E2A-HLF-expressing leukemia (22); Zfp521 (23); survivin (24); and death receptors DR4/DR5 (25).

Drosophila eyes absent homolog 2 protein belongs to the eyes absent (Eya) family of proteins and acts as a transcriptional co-activator. Eya proteins serve a critical role in fly eye development and are also involved in numerous processes, including organ development, innate immunity, and DNA damage repair. Eya proteins have threonine

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phosphatase and transactivation activity in the N-terminal domain and tyrosine phosphatase and protein-interacting activity in the C-terminal domain. Eya proteins are located in the cytoplasm and are translocated into the nucleus following binding to Six protein for transactivation (26-28). Human EYA transcriptional coactivator and phosphatase 2 (*EYA2*) is located on chromosome 20q13 (29). *EYA2* was reported to be upregulated in ovarian and breast cancer and astrocytoma (30-32). Eya2 was also reported to promote metastasis of breast cancer cells (31) and to promote the proliferation and invasion of human astrocytoma cells (32). By contrast, silencing of *EYA2* promotes tumor growth in pancreatic adenocarcinoma, indicating the tumor suppressive function of EYA2 (33). *Eya2* is differentially expressed in mouse long-term hematopoietic stem cells (34) and confers an aberrant self-renewal capacity in HSPCs (35). In addition, Eya2 is critically involved in leukemogenesis via zinc finger and BTB domain containing 16-retinoic acid receptor-α (*PLZF-RARA*) resulting from t(11;17)(q23;q21) in patients with acute promyelocytic leukemia (35).

To identify effective therapeutic targets in leukemia with *E2A-HLF*, it is important to clarify the molecular mechanism underlying E2A-HLF-mediated leukemogenesis. The present study investigated the upregulation of *Eya2* by E2A-HLF through promotor binding. It was also demonstrated that Eya2 has a crucial role in the aberrant self-renewal capacity conferred by E2A-HLF. *Eya2* knockdown experiments via retrovirally-expressed short hairpin (sh)RNA revealed the critical involvement of Eya2 in immortalizing HSPCs. The present findings therefore identified Eya2 as one of the key players downstream of oncogenic E2A-HLF.

Materials and methods

Mice. Mice were kept under standardized (temperature, 22-24˚C; humidity, 45-65%; 12 h light/12 h dark cycle; free access to food and water) and specific pathogen-free conditions until sacrifice. For purification of mouse HSPCs, bone marrow cells were harvested from one to two female C57BL/6N mice (8-12 weeks old; body weight, 17-21 g) which were purchased from Japan SLC, Inc. (Hamamatsu, Japan), for each experiment (n=13 in total). All animal studies were approved by the Animal Care Committees of Mie University (Tsu, Japan).

Reagents. G418 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and puromycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were used at final concentrations of 1 mg/ml and 1 μg/ml, respectively, for drug selection.

Construction of the plasmids and retroviral vectors. The retroviral vectors used in this study, pMYs-IRES-Neomycin^r (pMYs-IN) and pMXsU6-Kusabira Orange (KO), were previously described (36,37). The pMYs-E2A-HLF-IN vector was also described previously (36). To produce the E2A-pre-B-cell leukemia transcription factor 1 (*E2A-PBX1*) (38) fusion fragment, a portion of *PBX1* encoding amino acid residues 89-430 (39) generated by polymerase chain reaction (PCR) using cDNA derived from K562 cells (provided by Dr. Toshio Kitamura, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan), was inserted into pMYs-E2A-HLF-IN to replace that of *HLF*. *E2A-HLF* mutants were prepared as previously reported (40). Mutants lacking the AD1 domain, AD2 domain, bZIP domain, and a part of the basic region, respectively, were generated by site-directed mutagenesis using PCR with the wild-type *E2A-HLF* construct as a template, followed by cloning in a series of pMYs retroviral vectors (37). The *E2A-HLF* mutants were as follows: i) ΔAD1, which lacks 426 bp (Met-1 to Gly-142); ii) ΔAD2, which lacks the 405 bp *Pvu*II-*Nae*I restriction fragment (Leu-278 to Ala-412) in the E2A transactivation region; iii) ΔbZIP, which lacks 132 bp (Try-508 to Ala-551) in the bZIP domain of HLF; and iv) Δ509-518, which lacks 30 bp (Ala-509 to Ala-518) in the basic region of HLF. For the chromatin immunoprecipitation (ChIP) analysis, E2A-HLF was fused with the FLAG epitope tag at the N-terminus in pMYs-E2A-HLF-IN. PCR for construction was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, MA, USA), according to the manufacturer's protocol. Each insert fragment in the plasmid was validated by DNA sequence analysis. For the reporter assay, *E2A-HLF* and *E2A-PBX1* cDNAs were subcloned into pcDNA3.1+ expression vector (Invitrogen; Thermo Fisher Scientific, Inc.), respectively.

Purification of mouse HSPCs. Mouse HSPCs were purified as described (36). In brief, bone marrow mononuclear cells (BMMNCs) were prepared from 8- to 12-week-old C57BL/6N mice. Using a MACS cell separation system (Miltenyi Biotec, Inc., Auburn, CA, USA), Lin-depleted cells were isolated from BMMNCs, and c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) cells were purified from Lin-depleted cells using a FACSAria operated with FACSDiVa version 6.1.3 software (BD Biosciences, San Jose, CA, USA).

Retrovirus production and transduction. Plat-E packaging cells (41) were plated at a density of $4.5x10^5$/ml and transfected the following day with retroviral constructs using Polyethylenimine 'Max' (Polysciences, Inc., Warrington, PA, USA), according to the manufacturer's protocol. Retroviral supernatants of transfected Plat-E cells were harvested 48 h post-transfection following two medium changes. KSL and immortalized cells were transduced with retroviruses using RetroNectin (Takara Bio Inc., Otsu, Japan) for 48-72 h at 37˚C, as previously described (36).

Myeloid immortalization assay. Myeloid immortalization assays via serial replating were performed as previously described (36). In brief, every 5-7 days, colonies were counted, followed by replating of the harvested cells ($1x10^4$ cells/dish) in methylcellulose culture medium MethoCult™ M3234 (STEMCELL Technologies, Inc., Vancouver, BC, Canada) supplemented with 25 ng/ml mouse stem cell factor (SCF) (Miltenyi Biotec, Inc.), 10 ng/ml each of mouse IL-3 (Miltenyi Biotec, Inc.), human IL-6 (Miltenyi Biotec, Inc.), and mouse granulocyte macrophage-colony stimulating factor (GM-CSF) (Miltenyi Biotec, Inc.).

To evaluate the effects of knockdown of *Eya2* in the immortalized cells, the Eya2-depleted cells were plated in the same methylcellulose culture medium as that used in the immortalization assays. Relative colony-forming units (CFUs) were calculated as a percentage of the colony numbers compared

with the corresponding controls (normalized to 100%) in each experiment following culture for 5-7 days.

Fluorescence-activated cell sorting (FACS) analysis. An immunophenotypical analysis was performed using a FACSCalibur and BD Cell Quest Pro version 5.2.1 software (BD Biosciences), as previously described (42).

Gene silencing by RNA interference. The target sequences against the *Eya2* and *luciferase* genes were 5'-GTGTTTCAG AGACAATCAT-3' (shE09) and 5'-GCCTTATGCCGCCAT CTTG-3' (shE12), and 5'-GGCTATGAAGAGATACGCC-3' (shLuc). The shE09 and shE12 were two out of twelve sequences (shE01-shE12) designed for *Eya2* knockdown, which exerted the most efficient effects (35). To design a short hairpin structure, a loop sequence (5'-CTTCAAGAGAG-3') was used (35). Short hairpin RNA (shRNA) sequence(s) against *Eya2* or *luciferase* were cloned into the retroviral vector pMXsU6-KO (36). Each 9 μg DNA of pMXsU6-KO derivative was transfected into Plat-E cells (41) in a 10 cm dish to produce retroviruses, as described above. The filtered culture supernatant of Plat-E cells containing retroviruses 48 h post-transfection was used for transduction of *E2A-HLF*-immortalized mouse KSL cells ($2.5x10^5$) for 48 h at 37˚C using RetroNectin (Takara Bio, Inc.) in liquid culture containing SCF, IL-3, IL-6, and GM-CSF. The shRNA-transduced cells were then subjected to sorting by KO expression on the FACSAria operated by FACSDiVa version 6.1.3 software. The sorted KO^+ cells were cultured for 5-7 days for a colony-forming assay as previously described (36).

ChIP. ChIP was performed as previously described (36). In brief, the chromatin prepared from FLAG-tagged *E2A-HLF*-immortalized cells was precipitated using Dynabeads anti-Mouse IgG (Invitrogen; Thermo Fisher Scientific, Inc.) preincubated with a mouse monoclonal anti-FLAG (cat. no. M2; Sigma-Aldrich; Merck KGaA), a mouse monoclonal anti-RNA polymerase II (cat. no. CTD4H8; EMD Millipore, Billerica, MA, USA), or a mouse IgG1 antibody (BioLegend, Inc., San Diego, CA, USA). The purified DNA in the precipitates was subjected to quantitative polymerase chain reaction (qPCR).

Reverse transcription (RT), qPCR, RT-qPCR and RT-PCR. Total RNA was extracted using TRI Reagent LS (Molecular Research Center, Inc., Cincinnati, OH, USA). RT was performed with random hexamers using SuperScript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (36). The qPCR analyses were performed using the KOD SYBR qPCR Mix (for ChIP products; Toyobo Life Science, Osaka, Japan) or PowerSYBR® Green PCR Master Mix (for cDNA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) (36). For the RT-qPCR analysis, PCR was performed for 40 cycles (94˚C for 15 sec, 57˚C for 15 sec and 72˚C for 34 sec) in a total volume of 12 μl containing appropriately diluted cDNA. Following quantification of the expression of samples using the quantification cycle ($2^{-\Delta\Delta CT}$) method (43) and performing normalization relative to β-2-microglobulin (*B2m*), the relative expression was calculated. The sequences of the primers used (*Eya1*, *Eya2*, *Eya3*, *Eya4*, and *B2m*) have been previously described (35,36). For the ChIP-qPCR analysis, PCR was performed for 40 cycles (98˚C for 10 sec, 60˚C for 10 sec and 68˚C for 34 sec) in a total volume of 20 μl. When the CT values of the ChIP products had been measured and normalized to those of the corresponding input samples using the $2^{-\Delta\Delta CT}$ method, the percentages of samples relative to the input were calculated. The primer sets (*Eya2c-4*) for ChIP-qPCR were described previously (35). The promoter region of hemoglobin subunit β1 was used as a negative control. RT-PCR was performed as previously described (42). In brief, to detect the transcripts of *E2A-HLF*, standard PCR amplification of cDNA was performed for 30 cycles (98˚C for 10 sec and 68˚C for 40 sec) using LA Taq (Takara Bio Inc.). The primers specific for *E2A-HLF* were as follows: E2A S4, 5'-GATAGAAGACCA CCTGGACGAG-3'; E2A S2, 5'-GTGAGGACTACGGCA GGGAT-3'; HLF AS1, 5'-CCAGCTCCTTCCTCAAGTCAG-3'; and HLF ASx, 5'-gaaagaattcaCAGGGGCCCGTGCCTGG-3' (small letters, non-related sequence). To detect transcripts of *B2m*, PCR amplification of cDNA was performed for 26 cycles (94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec) using Quick TaqR HS DyeMix (Toyobo Life Science). The primers specific for *B2m* were as previously described (44). Together with 1 kb DNA ladder (New England Biolabs, Inc.) as a DNA size marker, the PCR products were electrophoresed on a 1% Tris-Acetate-EDTA agarose (Nacalai Tesque, Inc., Kyoto, Japan) gel, subsequently stained with ethidium bromide (VWR International, Radnor, PA, USA), and were visualized with an ultraviolet transilluminator system, Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's recommendation.

Western blot analyses. The expression of transgenes in transfected Plat-E cells was examined by western blot analysis, as previously described (45). In brief, the transfected cells were harvested with lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) and 2 mM phenylmethylsulfonyl fluoride. The lysates were mixed with an equal volume of 2X SDS sample buffer and boiled for 5 min. Western blot analyses of the samples obtained from the cells transfected with pMYs-IN, pMYs-E2A-HLF-IN, pMYs-E2A-PBX1-IN, pMYs-ΔAD1-IN, pMYs-ΔAD2-IN, pMYs-ΔbZIP-IN, and pMYs-Δ509-518-IN were performed using mouse monoclonal anti-E2A (Yae) (at 1:1,000 dilution for 1 h at room temperature; cat. no. sc-416; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-α-tubulin (at 1:10,000 dilution for 1 h at room temperature; cat. no. T-5168; Sigma-Aldrich; Merck KGaA) antibodies, followed by reaction with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (at 1:5,000 dilution for 1 h at room temperature; cat. no. 330; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

Luciferase reporter constructs and assays. To generate pGL3-mP, a minimal promotor (mP) sequence (derived from pGL 4.23; Promega Corporation, Madison, WI, USA) was inserted between the *Nhe*I and *Hin*dIII sites in the pGL3-Basic Vector (Promega Corporation). The putative promotor region of *Eya2* (-2219 to -226, relative to the ATG of the translation

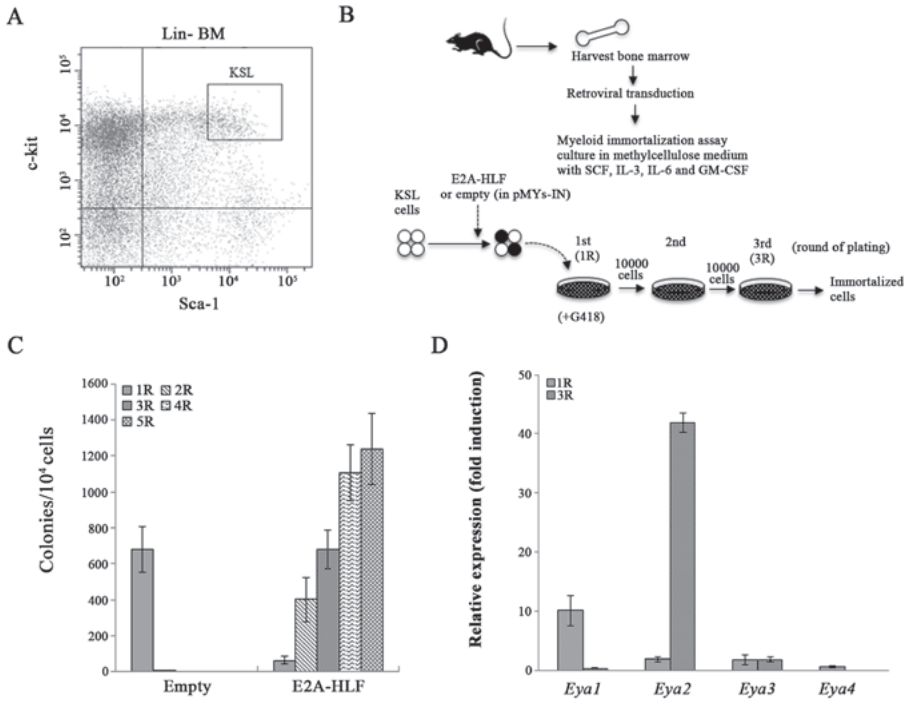

Figure 1. Myeloid immortalization of KSL cells by *E2A-HLF* in association with *Eya2* expression. (A) Lin-depleted (Lin$^-$) BM cells are displayed with the sorting gate for KSL cells. (B) The experimental strategy for myeloid immortalization assays of KSL cells with retroviral transduction of *E2A-HLF*. pMYs-IN was used as the backbone vector (empty). (C) Myeloid immortalization assays in KSL cells following retroviral transduction. The transduced cells were harvested 5-7 days post-transduction, followed by replating of $1x10^4$ cells. (D) The expression of *Eya* family genes by reverse transcription-quantitative polymerase chain reaction normalized to *B2m* in the myeloid immortalization assays. *E2A-HLF*-transduced cells were harvested at the end of the first round and the third round of plating in (B). The *Eya4* expression at 3R in *E2A-HLF*-transduced cells was below the detection level. The relative induction of each *Eya* family gene following transduction of *E2A-HLF* was calculated in comparison with transduction of the empty vector. Bar graphs present the mean ± standard deviation of three independent experiments. BM, bone marrow; *E2A-HLF*, immunoglobulin enhancer-binding factor/hepatic leukemia factor; *Eya*, EYA transcriptional coactivator and phosphatase; pMYs-IN, pMYs-IRES-Neomycin^r; R, round of plating.

initiation site) was inserted upstream of the mP sequence in pGL3-mP to generate pGL3-E2pro3. pGL3-E2pro3CSmut with the sequence CTATTCTAGT instead of CTTACCTAGT, a putative DNA-binding sequence of E2A-HLF that is similar to the consensus sequence (CS) GTTACGTAAT (9), was generated by PCR-mediated mutagenesis. All constructs for appropriate insertions were confirmed by DNA sequence analyses. Human leukemic K562 cells ($2.5x10^6$) were resuspended in 400 μl K-PBS (NaCl, 30.8 mM; KCl, 120.7 mM; Na_2HPO_4, 8.1 mM; KH_2PO_4, 1.46 mM) containing reporter (6.6 μg pGL3-E2pro3 or pGL3-E2pro3CSmut), equimolar amounts of effector (pcDNA-E2A-HLF, pcDNA-E2A-PBX1 or empty vector), and 0.5 μg internal control (phTK-RL; Promega Corporation) plasmid DNA. The reporter-to-effector molar ratio of the DNA was 2:1. These cells were electroporated at 170 V and 950 μF in a 4 mm cuvette using Gene Pulser Xcell (Bio-Rad Laboratories, Inc.), and were incubated in the culture medium for 48 h. Cells were harvested post-incubation and lysed in 100 μl lysis buffer (Promega Corporation). The activity of firefly and *Renilla* luciferases in each lysate was measured sequentially using a Dual Luciferase Assay System (Promega Corporation) on a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA, USA), according to the manufacturer's protocol.

Statistical analyses. Analyzed data are presented as mean ± standard deviation of three independent experiments. Comparisons of bar charts between two groups were performed using an unpaired one-tailed Student's t-test. As for the statistical comparisons of more than two groups, the assumption of homogeneity of variance was first tested using Bartlett's test. In cases where the variances were not equal at the 0.05 level, statistical differences were analyzed using a Friedman test as a non-parametric analysis of variance, followed by Steel's test as a post hoc multiple comparison test. All statistical tests were performed using R software version 3.1.2 (https://www.r-project.org/).

Results

Eya2 upregulation in E2A-HLF-immortalized cells. KSL cells were sorted from Lin-depleted bone marrow cells, retrovirally transduced with the *E2A-HLF* fusion oncogene, and subjected to a myeloid immortalization assay. *E2A-HLF* transduction immortalized KSL cells (Fig. 1A-C). Since it was recently revealed that Eya2 is important for inducing aberrant self-renewal activity in HSPCs (35), the expression of the *Eya* family genes *Eya1*, *Eya2*, *Eya3*, and *Eya4* was analyzed in *E2A-HLF*-transduced colony-forming cells. An RT-qPCR analysis revealed that *Eya2* was exclusively highly expressed among these genes following serial replating (Fig. 1D). Although the *Eya1* expression was increased compared with that of *Eya2* in the first-round colonies, the *Eya1* expression was much lower compared with that of *Eya2* in the third-round colonies. *Eya3* and *Eya4* were not highly expressed in either the first- or third-round colonies. These results suggested that an E2A-HLF fusion product induced

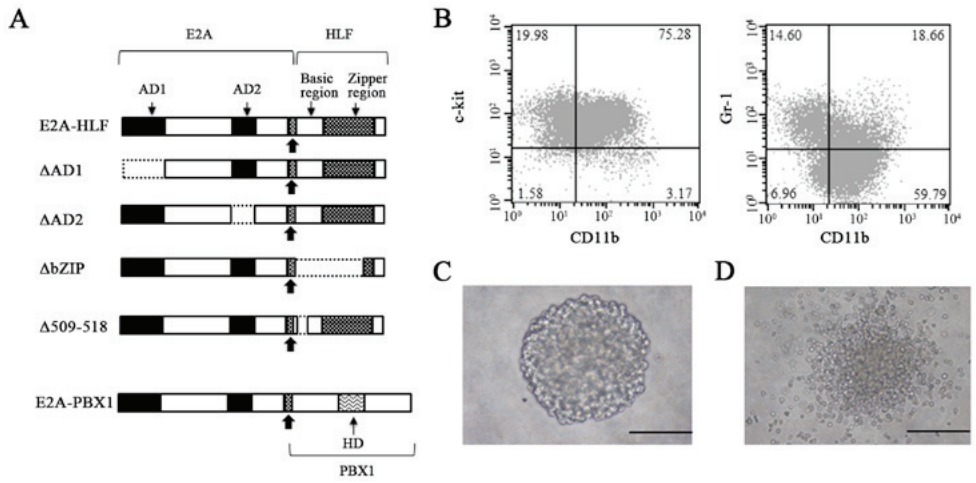

Figure 2. A schematic representation of the chimeric proteins E2A-HLF, E2A-HLF mutants, and E2A-PBX1 used in this study, and the characterization of the cells immortalized by *E2A-HLF*. (A) The chimeric oncoproteins E2A-HLF and E2A-PBX1 produced by the t(17;19) and t(1;19) translocations, respectively, retain the N-terminal transactivation domains of E2A. The E2A C-terminus including the bHLH DNA-binding and protein dimerization domain is replaced with the bZIP DNA-binding and dimerization domain of the HLF protein and a region of PBX1 containing a homeobox DNA-binding domain, respectively. Bold arrows indicate the fusion sites. The proportions of each domain are not to scale in this representation, in order to facilitate comparisons. (B) Immunophenotype of the cells immortalized by retroviral transduction of *E2A-HLF*. The typical morphology of the colonies generated by (C) E2A-HLF and (D) Δ509-518 at the end of the third round is presented. Colonies generated by the E2A-HLF mutant were smaller and less dense compared with those generated by the wild-type. Scale bar, 200 μm. E2A-HLF, immunoglobulin enhancer-binding factor/hepatic leukemia factor; PBX1, pre-B-cell leukemia transcription factor 1; CD, cluster of differentiation; AD activation domain; HD, homeodomain.

upregulation of *Eya2* in HSPCs, which may be associated with aberrant self-renewal activity in *E2A-HLF*-immortalized HSPCs.

E2A-HLF upregulates Eya2 expression in the aberrant self-renewal program. To investigate the molecular mechanism underlying the upregulation of *Eya2* by E2A-HLF in HSPCs, mouse KSL cells were retrovirally transduced with *E2A-HLF* and its mutants (Fig. 2A) and subjected to myeloid immortalization assays. The immortalized cells exhibited an immunophenotype of myeloid lineage with high expression of c-kit (Fig. 2B). Initial plating showed similar colony morphologies and reflected the transduction efficiency. In serial replating, *E2A-HLF*-transduced cells yielded and maintained increased numbers of dense colonies, whereas *E2A-HLF* mutant-transduced cells formed loose colonies that were smaller in size compared with those with *E2A-HLF* at the third round (Figs. 2C and D, and 3A) and failed to be immortalized (data not shown). A western blot analysis indicated similar expression of each mutant protein to that of wild-type E2A-HLF (Fig. 3B). The expression of the chimeric transcripts of *E2A-HLF* and *E2A-HLF* mutants was detected by RT-PCR of total RNA extracted from the transduced colonies at the first- and third-round plating, respectively. RT-PCR using primers E2A S4 and HLF AS1 in E2A-HLF, ΔAD1 and Δ509-518 generated 497, 497 and 467 bp products, respectively, while that using primers E2A S4 and HLF ASx in ΔbZIP generated a 426 bp product, and that using primers E2A S2 and HLF AS1 in ΔAD2 generated a 696 bp product (Fig. 3C).

ΔAD1 and ΔAD2 mutants lack each transactivation domain of E2A to abolish the transactivation ability while retaining DNA-binding and dimerization abilities, while ΔbZIP lacks the basic region and the leucine zipper domain of HLF to abolish DNA-binding and dimerization abilities, and Δ509-518 lacks a part of the basic region of HLF to abolish DNA-binding ability (Fig. 2A). Wild type *E2A-HLF* transduction immortalized KSL cells in serial colony replating (Figs. 1C, and 4A and B). Consistent with a previous report demonstrating that the two transactivation domains of E2A and the leucine zipper dimerization domain of HLF are essential for the transforming potential of the E2A-HLF in NIH3T3 cells (12), ΔAD2- and ΔbZIP-transduced cells failed to form colonies in serial replating. In addition, Δ509-518-transduced cells also failed to form colonies (Fig. 4B). ΔAD1-transduced cells formed some colonies at the third round, although they lost the ability at the fifth round (data not shown). Transduction of *E2A-PBX1*, a fusion oncogene generated by t(1;19)(q23;p13) (38), also induced immortalization of KSL cells (Fig. 4B). RT-qPCR analyses demonstrated that *Eya2* was highly expressed in the transduced cells with wild-type *E2A-HLF* or *E2A-PBX1*. The mutant ΔAD1- and ΔAD2-transduced cells exhibited elevated *Eya2* expression, albeit to a lesser extent compared with the wild-type. However, ΔbZIP- or Δ509-518-transduced cells showed no induction of *Eya2* expression (Fig. 4C). It is noteworthy that the colony-forming ability of E2A-HLF and its mutants roughly correlated with the level of *Eya2* expression. These results suggested that the two AD domains of E2A are required for complete activity of *Eya2* transactivation, while the DNA-binding and dimerization abilities of HLF are indispensable for *Eya2* transactivation in KSL-derived cells.

Eya2 is critical for the E2A-HLF-mediated myeloid immortalization of HSPCs. To corroborate the involvement of Eya2 in myeloid immortalization by E2A-HLF, *Eya2* was depleted in *E2A-HLF*-immortalized cells using shRNA expression (Fig. 5). The expression of *E2A-HLF* chimeric transcripts was not affected following shRNA transduction according to RT-PCR analysis of total RNA extracted from shRNA-transduced cells (Fig. 5B). shE09 and shE12 exhibited

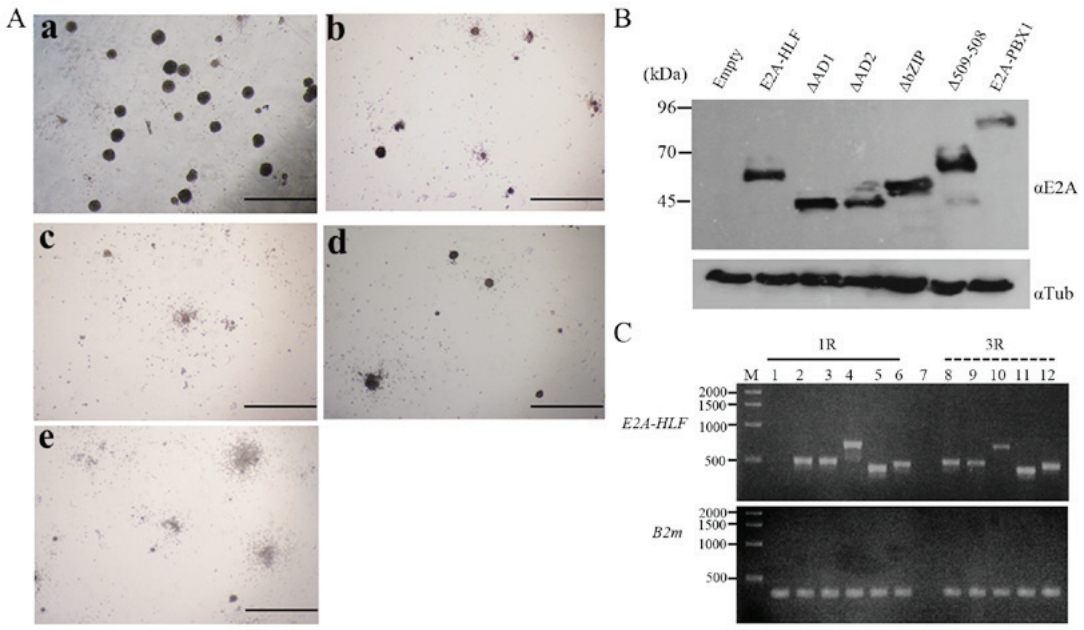

Figure 3. Colony formation in the methylcellulose assay by E2A-HLF and E2A-HLF mutants and the expression of these proteins. (A) Colonies generated by E2A-HLF and its mutants at the end of the third round of plating. Colonies generated by the mutants exhibited an altered morphology. (Aa) E2A-HLF; (Ab) ΔAD1; (Ac) ΔAD2; (Ad) ΔbZIP; (Ae) Δ509-518. Colonies were viewed with an Olympus CKX41 microscope. Scale bar, 200 μm. (B) The expression of wild-type and mutant E2A-HLF chimeric proteins was assessed by western blot analysis. Lysates extracted from Plat-E cells (41) transfected with empty vector (pMYs-IN), pMYs-E2A-HLF-IN, E2A-HLF mutants (pMYs-ΔAD1-IN, pMYs-ΔAD2-IN, pMYs-ΔbZIP-IN, and pMYs-Δ509-518-IN), and pMYs-E2A-PBX1-IN were blotted with the anti-E2A antibody (αE2A), followed by reprobing with αTub antibody as an internal control. (C) The expression of *E2A-HLF* by reverse transcription-polymerase chain reaction in the cells from the first- and third-round cultures. B2m was used as an internal standard. M, 1 kb DNA ladder; lane 1, mock (pMYs-IN); lane 2, *E2A-HLF*; lane 3, Δ*AD1*; lane 4, Δ*AD2*; lane 5, Δ*bZIP*; lane 6, Δ*509-518*; lane 7, negative control (water instead of cDNA mixture); lane 8, *E2A-HLF*; lane 9, Δ*AD1*; lane 10, Δ*AD2*; lane 11, Δ*bZIP*; lane 12, Δ*509-518*. E2A-HLF, immunoglobulin enhancer-binding factor/hepatic leukemia factor; PBX1, pre-B-cell leukemia transcription factor 1; αTub, α-tubulin; R, round of plating; *B2m*, β-2-microglobulin.

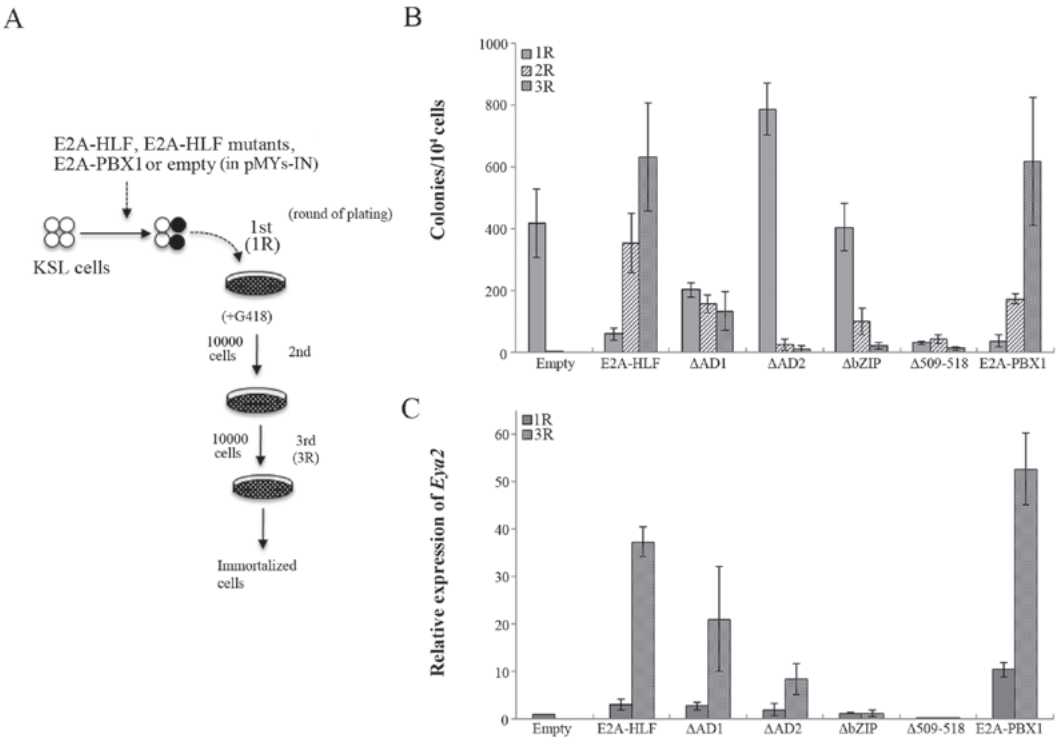

Figure 4. *Eya2* expression in *E2A-HLF*-mediated immortalization of hematopoietic stem/progenitor cells. (A) The experimental strategy for the myeloid immortalization assays of KSL cells. The cells were retrovirally transduced with empty vector, *E2A-HLF*, its mutants, and *E2A-PBX1* in pMYs-IN. (B) Myeloid immortalization assays in KSL cells following retroviral transduction. The transduced cells were harvested 5-7 days post-transduction, followed by replating of $1x10^4$ cells. (C) Expression levels of *Eya2* were measured by reverse transcription-quantitative polymerase chain reaction and normalized to *B2m* in colony-forming cells at the end of the first and third round of plating in panel (A). Bar graphs illustrate the mean ± standard deviation of three independent experiments. R, round of plating; E2A-HLF, immunoglobulin enhancer-binding factor/hepatic leukemia factor; PBX1, pre-B-cell leukemia transcription factor 1; AD, activation domain; *B2m*, β-2-microglobulin.

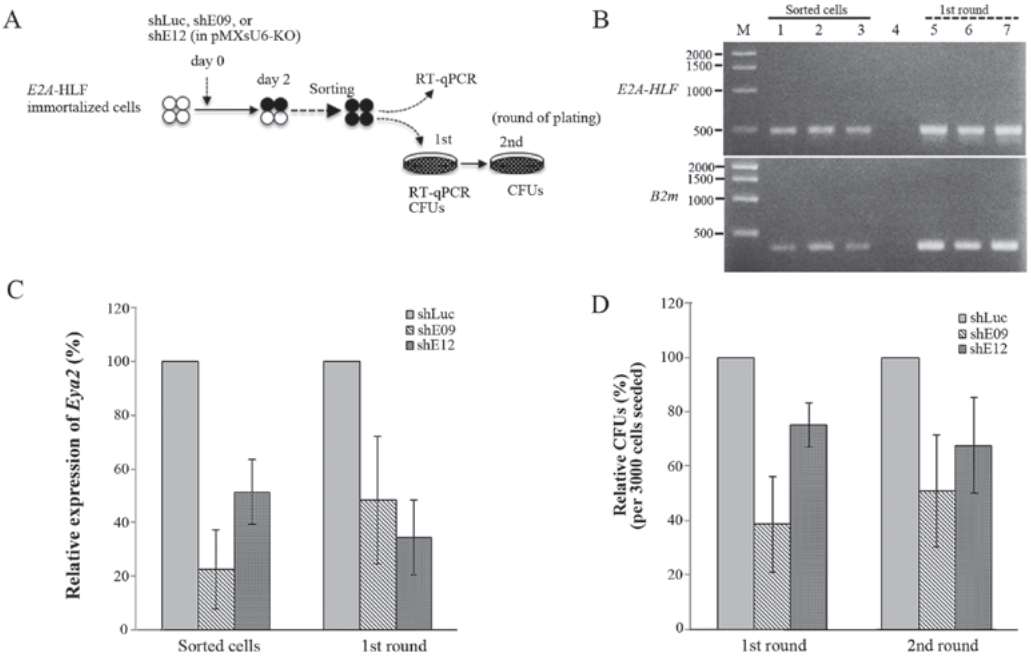

Figure 5. Suppressive effects of Eya2 depletion on the clonogenicity of *E2A-HLF*-immortalized KSL cells. (A) Experimental strategy for the analysis of *E2A-HLF*-immortalized cells with Eya2 depletion by retroviral transduction of the shRNA/KO co-expresser in pMXsU6-KO. (B) The expression of *E2A-HLF* by RT-PCR in the sorted cells and colony-forming cells at the end of the first-round of plating. *B2m* was used as an internal standard. M, 1 kb DNA ladder; lane 1, shLuc; lane 2, shE09; lane 3, shE12; lane 4, negative control, lane 5, shLuc; lane 6, shE09; lane 7, shE12. (C) The expression of *Eya2* by RT-qPCR in the cells sorted from shRNA-transduced *E2A-HLF*-immortalized cells and subsequent colony-forming cells at the end of the first round of plating. (D) Relative CFUs of the cells at the end of the first and second round of plating. Bar graphs illustrate the mean ± standard deviation of three independent experiments. CFU, colony-forming unit; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; sh, short hairpin; Luc, luciferase; *E2A-HLF*, immunoglobulin enhancer-binding factor/hepatic leukemia factor; PBX1, pre-B-cell leukemia transcription factor 1; Eya2, EYA transcriptional coactivator and phosphatase 2; *B2m*, β-2-microglobulin; KO, Kusabira-Orange.

effective depletion of endogenous *Eya2* expression in the E2A-HLF-immortalized cells (Fig. 5C), and *Eya2* knockdown by shE09 and shE12 reduced the clonogenicity to ~40 and 70% of that in the control at the first round of plating, respectively (Fig. 5D). It was not possible to perform rescue experiments on *Eya2*-knockdown HSPCs that were immortalized with *E2A-HLF* by forced expression of *Eya2* without the shRNA target sequence, likely due to the genotoxic stress induced by *Eya2* overexpression, as the forced expression of *Eya2* resulted in markedly reduced colony-forming efficiency in *E2A-HLF*-immortalized cells (data not shown).

E2A-HLF upregulates the Eya2 expression by binding to the promoter region. It was previously observed that, in *Plzf*-mediated immortalization, Plzf binds to the putative *Eya2* promoter region around exon 1c of *Eya2* (35), in accordance with public data (46) from ChIP sequencing. Therefore, a ChIP-qPCR analysis of FLAG-tagged *E2A-HLF*-immortalized HSPCs with the primers around exon 1c was performed (Fig. 6). The analyses indicated increased binding of E2A-HLF to the *Eya2* promoter region in the immortalized cells, accompanied by RNA pol II binding signals (Fig. 6A). These results suggested that E2A-HLF drives the aberrant expression of *Eya2* in HSPCs by promoter binding. Subsequently, the effect of E2A-HLF expression on *Eya2* promoter activity was confirmed using a reporter assay. The luciferase assay in K562 cells demonstrated that E2A-HLF and E2A-PBX1 activated the reporter gene expression through the *Eya2* promoter region (Fig. 6C), and transactivation through the *Eya2* promoter region was significantly reduced by the mutation of the putative E2A-HLF binding consensus sequence (Fig. 6B and D). Taken together, these findings suggested that E2A-HLF upregulates *Eya2* transcription through the E2A-HLF binding consensus sequence.

Discussion

To clarify the mechanism underlying E2A-HLF-mediated leukemogenesis, the present study examined the mechanism whereby E2A-HLF leads to the transformation of HSPCs, and explored therapeutic targets for novel treatments.

Since E2A-HLF alone is reportedly unable to immortalize mouse hematopoietic cells *in vitro* under lymphoid conditions on irradiated stromal cells, unless the anti-apoptotic molecule BCL-2 is forcibly co-expressed (47), the present study employed a myeloid condition to assess the transforming potential of E2A-HLF on mouse HSPCs *in vitro*, where *E2A-HLF* expression alone is sufficient to immortalize mouse HSPCs. It was observed that E2A-HLF binds to the promotor of *Eya2* to elevate *Eya2* expression in HSPCs where endogenous *Eya2* is preferentially expressed (34). The present study also identified the cis-acting element that resembles the previously reported DNA-binding consensus sequence (10) within the *Eya2* promoter region in transactivation by E2A-HLF. It was recently reported that the forced expression of *Eya2* using a retroviral vector induces aberrant self-renewal in mouse HSPCs (35). The suppressive effect induced by *Eya2* depletion on the clonogenicity of *E2A-HLF*-immortalized mouse HSPCs

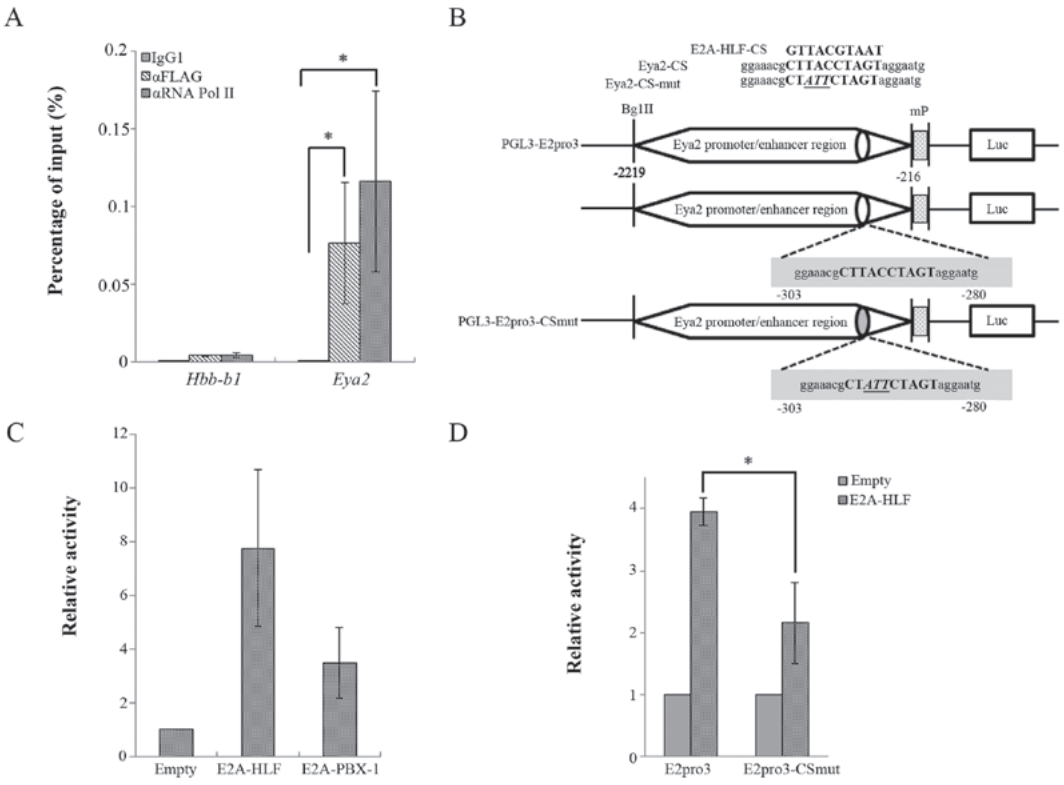

Figure 6. E2A-HLF upregulates *Eya2* expression by binding to the promoter region. (A) A ChIP-qPCR analysis on the *Eya2* promoter region in *E2A-HLF*-immortalized mouse hematopoietic stem/progenitor cells. The relative binding activity of E2A-HLF (detected by the anti-Flag antibody) and RNA polymerase II around exon 1c of *Eya2* in KSL cells immortalized by FLAG-tagged E2A-HLF is presented. The promoter region of *Hbb-b1* was examined as a negative control. Statistical analysis was performed using a Friedman test, followed by a Steel's test for post hoc nonparametric multiple comparisons. (B) A schematic representation of two reporter constructs for the luciferase assay. Mutations in the consensus sequence (9) are underlined. The position of the 5'end of the protein coding sequence in exon 2 of mouse *Eya2* is numbered +1. The CS of E2A-HLF binding is located within the amplified region by ChIP-qPCR. (C) The effects of E2A-HLF and E2A-PBX1 on the *Eya2* promoter activity in the luciferase assay in K562 cells. (D) A luciferase assay using a wild-type *Eya2* and a mutated reporter construct containing a putative E2A-HLF-binding CS. Statistical analysis was performed using a t-test. *P<0.05. Bar graphs illustrate the mean ± standard deviation of three independent experiments. ChIP, chromatin immunoprecipitation; *Eya2*, EYA transcriptional coactivator and phosphatase 2; mut, mutant; E2A-HLF, immunoglobulin enhancer-binding factor/hepatic leukemia factor; PBX1, pre-B-cell leukemia transcription factor 1; Luc, luciferase; *Hbb-b1*, hemoglobin subunit β1; Eya2, EYA transcriptional coactivator and phosphatase 2; qPCR, quantitative polymerase chain reaction; CS, consensus sequence.

in the present study suggested the involvement of Eya2 in the aberrant self-renewal capacity of *E2A-HLF*-immortalized HSPCs.

Previous studies revealed the anti-apoptotic activity of E2A-HLF in IL-3-dependent murine pro-B cells through the AD1 and AD2 transactivation domains of E2A, but not the bZIP domain (13,19,40). However, the molecular mechanism underlying the function of E2A-HLF on the self-renewal and/or cell proliferation of hematopoietic cells is poorly understood. E2A-HLF was previously demonstrated to transactivate the *LMO2* oncogene through the AD1 and AD2 domains of E2A and the basic region of HLF (17). Similar to this finding, the present study revealed that the two transactivation domains of E2A and the basic region of HLF are essential for the E2A-HLF-mediated transformation of mouse HSPCs. The present study focused on Eya2 as one of the key molecules downstream of E2A-HLF. Eya2, as an oncogenic molecule, has been demonstrated to be involved in solid tumors with a poor prognosis (30-32). It was recently observed that Eya2 is critical for leukemogenesis by *PLZF-RARA* (35). The present study further indicated that Eya2 is involved in *E2A-HLF*-mediated leukemogenesis. The same may be true for E2A-PBX1, which also activated *Eya2* expression in the present study. Therefore, Eya2 may be involved more generally in leukemogenesis, and further studies using clinical samples may be helpful to verify this hypothesis. Of note, *Eya2* knockout mice (48) appear to have no severe abnormalities, suggesting that Eya2 may be a potential target of molecular therapy without major adverse effects for certain subtypes of leukemia.

In conclusion, the present study demonstrated the critical role of Eya2 in the *E2A-HLF*-mediated transformation of mouse HSPCs *in vitro*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RO and TN designed the research. BDM and RO performed the experiments. BDM, RO, and TN analyzed the results. BDM and TN wrote the manuscript.

Ethics approval and consent to participate

All animal studies were approved by the Animal Care Committees of Mie University (Tsu, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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