Identification of cooperative genes for E2A-PBX1 to develop acute lymphoblastic leukemia

Yasuyuki Sera,1,*, Norimasa Yasamaki,1,*, Hideaki Oda,2 Akiko Nagamachi,2 Linda Wolff,4 Takeshi Inukai,3 Toshiya Inaba2 and Hiroaki Honda1

1Department of Disease Model, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; 2Department of Pathology, Tokyo Women's Medical University, Tokyo, Japan; 3Department of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; 4Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA; 5Department of Pediatrics, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan

Key words
Acute lymphoblastic leukemia, conditional knock-in mice, E2A-PBX1, retroviral insertional mutagenesis, Zfp521/ZNF521

Correspondence
Hiroaki Honda, Department of Disease Model, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.
Tel: +81-82-257-5819; Fax: +81-82-257-1556; E-mail: hhonda@hiroshima-u.ac.jp

Funding Information
Ministry of Education, Science and Culture of Japan.

These authors contributed equally to this work.

Received December 20, 2015; Revised March 22, 2016; Accepted April 9, 2016

Cancer Sci 107 (2016) 890-898
doi: 10.1111/cas.12945

E2A-PBX1 is a chimeric gene product detected in t(1;19)-bearing acute lymphoblastic leukemia (ALL) with B-cell lineage. To investigate the leukemogenic process, we generated conditional knock-in (cKI) mice for E2A-PBX1, in which E2A-PBX1 is inducibly expressed under the control of the endogenous E2A promoter. Despite the induced expression of E2A-PBX1, no hematopoietic disease was observed, strongly suggesting that additional genetic alterations are required to develop leukemia. To address this possibility, retroviral insertional mutagenesis was used. Virus infection efficiently induced T-cell, B-cell, and biphenotypic ALL in E2A-PBX1 cKI mice. Inverse PCR identified eight retroviral common integration sites, in which enhanced expression was observed in the Gfi1, Mycn, and Pim1 genes. In addition, it is of note that viral integration and overexpression of the Zfp521 gene was detected in one tumor with B-cell lineage; we considered that Zfp521 may play a clinically relevant role in E2A fusion genes to develop B-lineage ALL.

Chromosomal abnormalities are a recurrent feature of human cancers. In human leukemias, a number of disease subtype-specific chromosomal translocations are detected.1–3 These events induce aberrant expression of a translocation-associated gene or generate a chimeric gene product fusing two different genes on different chromosomes, which is considered to play a critical role in the disease pathogenesis.

The E2A gene (also known as TCF3) encodes a basic helix-loop-helix transcription factor belonging to E-box DNA-binding proteins, which plays an essential role in B-cell development.2–3 E2A is located on chromosome 19 and is the target in subsets of acute lymphoblastic leukemia (ALL) with B-cell lineage.4–5 Two different chromosomal translocations involving E2A have been identified, t(1;19)(q23;p13) and t(17;19)(q22;p13), in which the E2A gene is fused to the PBX1 gene on chromosome 1 and the HLF gene on chromosome 17, thereby generating E2A-PBX1 and E2A-HLF chimeric gene products, respectively.3–5 In E2A-PBX1, the transactivation domain of E2A is fused to the homeodomain of PBX1,5,6 and in E2A-HLF, the same domain of E2A is fused to the basic region/leucine zipper domain of HLF.5–6 Thus, in both fusion proteins, the DNA-binding ability depends on the C-terminal PBX1- or HLF-derived region, whereas the transcription activation capacity resides in the N-terminal E2A-derived region.

To clarify the role of E2A-PBX1 in leukemogenesis and to create a mouse model for E2A-PBX1-positive ALL, several different approaches have been made. Kamps et al. transduced E2A-PBX1-expressing retrovirus to hematopoietic progenitor cells and transplanted the cells into syngeneic mice.5 Although the recipient mice developed leukemia, the disease was exclusively classified as acute myeloid leukemia.5 Dedera et al. generated transgenic (Tg) mice expressing E2A-PBX1 under the control of immunoglobulin (Ig) heavy-chain promoter.7 The transgenic mice developed leukemia but the disease was mainly diagnosed as T-cell ALL.7 Subsequently, Bijl et al. created lymphoid-specific transgenic mice for E2A-PBX1 and crossed the mice with CD3e-deficient mice to prevent the development T-cell ALL.8 The compound (E2A-PBX1 Tg and CD3e-deficient) mice mainly developed B-cell malignancies, and the disease onset was accelerated with retroviral insertional mutagenesis, possibly in cooperation with deregulated expression of Hoxa genes.8 These results indicated that E2A-PBX1 possesses oncogenic potential in various types of hematopoietic progenitors and suggest that E2A-PBX1 renders oncogenicity to myeloid and T-lymphoid cells rather than leukemia.
than B-lymphoid cells. Therefore, the mechanism of how E2A-PBX1 is detected in B-lineage ALL in the human is not yet clarified.

To address this issue and to create a more clinically relevant model for E2A-PBX1-positive leukemia, we generated conditional knock-in (cKI) mice for E2A-PBX1 in which E2A-PBX1 is inducibly expressed under the control of the native E2A promoter.

Materials and Methods

Construction of a targeting vector and generation of cKI mice. The methods of construction of the cKI vector and generation of cKI mice were essential the same as previously described,(9) except that Flag-tagged human E2A-PBX1 cDNA was used instead of human E2A-HLF cDNA. WT/EPKINeo+ mice were crossed with MxCre+ mice to generate WT/EPKINeo+, MxCre+ mice. Cre activation was achieved by i.p. treatment with 500 μg polyinosinic-polycytidylic acid; Tg, transgenic.

Southern blot and genome PCR. Southern blot analyses and genome PCR were carried out as previously described.(9)

Immunoprecipitation and Western blot analyses. Immunoprecipitation and Western blot analyses were carried out as previously described.(9) The anti-Flag antibody was purchased from Sigma (#F7425).

Flow cytometric and gene rearrangement analyses. For flow cytometric analysis, cells were stained with phycoerythrin.

Fig. 1. Generation of E2A-PBX1 conditional knock-in (cKI) mice and acquired expression of E2A-PBX1. (a) Schematic illustration of the conditional KI strategy. Part of the non-coding region of exon 2, the coding region of exon 2, intron 2, and part of the coding region of exon 3 were replaced with a floxed neomycin resistance gene (Neo), followed by Flag-tagged E2A-PBX1 cDNA, IRES-GFP (Ig), and a polyadenylation signal (pA). Restriction enzymes: C, ClaI; H, HindIII; N, Nael; X, XbaI. The positions of the 5' probe for Southern blot analysis and P1 and P2 primers for the 3’ genome PCR are shown. (b) Results of 5’ Southern blot and 3’ genomic PCR to detect homologous recombination. Positions of germline (GL) and KI allele-derived bands by 5’ Southern blot and the PCR product by 3’ genomic PCR are indicated by arrows and an arrowhead, respectively. (c) Acquired E2A-PBX1 expression. Proteins extracted from the spleen of WT/EPKINeo+ and WT/EPKINeo+ mice were immunoprecipitated with an anti-E2A antibody and the immunoprecipitated proteins were blotted with an anti-Flag antibody. The positions of E2A-PBX1 protein and Ig are indicated by arrows. DTA, diphtheria toxin A gene; Neo, neomycin resistance gene; Ip, immunoprecipitant; pBS, pBluescript; plpC, polyinosinic-polycytidylic acid; Ig, transgenic.
Preparation and infection of MOL4070A retrovirus were carried out as previously described. Newborn mice were inoculated i.p. with virus solution containing approximately 1 × 10^5 MOL4070A particles. Retroviral integration sites were identified by inverse PCR (iPCR) as previously described. The primer sequences are as follows: mouse Evi5, 5′-GACCTATTGATTCTCTGGGGAAG-3′ and 5′-AACCAACCTCAGTCCACTAAG-3′; mouse Ccnd3, 5′-CTTGGCATCTATAAGGACCAGGC-3′ and 5′-CAACTCTGTGGCTCATCCGAG-3′; mouse Chflα1, 5′-CCCCCATCTCACCCAATGGCTTC-3′ and 5′-GGGAAAGTTGTTGCTTCCGTGAG-3′; mouse Ikzf1, 5′-CGTTGTAAGGCAATCCCCAAAT GTG-3′ and 5′-CTTACGTTGCGACCATTGCTTG-3′; mouse Gfi1, 5′-GTGGAGTCGGAATTCAGGGCTTG-3′ and 5′-GAGCACGTGGACACGCTTG-3′; mouse Pecam1, 5′-GTGCTAGTAAAGGTCGATGTCCAG-3′ and 5′-CGGCAATGCCCTGTTCTTCCATG-3′; mouse Mycn, 5′-GTCACCGTAGAGAAGACGCCTAC-3′ and 5′-GGTAGGTTGCGACATAGTTGTTG-3′; mouse Pim1, 5′-AAAAAGGATGTGGCTATTG-3′ and 5′-GGTGATTTGGGTGACTGAC-3′ and 5′-GGTGTGCATTGCTGTCCCTCTGATC-3′; mouse Zfp521, 5′-CCAGGTGTTGGTGGTCC-3′ and 5′-GTCACCGTAGAGAAGACGCCTAC-3′; mouse Cbfa2t3 h, 5′-AAAATGGGTGCTGTCCCTG-3′ and 5′-GAATGGGATATTCAGG-3′ and 5′-GAATGGGATATTCAGG-3′.

Quantitative real-time PCR. Total cellular RNA was extracted using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and quantitative real-time PCR (qPCR) was carried out as previously described. The primer sequences are as follows: mouse Evi5, 5′-GACCTATTGATTCTCTGGGGAAG-3′ and 5′-AACCAACCTCAGTCCACTAAG-3′; mouse Ccnd3, 5′-CTTGGCATCTATAAGGACCAGGC-3′ and 5′-CAACTCTGTGGCTCATCCGAG-3′; mouse Chflα1, 5′-CCCCCATCTCACCCAATGGCTTC-3′ and 5′-GGGAAAGTTGTTGCTTCCGTGAG-3′; mouse Ikzf1, 5′-CGTTGTAAGGCAATCCCCAAAT GTG-3′ and 5′-CTTACGTTGCGACCATTGCTTG-3′; mouse Gfi1, 5′-GTGGAGTCGGAATTCAGGGCTTG-3′ and 5′-GAGCACGTGGACACGCTTG-3′; mouse Pecam1, 5′-GTGCTAGTAAAGGTCGATGTCCAG-3′ and 5′-CGGCAATGCCCTGTTCTTCCATG-3′; mouse Mycn, 5′-GTCACCGTAGAGAAGACGCCTAC-3′ and 5′-GGTAGGTTGCGACATAGTTGTTG-3′; mouse Pim1, 5′-AAAAAGGATGTGGCTATTG-3′ and 5′-GGTGATTTGGGTGACTGAC-3′ and 5′-GGTGTGCATTGCTGTCCCTCTGATC-3′; mouse Zfp521, 5′-CCAGGTGTTGGTGGTCC-3′ and 5′-GTCACCGTAGAGAAGACGCCTAC-3′; mouse Cbfa2t3 h, 5′-AAAATGGGTGCTGTCCCTG-3′ and 5′-GAATGGGATATTCAGG-3′ and 5′-GAATGGGATATTCAGG-3′.

Human leukemic cell lines. Human B-progenitor cell lines with or without t(1;19) were used in this study. The cell lines

---

(a) Survival curves of control (Ctrl, black), EPIK (green), Ctrl+MOL4070A (blue), and EPKI+MOL4070A (red) mice and their symptomatic incidence are shown. The time points of MOL4070A infection and polyinosinic-polycytidylic acid (pIpC) injection are indicated by arrows and the diseased mice in the EPKI+MOL4070A group are numbered. (b) Pathological analysis of a tumor that developed in an EPKI-MOL4070A mouse. The infiltrated leukemic cells are indicated by arrows. (c) Flow cytometric analysis of EPKI-MOL4070A tumors. The representative results of T-cell (no. 3), B-cell (no. 6), and biphenotypic (no. 1) acute lymphoblastic leukemia (ALL) are shown. (d) Gene rearrangement analysis. DNAs extracted from tumor tissues and a control spleen (C Spl) were blotted with an IgH or T-cell receptor β (TCRβ) probe. The germline (GL) and rearranged bands are indicated by arrows and arrowheads, respectively.
Southern blot using a 5(ES) clones with homologous recombination identified by the non-coding region of exon 2 (Fig. 1a). Embryonic stem strategy successfully generated cKI mice for The pIpC-treated eases but developed ALL by retroviral insertional mutagene-

WT/EPKI mice were highly malignant, as evidenced by marked proliferation of immature blast cells in the spleen and massive infiltration of leukemic cells in the non-hematopoietic tissues, such as the lung, liver, and kidney (Fig. 2b).

Macroscopically, EPKI+MOL4070A mice showed splenomegaly, frequently associated with thymic enlargement and lymph node swelling (Table 1). To determine the lineage(s) and clonality of the leukemic cells, flow cytometric and gene rearrangement analyses were carried out. The results of flow cytometric analysis revealed that the leukemic cells expressed Thy1.2 (T-cell marker), B220 (B-cell marker), or both, but none was positive for Mac1 or Gr1 (myeloid markers) (Fig. 2c, Table 1), indicating that all the leukemias were committed to the lymphoid lineage. In addition, Southern blot analysis showed that most of the EPKI+MOL4070A tumors carried rearrangements at either or both IgH and T-cell receptor β (TCRβ) loci (Fig. 2d, arrowheads) and the rearranged patterns mostly correspond to the results of the flow cytometric analysis (Table 1). Thus, the leukemias developed in EPKI+-MOL4070A mice were diagnosed as T-cell, B-cell, or biphenotypic ALL and were mainly clonal in origin. These findings indicated that the expression of E2A-PBX1 predisposes hematopoietic cells to malignant transformation and develops

Results

Generation of cKI mice for E2A-PBX1 and acquired expression of E2A-PBX1. To investigate the leukemogenic role of E2A-PBX1 and to create an animal model that mimics human leukemia with t(1;19)(q23;p13), we aimed to generate genetically engineered mice in which E2A-PBX1 is inducibly expressed under the control of the native E2A promoter. To this end, we designed a knock-in vector in which a floxed neomycin resistance (Neo) gene, Flag-tagged E2A-PBX1 cDNA, internal ribosomal entry site (IREs)-GFP (Ig), and a pA was inserted in the non-coding region of exon 2 (Fig. 1a). Embryonic stem (ES) clones with homologous recombination identified by Southern blot using a 5’ probe and genomic PCR using a 3’ primer set were used to create chimeric mice (Fig. 1b), which transmitted the mutant allele and generated heterozygous (WT/EPKI) mice.

The WT/EPKI+Neo+ mice do not express E2A-PBX1 protein because of the existence of the Neo gene between the non-coding region of exon 2 and E2A-PBX1 cDNA (Fig. 1a, line 3). In contrast, crossing WT/EPKI+Neo+ mice with MxCre+ mice and treating the WT/EPKI+Neo+, MxCre+ compound mice with pIpC produce WT/EPKI+Neo+ mice (Fig. 1a, line 4), which in turn express E2A-PBX1 protein due to the excision of the Neo gene (Fig. 1a, line 4). To verify this, proteins extracted from the spleen of WT/EPKI+Neo+ and WT/EPKI+Neo+ mice were immuno-

Table 1. Characteristics of EPKI+MOL4070A leukemic mice

| Mouse No. | Age at disease (days) | PB parameters | Macroscopic tumor sites | Surface markers | Gene rearrangements | Diagnosis |
|-----------|-----------------------|---------------|-------------------------|----------------|--------------------|-----------|
|           | WBC (x 10^3/μL) | Hb (g/dL) | Plt (x 10^5/μL) |                |                  |           |
| 1         | 150                   | 27.1        | 14.2                 | 17.2           | Spl                | Thy1.2*, B220* | G/R G/R Biphentypic ALL |
| 2         | 164                   | 13.3        | 11.3                 | 12.8           | Spl, LN            | Thy1.2*, B220* | G/R G/R Biphentypic ALL |
| 3         | 182                   | 64.1        | 9.7                  | 25.0           | Thy, Spl           | Thy1.2*       | G/R G/R Biphentypic ALL |
| 4         | 190                   | ND          | ND                   | ND             | Spl                | ND           | G/R G/R Biphentypic ALL |
| 5         | 200                   | 22.3        | 12.9                 | 28.2           | Spl, LN            | Thy1.2*, B220* | G/R G/R Biphentypic ALL |
| 6         | 227                   | 13.1        | 10.0                 | 73.7           | Spl, LN            | B220*         | G/R G/R Biphentypic ALL |
| 7         | 235                   | 17.2        | 13.4                 | 26.7           | Spl, LN            | Thy1.2*, B220* | G/R G/R Biphentypic ALL |
| 8         | 250                   | ND          | ND                   | ND             | Spl, LN            | Thy1.2*       | G/R G/R Biphentypic ALL |
| 9         | 277                   | 38.3        | 11.8                 | 14.3           | Spl, LN            | Thy1.2*       | G/R G/R Biphentypic ALL |
| 10        | 290                   | 82.1        | 13.5                 | 37.6           | Thy, Spl, LN       | Thy1.2*       | G/R G/R Biphentypic ALL |
| 11        | 318                   | 45.2        | 10.3                 | 7.1            | Spl                | Thy1.2*       | G/R G/R Biphentypic ALL |
| 12        | 318                   | 65.2        | 13.6                 | 33.5           | Spl, LN            | Thy1.2*, B220* | G/R G/R Biphentypic ALL |
| 13        | 328                   | 10.4        | 12.2                 | 13.5           | Spl, LN            | Thy1.2*, B220* | G/R G/R Biphentypic ALL |

ALL, acute lymphoblastic leukemia; G, germline; Hb, hemoglobin; LN, lymph node; ND, not done; PB, peripheral blood; Plt, platelet count; R, rearranged; s/o, suspected of; Spl, spleen; TCRβ, T-cell receptor β; Thy, thymus; WBC, white blood cells. *Found dead.
Identification of common integration sites in leukemias developed in EPKI+MOL4070A mice. To identify gene(s) whose altered expression cooperated with E2A-PBX1 to develop leukemia, genomic DNAs extracted from leukemic samples of EPKI+MOL4070A mice were subjected to iPCR, the method to isolate integration site (CIS), which were EPKI+, Ccnd3, Cbfα2t3 h, Ikzf1, and Pecam1 genes, enhanced expression (>2-fold) was detected in the Gfi1, Mycn, and Pim1 genes. In the Gfi1 gene, most of the tumors, including virus-integrated nos. 2 and 12 showed more than 5-fold upregulation (Fig. 3, left bottom panel). In the Mycn gene, among three virus-integrated tumors (nos. 3, 10, and 13), two samples (nos. 10 and 13) showed significantly enhanced (>200-fold) expression, along with one insertion-undetectable case (no. 12) (Fig. 3, right-hand panel, row 3). In addition, concerning the Pim1 gene, in the two virus-integrated tumors (nos. 5 and 10), one sample (no. 10) showed more than 5-fold upregulation (Fig. 3, bottom right panel). These results strongly suggested that, among the eight CIS genes, integrations in the Gfi1, Mycn, and Pim1 genes enhanced the expression patterns and possibly contribute to development and progression of leukemias in EPKI+MOL4070A mice.

Fig. 3. Analysis of retroviral integration sites and altered gene expression patterns in tumors of EPKI+ML4070A mice. In the schematic illustrations of common integration site (CIS) genes (upper panel of each gene figure), exons are boxed, coding regions are filled, and viral integration sites are indicated by vertical arrows with the related mouse numbers. In the quantitative real-time PCR analysis (lower panel of each gene figure), exons are boxed, coding regions are filled, and viral integration sites are indicated by vertical arrows with the related mouse numbers. In the quantitative real-time PCR analysis (lower panel of each gene figure), exons are boxed, coding regions are filled, and viral integration sites are indicated by vertical arrows with the related mouse numbers.
Identification of Zfp521 as the major retroviral integration site in a leukemic mouse with B-lineage phenotype and cooperative oncogenicity of overexpressed Zfp521 with E2A-PBX1 in B-cell tumorigenesis. Among the EPKI+MOL4070A leukemic mice, no. 6 attracted our attention with two reasons: (i) the leukemia of no. 6 was diagnosed as B-lineage ALL (Fig. 2c, Table 1), which correlates with the phenotype of human E2A-PBX1-positive leukemia; and (ii) Zfp521 (also known as Evi3) was detected as a viral integration site (Table S1, double asterisks and bold type), which we previously identified as a CIS in our E2A-HLF cKI RIM study and reported as a cooperative oncogenicity of overexpressed Zfp521 and EPKI in cKI mice as well as in E2A-HLF cKI mice.

The viral integration site of no. 6 in the Zfp521 gene was located in the promoter region, which was very close to those detected in two EPKI-MOLV B-lineage ALL samples (EHKI-2 and EHKI-4) (Fig. 4a). To investigate whether cells with Zfp521 integration account for the majority of the tumor, Southern blot analysis was carried out using a genomic probe adjacent to the viral integration sites (Fig. 4a, probe E). A rearranged band was detected in no. 6 as well as EHKI-2 and EHKI-4 (Fig. 4b, arrowheads), indicating that Zfp521 was the major integration site in these three tumors. We then examined the expression alteration of the Zfp521 gene by virus integration. As shown in Figure 4(c), more than 2-fold upregulation of Zfp521 expression was detected in no. 6 as well as EHKI-2 and EHKI-4.

To directly verify the in vivo cooperative oncogenicity of overexpressed Zfp521 with E2A-PBX1, we crossed EPKI mice with E2A/SV/Zfp521 (Zfp521) Tg mice that express Zfp521 at a high level in lymphoid cells. The survival curves of plpC-treated offspring are shown in Figure 4(d). During approximately 5 months of observation, more than half of the EPKI/Zfp521 Tg compound mice developed acute leukemia, while...
none of the EPKI or Zfp521 Tg alone mice showed hematological abnormalities. The flow cytometric and gene rearrangement analyses of the leukemic tissues indicated that the leukemias were of B-cell lineage (Fig. 4e). These results showed that overexpressed Zfp521 synergized with EPKI and contributed to B-cell tumorigenesis.

**Enhanced expression of ZNF521 in human leukemic cell lines with t(1;19).** We finally investigated the clinical relevance of overexpression of Zfp521 in human leukemias bearing t(1;19). To this end, the expression levels of ZNF521, the human counterpart of Zfp521, were examined in t(1;19)-positive ALL cell lines and the results were compared with those in B-lineage ALL lines without t(1;19). As shown in Figure 5, although the expression levels of ZNF521 varied among lines, the mean expression level in ALL lines with t(1;19) was apparently higher than that in control lines without t(1;19) and marked upregulation (more than 10-fold of the mean of control cell lines) were observed in three lines (Fig. 5, arrows). These results strongly suggested that overexpression of ZNF521 would be implicated, at least in several cases, in the pathogenesis of t(1;19)-positive B-lineage ALL.

**Discussion**

**E2A-PBX1** is detected in approximately 5% of ALL patients and associated with B-cell phenotype.\(^{(18)}\) To clarify the leukemogenic mechanism and to create a mouse model for E2A-PBX1-positive human leukemia, we generated mice in which E2A-PBX1 was inducibly expressed under the control of the native E2A promoter (Fig. 1a). Despite the induced expression of E2A-PBX1 in the hematopoietic tissue (Fig. 1c), no disease developed during the observation period (Fig. 2a), indicating that expression of E2A-PBX1 per se is not sufficient to develop hematopoietic malignancies. This finding is in good agreement with our previous study using E2A-HLF cKI mice\(^{(39)}\) and other cKI studies for leukemia-associated fusion genes, such as RUNX1/ETO and MLL/CBP.\(^{(19,20)}\) To identify cooperative gene(s) for E2A-PBX1 to exert its fully oncogenic potential, E2A-PBX1 cKI mice were subjected to RIM study, by which we have successfully isolated cooperative genes in various types of genetically engineered mice.\(^{(9,13,14,21–23)}\) By MOL4070A infection, all the E2A-PBX1 cKI mice developed leukemia; in contrast, only one control mouse showed hematopoietic malignancy (Fig. 2a), indicating that E2A-PBX1 confers high susceptibility to leukemia development. It is of note that the leukemia that developed in control+MOL4070A was of T-cell lineage (not shown), whereas the leukemias that developed in EPKI+MOL4070A mice were diagnosed as either T-cell, B-cell, or biphenotypic ALL (Fig. 2c,d, Table 1). Thus, it is strongly suggested that the expression of E2A-PBX1 primed virus-integrated cells to B-lineage (ALL) and addition to T-lineage ALL, as reported in previous studies.\(^{(7,8)}\)

Using iPCR, we detected eight retroviral common integration sites, Evi5, Cnd3, Cbfa2t3 h, Gfi1, Ikek1, Pacman1, Mync, and Pim1 (Table S1), which have already been identified by retrovirus-tagged mouse mutagenesis studies using various types of genetically engineered mice (Retrovirus and Transposon tagged Cancer Gene Database, http://variation.osu.edu/rtgd/). Quantitative real-time PCR showed that three of them, Gfi1, Mync, and Pim1, exhibited high expression patterns (Fig. 3), leading to the idea that deregulated expression of these genes contributes to the leukemogenic process(es).

**Gfi1** encodes a transcription factor and was originally identified as the gene that conferred interleukin-2 (IL-2)-independent growth ability to T-lymphocytes.\(^{(24)}\) Subsequent studies reported Gfi1 as a frequent viral integration site in T-lymphoid tumors in MMLV-infected mice.\(^{(25,26)}\) Although targeted expression of Gfi1 did not efficiently induce leukemia in mice, it cooperates with other genes, such as Myc or Pim1, to develop T-cell malignancies.\(^{(27)}\) We found that overexpression of Gfi1 (>5-fold) in E2A-PBX1 + MOL4070A mice with T-cell lineage at high frequency (10/13), indicating that Gfi1 could be a new partner for E2A-PBX1 to develop T-cell leukemia.

**Mync** is a member of the MYC family of transcription factors. Mync was originally identified as a gene amplified in neuroblastoma cells and subsequently found to be overexpressed in various types of cancers.\(^{(28)}\) The contribution of N-myc to leukemia development was verified by transgenic and bone marrow transplantation (BMT) studies; targeted expression of N-myc by lymphoid-specific transgenic enhancer/promoter induced B-lymphoid malignancies,\(^{(29–31)}\) and retrovirus-mediated transfer of N-myc into hematopoietic progenitor cells developed acute myeloid leukemia.\(^{(32)}\) In addition, in a RIM study, N-myc was found as a viral integration site and accelerated the onset of T-cell ALL in N-ras and Pim1 Tg mice.\(^{(33,34)}\) Therefore, it may be possible that overexpressed N-myc cooperates with E2A-PBX1 and enhances its oncogenic activity to develop ALL.

**Pim1** encodes a serine–threonine kinase that is involved in the regulation of apoptosis, metabolism, and the cell cycle.\(^{(35)}\) A RIM study applied to E2A-PBX1 Tg mice\(^{(7)}\) identified Pim1 as a new partner for E2A-PBX1 to develop T-cell leukemia.
as a frequent target of retrovirus insertions in mice with accelerated diseases. Therefore, our study provided further evidence that deregulated expression of Pim1 cooperates with E2A-PBX1 to develop aggressive T-cell ALL. Given that Pim1 contributes to factor independency and E2A-PBX1-positive hematopoietic cells tend to undergo apoptosis, especially under cytokine-depleted conditions, it is postulated that Pim1 confers cytokine-independent growth ability to E2A-PBX1-positive cells and induces a more malignant phenotype.

Of particular interest is the identification of Zfp521 in an EPKI-MOL4070A tumor with B-cell phenotype (no. 6; Fig. 4, Table S1). Zfp521 was originally cloned as a retroviral integration site in AKXD mice, which encodes a transcription factor with multiple zinc fingers and is highly expressed in hematopoietic progenitor cells. Recent studies have reported the overexpression of ZNF521, the human counterpart of Zfp521 (also known as EHZF), in human B-cell lymphoblastic lymphoma samples and the detection of the Pax5/ZNF521 fusion transcripts in B ALL samples. Thus, it is strongly postulated that deregulated and/or structurally altered ZNF521 is implicated in B-cell malignancy. Our findings, together with our previous study, indicate that overexpressed Zfp521 cooperated with E2A-involving fusion genes to develop B-lineage ALL and that enhanced expression of ZNF521 is detected in human B-lineage ALL samples bearing t(1;19) or t(17;19) (Figs 4,5). Although the mechanism(s) of how deregulated Zfp521 contributes to B-cell leukemogenesis has not fully been understood, studies have reported that Zfp521 impairs normal B-cell development by inhibiting the function of EBF1, a transcription factor required for B-cell development. In addition, Zfp521 was shown to enhance pre-B-cell receptor signaling and interfere with the IL-7/IL-7 receptor-mediated maturation pathway. Therefore, it is postulated that Zfp521/ZNF521 contributes to the development of B-lineage ALL by expanding the pre-B cell population and impairing the terminal differentiation toward mature B cells.

Recently, generation and analysis of another cKI mouse for E2A-PBX1 was reported. The researchers knocked-in PBX1 cDNA coupled with IRES-GFP downstream of the E2A gene and inserted two loxP sites together with the Neo resistance gene, where one loxP was located between exons 12 and 13 and another between the last exon of the E2A gene and PBX1 cDNA. By crossing the knock-in mice with various types of Cre (McCre+, Mbc1Cre+, and CD19Cre+) Tg mice, the compound mice (named as conditional E2A-PBX1 Tg mice) expressed E2A-PBX1, as in our study. However, unlike our EKPI mice, the conditional E2A-PBX1 Tg mice spontaneously developed leukemias with B-cell lineage. The phenotypic discrepancy between the two studies remains unknown, but one possibility is the difference in the knock-in strategy. We directly knocked-in E2A-PBX1 cDNA in the 5′ non-coding region of the E2A gene, but Duque-Afonso et al. retained the exon/intron structure of the E2A gene and knocked-in PBX1 cDNA downstream of the E2A gene. Thus, it is strongly suggested that unidentified regulatory region(s) in the exon/intron(s) of the E2A gene may play a pivotal role in the spontaneous development and lineage-determination of E2A-PBX1-positive (and presumably E2A-HLF-positive) leukemias. They showed that secondary mutations, including loss of Pax5, were frequently detected in the leukemic tissues, therefore it is conceivable that additional genetic aberrations are necessary for E2A-PBX1 to fully exert its leukemogenic potential.

In this report, we generated E2A-PBX1 cKI mice, isolated cooperative candidate genes, identified Zfp521 as a partner to develop B-lineage ALL, and showed the frequent overexpression of ZNF521 in t(1;19)-positive ALL samples. Our results, together with the findings of other groups, provide evidence that multistep gene alterations are required for E2A-PBX1 to develop ALL and prove that RIM study is a valuable tool for identifying genes whose altered expression contributes to malignant transformation of hematopoietic cells.

Acknowledgments
We thank Yuki Sakai, Sawako Ogata, and Rika Tai for animal care, mouse genotyping, and molecular experiments. We also thank Drs. Soren Warming, Neal G. Copeland, and Nancy A. Jenkins for providing us with mouse Zfp521 cDNA. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

Disclosure Statement
The authors have no conflict of interest.

References
1 Look AT. Oncogenic transcription factors in the human acute leukemias. Science 1997; 278: 1059–64.
2 Murre C. Regulation and function of the E2A proteins in B cell development. Adv Exp Med Biol 2007; 596: 1–7.
3 Hunger SP, Galili N, Carroll AJ, Crist WM, Link MP, Cleary ML. The t(1;19)(q23;p13) results in consistent fusion of E2A and PBX1 coding sequences in acute lymphoblastic leukemias. Blood 1991; 77: 687–93.
4 Kamps MP, Look AT, Baltimore D. The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. Genes Dev 1991; 5: 358–68.
5 Inaba T, Roberts WM, Shapiro LH et al. Fusion of the leucine zipper gene HLF to the E2A gene in human acute B-lineage leukemia. Science 1992; 257: 1–4.
6 Kamps MP, Baltimore D. E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol Cell Biol 1993; 13: 351–7.
7 Dedera DA, Waller EK, LeBrun DP et al. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell 1993; 74: 833–43.
8 Bijl J, Sauvageau M, Thompson A, Sauvageau G. High incidence of proviral integrations in the Hoxa locus in a new model of E2A-PBX1-induced B-cell leukemia. Genes Dev 2005; 19: 224–33.
9 Yamashita N, Miyazaki K, Nagamachi A et al. Identification of Zfp521/ZNF521 as a cooperative gene for E2A-HLF to develop acute B-lineage leukemia. Oncogene 2010; 29: 1963–75.
10 Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. Science 1995; 269: 1427–9.
11 Rosenbaum H, Harris AW, Bath ML et al. An E mu-v-abl transgene elicits plasmacytomas in concert with an activated myc gene. EMBO J 1990; 9: 897–905.
12 Kamping S, Liu P, Suzuki T et al. Evi3, a common retroviral integration site in murine B-cell lymphoma, encodes an EF1alpha-related Kruppel-like zinc finger protein. Blood 2003; 101: 1934–40.
13 Mizuno T, Yamashita N, Miyazaki K et al. Overexpression/enhanced kinase activity of BCR/ABL and altered expression of Notch1 induced acute leukemia in p210BCR/ABL transgenic mice. Oncogene 2008; 27: 3465–74.
14 Nagamachi A, Matsui H, Asou H et al. Haploinsufficiency of SAMD9L, an endosome fusion facilitator, causes myeloid malignancies in mice mimicking human diseases with monosomy 7. Cancer Cell 2013; 24: 305–17.
15 Hirose K, Irukao T, Kikuchi J et al. Aberrant induction of LMO2 by the E2A-HLF chimeric transcription factor and its implication in leukemogenesis of B-precursor ALL with t(17;19), Blood 2010; 116: 962–70.
16 Nakamura T. Retroviral insertional mutagenesis identifies oncogene cooperation. Cancer Sci 2005; 96: 7–12.
17 Wolf L, Koller R, Hu X, Anver MR. A Moloney murine leukemia virus-based retrovirus with 4070A long terminal repeat sequences induces a high
incidence of myeloid as well as lymphoid neoplasms. J Virol 2003; 77: 4965–71.
18 Aspland SE, Bendall HH, Murre C. The role of E2A-PBX1 in leukemogene-
sis. Oncogene 2001; 20: 5708–17.
19 Higuchi M, O’Brien D, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a human model of human (8;21) acute myeloid leu-
kemia. Cancer Cell 2002; 1: 63–74.
20 Wang J, Iwasaki H, Krivtsov A et al. Conditional MLL-CBP targets GMP and models therapy-related myeloproliferative disease. EMBO J 2005; 24: 368–81.
21 Miyazaki K, Yamasaki N, Oda H et al. Enhanced expression of p210BCR/ ABL and aberrant expression of Zifp423/ZNF423 induce blast crisis of chronic myelogenous leukemia. Blood 2009; 113: 4702–10.
22 Nishibe R, Watanabe W, Ueda T et al. E2A-PBX1, a p210Cip1/Waf1-interacting protein, functions as a tumor suppressor in vivo. FERS Lett 2013; 587: 1529–35.
23 Ueda T, Nagamachi A, Takubo K et al. Fbxl10 overexpression in murine hematopoietic stem cells induces leukemia involving metabolic activation and upregulation of Nsg2. Blood 2015; 125: 3437–46.
24 Gilks CB, Bear SE, Grimes HL, Tschiklis PN. Progression of interleukin-2 (IL-2)-dependent rat T cell lymphoma lines to IL-2-independent growth fol-
lowing activation of a gene (Gfi-1) encoding a novel zinc finger protein. Mol Cell Biol 1993; 13: 1759–66.
25 Zörnig M, Schmidt T, Karoukny H, Grzeschiczek A, Móroy T. Zinc fin-
ger protein GFI-1 cooperates with myc and pim-1 in T-cell lymphoma-
genesis by reducing the requirements for IL-2. Oncogene 1996; 12: 1789–801.
26 Scheijen B, Jonkers J, Acton D, Berns A. Characterization of pal-1, a com-
mon proviral insertion site in murine leukemia virus-induced leukemias of c-myc and Pim-1 transgenic mice. J Virol 1997; 71: 9–16.
27 Schmidt T, Karoukny H, Gau E, Zevnik B, Elsässer HP, Móroy T, Zinner finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. Oncogene 1998; 17: 2661–7.
28 Beltran H. The N-myc Oncogene: maximizing its Targets, Regulation, and Therapeutic Potential. Mol Cancer Res 2014; 12: 815–22.
29 Rosenbaum H, Webb E, Adams JM, Cory S, Harris AW. N-myc transgene promotes B lymphoid proliferation, elicits lymphomas and reveals cross-reg-
ulation with c-myc. EMBO J 1989; 8: 749–55.
30 Dildrop R, Ma A, Zimmerman K et al. IgH enhancer-mediated deregulation of N-myc gene expression in transgenic mice: generation of lymphoid neo-
plasias that lack c-myc expression. EMBO J 1989; 8: 1121–8.
31 Sheppard RD, Samant SA, Rosenberg M, Silver LM, Cole MD. Transgenic N-myc mouse model for indolent B cell lymphoma: tumor characterization and analysis of genetic alterations in spontaneous and retrovirally accelerated tumors. Oncogene 1998; 17: 2073–85.
32 Kawagoe H, Kandilci A, Kranenburg TA, Grosveld GC. Overexpression of N-Myc rapidly causes acute myeloid leukemia in mice. Cancer Res 2007; 67: 10677–85.
33 van Lohuizen M, Verbeek S, Krimpenfort P et al. Predisposition to lym-
phomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. Cell 1989; 56: 672–82.
34 Haupt Y, Harris AW, Adams JM. Retroviral infection accelerates T lymphomagenesis in E mu-N-ras transgenic mice by activating c-myc or N-myc. Oncogene 1992; 7: 981–6.
35 Narlik-Grassow M, Blanco-Aparicio C, Carnero A. The PIM family of ser-
ine/threonine kinases in cancer. Med Res Rev 2014; 34: 136–59.
36 Feldman BJ, Reid TR, Cleary ML. Pim1 cooperates with E2a-Pbx1 to facil-
tate the progression of thymic lymphomas in transgenic mice. Oncogene 1997; 15: 2735–42.
37 Nosaka T, Kitamura T. Pim-1 expression is sufficient to induce cytokine independence in murine hematopoietic cells, but is dispensable for BCR- ABL-mediated transformation. Exp Hematol 2002; 30: 697–702.
38 Hiratsuka T, Takei Y, Ohmori R et al. ZFP521 contributes to pre-B-cell lymphomagenesis through modulation of the pre-B-cell receptor signaling pathway. Oncogenesis 2015; doi:10.1038/onc.2015.385.
39 Nebral K, König M, Harder L, Siebert R, Haas OA, Strehl S. Identification of PML as novel PAX5 fusion partner in childhood acute lymphoblastic leu-
kaemia. Br J Haematol 2007; 139: 269–74.
40 Mullighan CG, Goorha S, Radtke I et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature 2007; 446: 578–64.
41 Hentges KE, Weiser KC, Schountz T, Woodward LS, Morse HC, Justice MJ. Evi3, a zinc-finger protein related to EBFAZ, regulates EBF activity in B-cell leukaemia. Oncogene 2005; 24: 1220–30.
42 Mega T, Lupia M, Amadio N et al. Zinc finger protein 521 antagonizes early B-cell factor 1 and modulates the B-cell differentiation of pri-
mary hematopoietic progenitors. Cell Cycle 2011; 10: 2129–39.
43 Lin H, Groschedl R. Failure of B-cell differentiation in mice lacking the transcrip-
tion factor EBF. Nature 1995; 376: 263–7.
44 Duque-Afonso J, Feng J, Scherer F et al. Comparative genomics reveals multistep pathogenesis of E2A-PBX1 acute lymphoblastic leukemia. J Clin Invest 2015; 125: 3667–80.
45 Bijl J, Krosl J, Lebert-Ghali CE, Vacher J, Mayotte N, Sauvageau G. Evi-
dence for Hox and E2A-PBX1 collaboration in mouse T-cell leukaemia. Oncogene 2008; 27: 6356–64.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Retroviral integration sites identified by iPCR