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

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

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***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 previously identified *Zfp521* as a cooperative gene with *E2A-HLF*, another *E2A*-involving fusion gene with B-lineage ALL. The cooperative oncogenicity of *E2A-PBX1* with overexpressed *Zfp521* in B-cell tumorigenesis was indicated by the finding that *E2A-PBX1* cKI, *Zfp521* transgenic compound mice developed B-lineage ALL. Moreover, upregulation of *ZNF521*, the human counterpart of *Zfp521*, was found in several human leukemic cell lines bearing t(1;19). These results indicate that *E2A-PBX1* cooperates with additional gene alterations to develop ALL. Among them, enhanced expression of *ZNF521* 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] 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] *E2A* is located on chromosome 19 and is the target in subsets of acute lymphoblastic leukemia (ALL) with B-cell lineage.[1] 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*,[3,4] and in *E2A-HLF*, the same domain of *E2A* is fused to the basic region/leucine zipper domain of HLF.[5] 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-PBX*1-expressing retrovirus to hematopoietic progenitor cells and transplanted the cells into syngeneic mice.[6] Although the recipient mice developed leukemia, the disease was exclusively classified as acute myeloid leukemia.[6] 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 *CD3ε*-deficient mice to prevent the development T-cell ALL.[8] The compound (*E2A-PBX1* Tg and *CD3ε*-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

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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/EPKI*$^{Neo+}$ mice were crossed with *MxCre*$^{+}$[10] mice to generate *WT/EPKI*$^{Neo+}$, *MxCre*$^{+}$ mice. Cre activation was achieved by i.p. treatment with 500 μg polyinosinic–polycytidylic acid (pIpC) (Sigma, St. Louis, MO, USA) three times at 2 day intervals. Generation of *EμSV/Zfp521* Tg mice was as previously described.[9] In brief, a DNA fragment that contains *EμSV* enhancer/promoter,[11] *HA*-tagged *Zfp521* cDNA,[12] *SV40* splicing, and *polyA (pA)* signals was microinjected into the pronuclei of mouse eggs. *WT/EPKI*$^{Neo+}$, *MxCre*$^{+}$ mice were crossed with *EμSV/Zfp521* Tg mice, and resultant offspring were subjected to pIpC treatment. Mice that have been back-crossed to the C57BL/6N-Ly5.2 background at least seven times were used for experiments. All the experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Hiroshima University Animal Research Committee.

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

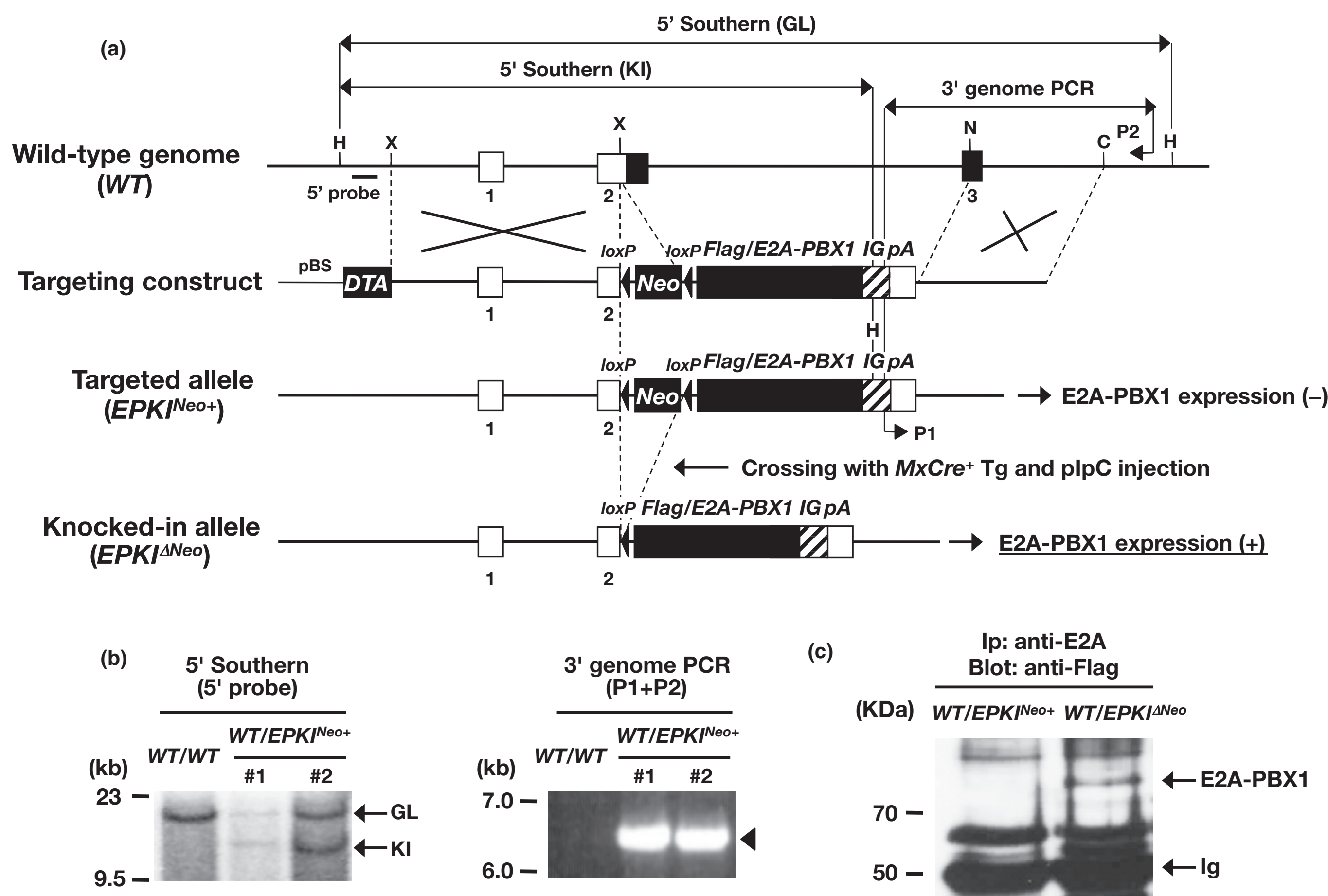

Fig. 1. Generation of *E2A-PBX1* conditional knock-in (cKI) mice and acquired expression of *E2A-PBX1*. (a) Schematic illustration of the conditional KI strategy. Part of the non-coding region of exon 2, the coding region of exon 2, intron 2, and part of the coding region of exon 3 were replaced with a floxed *neomycin resistance* gene (*Neo*), followed by *Flag*-tagged *E2A-PBX1* cDNA, *IRES-GFP* (*IG*), and a *polyadenylation* signal (*pA*). Restriction enzymes: C, *Cla*I; H, *Hind*III; N, *Nae*I; X, *Xba*I. 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/EPKI*$^{Neo+}$ and *WT/EPKI*$^{\Delta Neo}$ 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; pIpC, polyinosinic–polycytidylic acid; Tg, transgenic.

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(PE)- or allophycocyanin (APC)-conjugated anti-Gr1, anti-Mac1, anti-Thy1.2, and anti-B220 antibodies (Pharmingen, San Diego, CA, USA) and analyzed on BD FACSCanto II (BD Biosciences, San Jose, CA, USA) as previously described.[9] For gene rearrangement analysis, DNA was digested with *Eco*RI and blotted with an indicated probe as previously described.[9]

Infection with MOL4070A and identification of retroviral integration sites. Preparation and infection of MOL4070A retrovirus were carried out as previously described.[13] 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.[13]

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.[14] The primer sequences are as follows: mouse *Evi5*, 5′-GACTCTTGGATTCTCTGGGGAAG-3′ and 5′-AACCAACCTCACGGTCCACTAAG-3′; mouse *Ccnd3*, 5′-CTTTGCATCTATACGGACCAGGC-3′ and 5′-CAACTCTGTGAGCTCATCCGCAG-3′; mouse *Cbfa2t3 h*, 5′-CCCCATCTACACCCAATGGCTTC-3′ and 5′-GGAAAGTTGGTGGCTTCCTGGAG-3′; mouse *Ikzf1*, 5′-CGTTGGTAAGCCTCACAAATGTG-3′ and 5′-CTTACGTTTGGCGACATTGCTTG-3′; mouse *Gfi1*, 5′-GTGGAGTCGGAGCTGCTTTGCAC-3′ and 5′-GAGCAGATGTGTGGACAGCGTGG-3′; mouse *Pecam1*, 5′-GTCGAGTAACGAGGTACAGTCTG-3′ and 5′-CGCAATGAGCCCTTTCTTCCATG-3′; mouse *Mycn*, 5′-GTCACCGTAGAGAAGAGACGTTC-3′ and 5′-GGTGAGGGTGCAGCATAGTTGTG-3′; mouse *Pim1*, 5′-TAAATGGTGCCTGTCCCTGAGAC-3′ and 5′-GTTGTCATTGCTGTCCCTGCATC-3′; mouse *Zfp521*, 5′-CCAGGTGTTTGAGTCACTGAGC-3′ and 5′-GAAATCACACCCTTCTCCATGG-3′; and human *ZNF521*, 5′-TACAATGAATGGGATATTCAGG-3′ and 5′-TCCCATCCCTTCGAAGCTGTGC-3′.

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

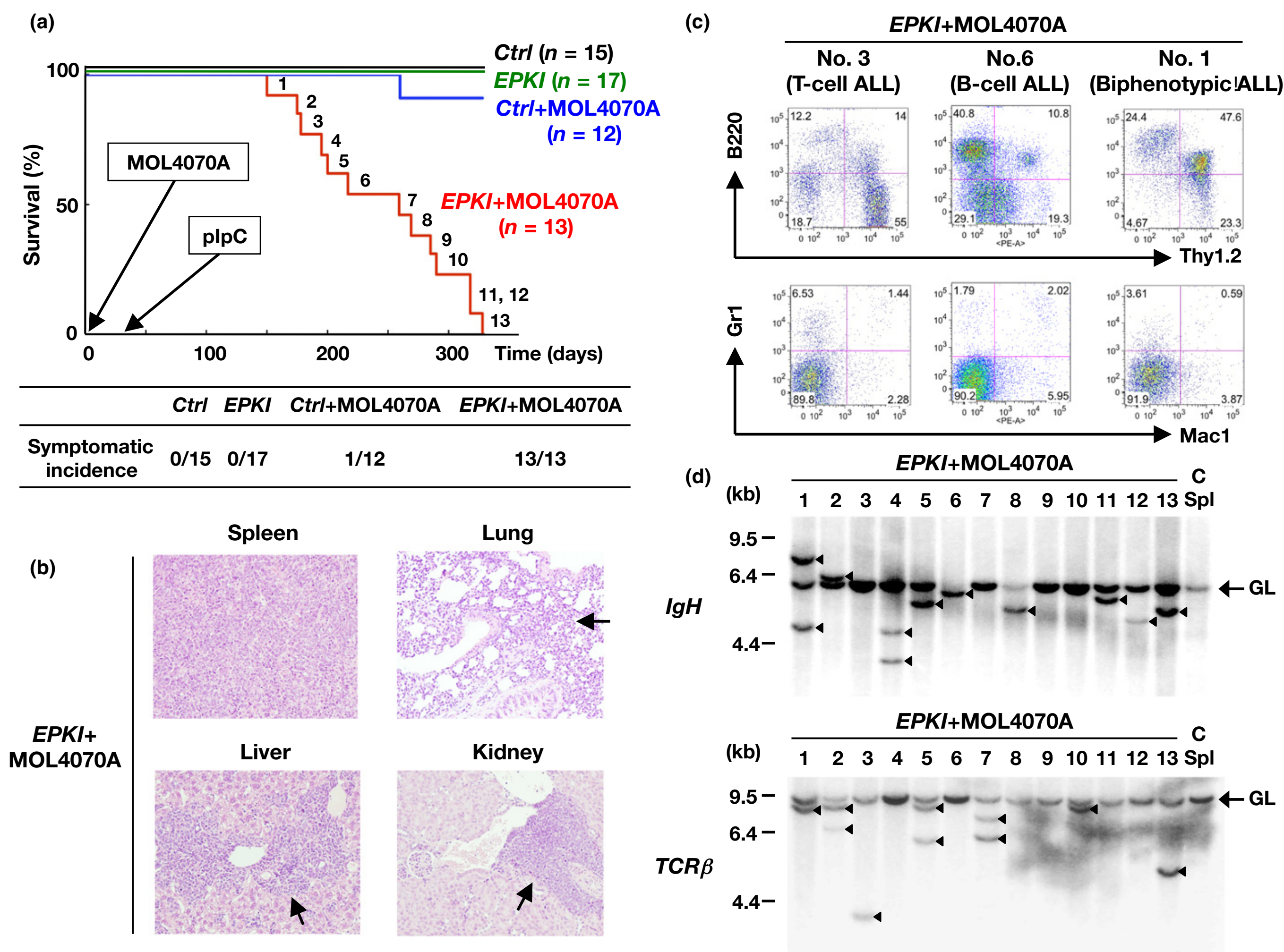

Fig. 2. Analysis of leukemias developed in MOL4070A-infected *EPKI* mice. (a) Survival curves of *control* (*Ctrl*, black), *EPKI* (green), *Ctrl*+MOL4070A (blue), and *EPKI*+MOL4070A (red) mice and their symptomatic incidence are shown. The time points of MOL4070A infection and polyinosinic–polycytidylic 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*+ML4070A 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 a *IgH* or *T-cell receptor β* (*TCRβ*) probe. The germline (GL) and rearranged bands are indicated by arrows and arrowheads, respectively.

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with t(1;19) were KOPNK, KOPN34, KOPN36, KOPN54, KOPN60, KOPN63, YAMN90R, YAMN92, Kasumi2, and 697, and those without t(1;19) are KOPN55bi, KOPN57bi, KOPN72bi (these three lines are Ph-positive), Reh (t(12;21)-positive), and Nalm6.[15]

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*$^{Neo+}$) 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*$^{\Delta Neo}$ 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*$^{\Delta Neo}$ mice were immunoprecipitated with an anti-*E2A* antibody and immunoprecipitants were blotted with an anti-Flag antibody. As expected, *E2A-PBX1* protein (molecular weight approximately 85 kDa) was detected in the spleen of a *WT/EPKI*$^{\Delta Neo}$ mouse but not in that of a *WT/EPKI*$^{Neo+}$ mouse (Fig. 1c), indicating that our targeting strategy successfully generated cKI mice for *E2A-PBX1*.

***WT/EPKI*$^{Neo+}$*, MxCre*$^{+}$ mice did not show hematopoietic diseases but developed ALL by retroviral insertional mutagenesis.** The pIpC-treated *WT/EPKI*$^{Neo+}$, *MxCre*$^{-}$ and *WT/EPKI*$^{Neo+}$, *MxCre*$^{+}$ mice (hereafter referred to as *control* and *EPKI* mice, respectively) were subjected to routine examination of peripheral blood parameters and continuously observed for any sign of illness. However, during 1 year of observation, no abnormality was detected in either type of mice (Fig. 2a, black and green lines). These results indicated that *E2A-PBX1* expression induced under the control of the *E2A* promoter alone is not sufficient to develop hematopoietic diseases and strongly suggest that additional genetic changes are required to produce a fully malignant phenotype.

To address this possibility, retroviral insertional mutagenesis (RIM) was used.[16] Neonatal mice were infected with replication-competent MOL4070LTR mouse leukemia retrovirus, a derivative of Moloney murine leukemia virus (MMLV).[17] During the same observation period (1 year), all the *EPKI* mice infected with MOL4070A died of leukemias (Fig. 2a, red line; disease incidence, 13/13), whereas only one control mouse infected with MOL4070A developed leukemia (Fig. 2a, blue line; disease incidence, 1/12). The leukemias developed in *EPKI*+MOL4070A 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

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 ($\times10^3/\mu L$)	Hb (g/dL)	Plt ($\times10^4/\mu L$)			*IgH*	*TCRβ*	
1	150	27.1	14.2	17.2	Spl	Thy1.2$^+$, B220$^+$	G/R	G/R	Biphenotypic ALL
2	164	13.3	11.3	12.8	Spl, LN	Thy1.2$^+$, B220$^+$	G/R	G/R	Biphenotypic ALL
3	182	64.1	9.7	25.0	Thy, Spl	Thy1.2$^+$	G/G	G/R	T-lineage ALL
4†	190	ND	ND	ND	Spl	ND	G/R	G/G	B-lineage ALL s/o
5	200	22.3	12.9	28.2	Spl, LN	Thy1.2$^+$, B220$^+$	G/R	G/R	Biphenotypic ALL
6	227	13.1	10.0	73.7	Spl, LN	B220$^+$	R/R	G/G	B-lineage ALL
7	235	17.2	13.4	26.7	Spl, LN	Thy1.2$^+$, B220$^+$	G/G	G/R	Biphenotypic ALL
8	250	ND	ND	ND	Spl, LN	Thy1.2$^+$	G/R	G/G	T-lineage ALL s/o
9	277	38.3	11.8	14.3	Spl	Thy1.2$^+$	G/G	G/G	T-lineage ALL
10	290	82.1	13.5	37.6	Thy, Spl, LN	Thy1.2$^+$	G/G	G/R	T-lineage ALL
11	318	45.2	10.3	7.1	Spl	Thy1.2$^+$, B220$^+$	G/R	G/G	Biphenotypic ALL
12	318	65.2	13.6	33.5	Thy, Spl	Thy1.2$^+$, B220$^+$	G/R	G/G	Biphenotypic ALL
13	328	10.4	12.2	13.5	Spl, LN	Thy1.2$^+$, B220$^+$	G/R	G/R	Biphenotypic 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.

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various types of ALL in cooperation with additional gene alterations.

Identification of common integration sites in leukemias developed in *EPKI*+MOL4070 mice. To identify gene(s) whose altered expression cooperated with *E2A-PBX1* to develop leukemia, genomic DNAs extracted from leukemic samples of *EPKI*+-MOL4070 mice were subjected to iPCR, the method to isolate virus integration sites.[16] In the iPCR products obtained from *EPKI*+MOL4070 tumors, we identified eight retroviral common integration sites (CIS), which were *Evi5 (ecotropic viral integration site 5)*, *Ccnd3 (cyclin D3)*, *Cbfa2t3 h (core-binding factor, runt domain, α subunit 2)*, *Gfi1 (growth factor independent 1)*, *Ikzf1 (IKAROS family zinc finger 1 (Ikaros))*, *Pecam1 (platelet/endothelial cell adhesion molecule 1)*, *Mycn (v-myc myelocytomatosis viral related oncogene, neuroblastoma derived)*, and *Pim1 (proviral integration site 1)* (Table S1, asterisks and bold type).

The viral integration sites were found in 5′ upstream and 3′ downstream regions, in introns and occasionally in exons (Fig. 3). Viral integrations are considered to contribute to disease progression through altering the expression of the affected genes.[16] To investigate this possibility, expression patterns of the CIS genes in tumor tissues were examined by qPCR and compared to that in a control spleen. As shown in Figure 3, although expressional change was less than 2-fold in the *Evi5*, *Ccnd3*, *Cbfa2t3 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.

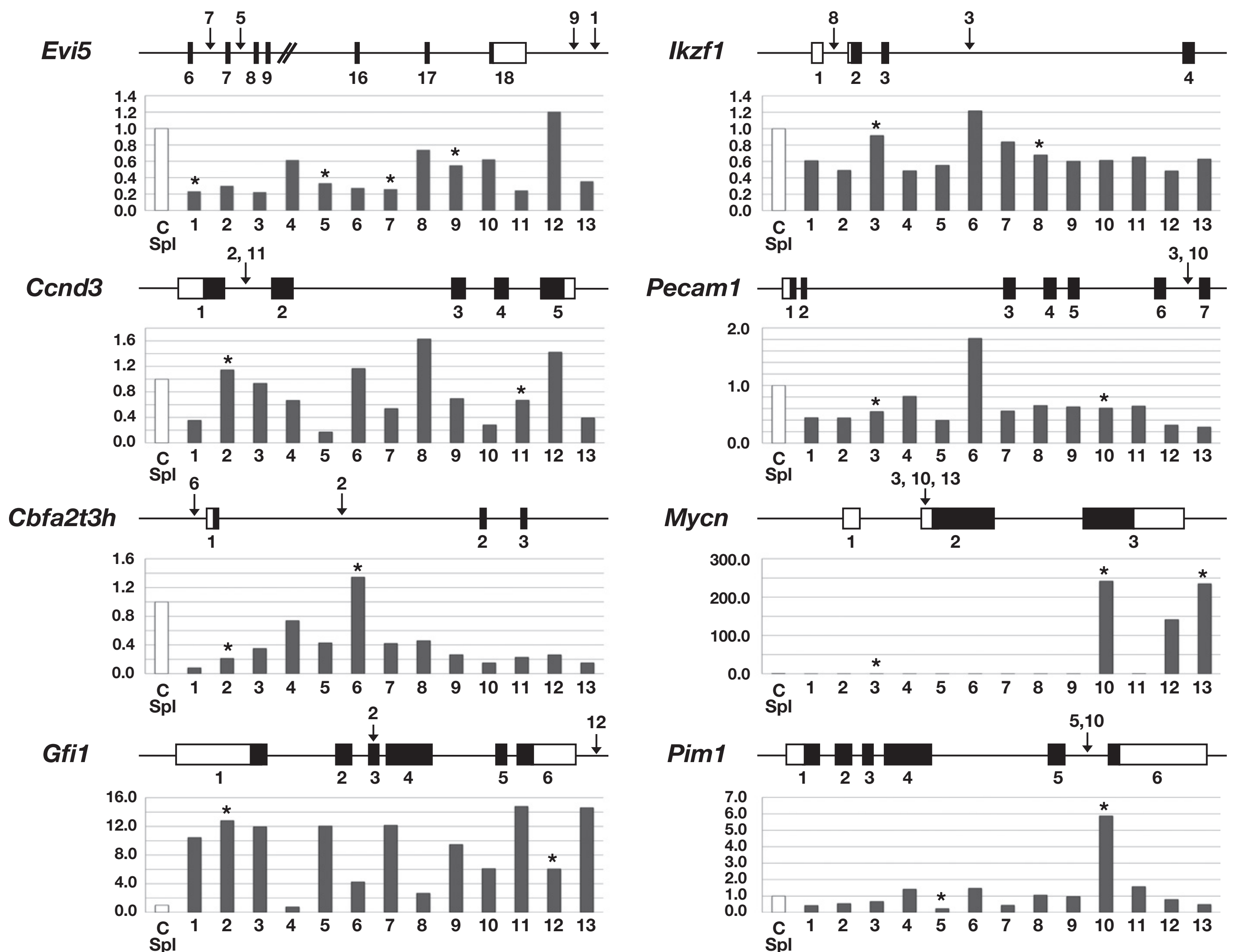

Fig. 3. Analysis of retroviral integration sites and altered gene expression patterns in tumors of *EPKI*+ML4070A mice. In the schematic illustrations of common integration site (CIS) genes (upper panel of each gene figure), exons are boxed, coding regions are filled, and viral integration sites are indicated by vertical arrows with the related mouse numbers. In the quantitative real-time PCR analysis (lower panel of each gene figure), the expression levels of common integration sites in *EPKI*+ML4070A tumors relative to that in a control spleen (C Spl) are shown. *Tumors with viral integrations.

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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;[18] 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 gene with *E2A-HLF* to develop B-lineage ALL.[9] Therefore, it is strongly postulated that *Zfp521* may participate in the development of B-lineage leukemia in *EPKI* 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 *EHKI*+MMLV B-lineage ALL samples (*EHKI-2* and *EHKI-4*) (Fig. 4a).[9] 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).[9] 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 *EμSV/Zfp521* (*Zfp521*) Tg mice that express *Zfp521* at a high level in lymphoid cells.[9] The survival curves of pIpC-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

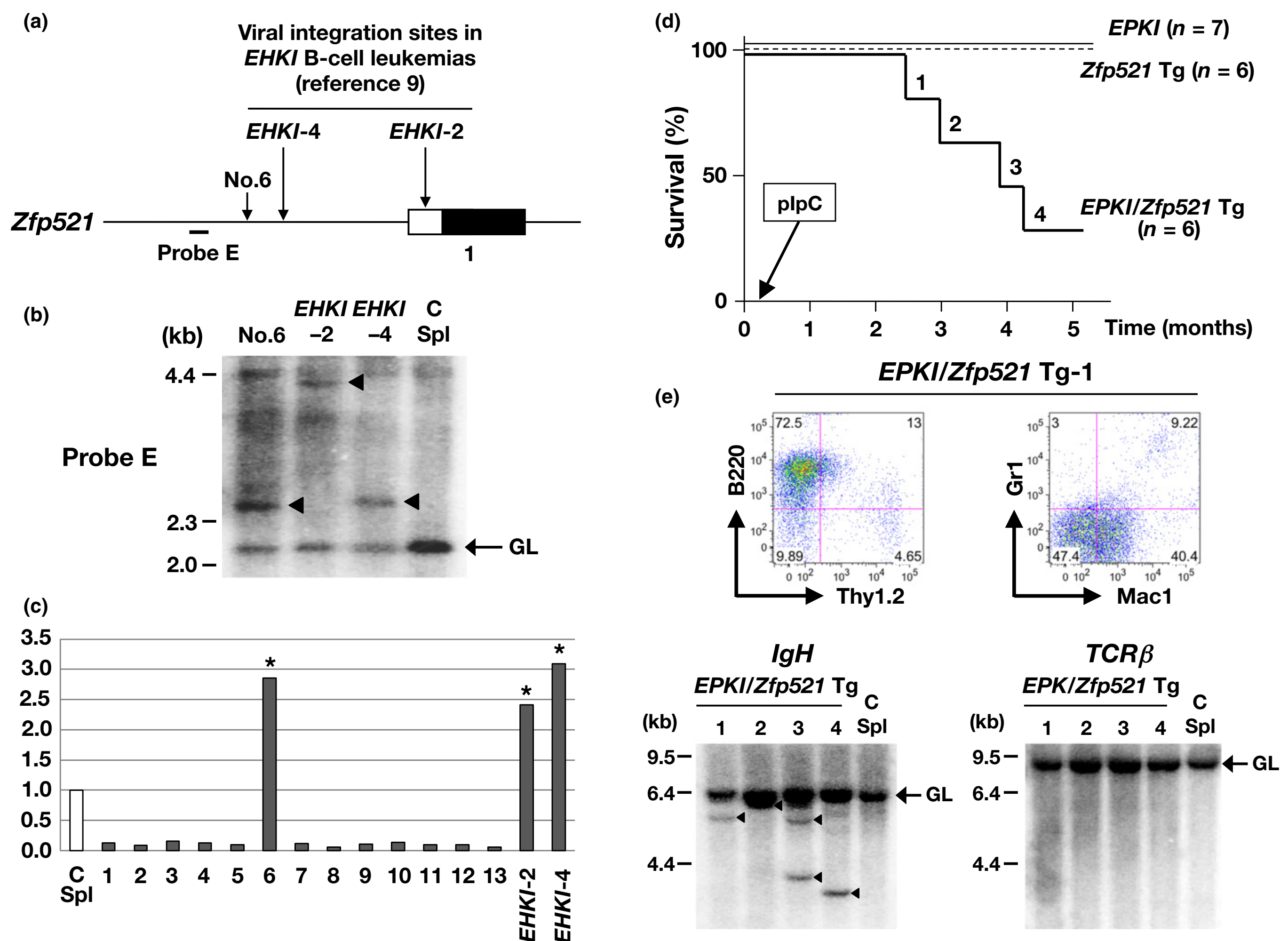

Fig. 4. Analysis of *Zfp521* in no. 6 and *EHKI*-2 and *EHKI*-4 tumors. (a) Schematic illustration of the viral integration sites. Exon 1 is boxed and the coding region is filled. The viral integration sites in no. 6, *EHKI*-2, and *EHKI*-4 are indicated by vertical arrows. The position of the probe for Southern blot (probe E) is also shown. (b) Southern blot to detect gene rearrangements by viral integration. The germline (GL) and rearranged bands are indicated by an arrow and arrowheads, respectively. (c) Quantitative real-time PCR analysis for *Zfp521* expression. The expression levels of *Zfp521* in *EPKI*+MOL4070A tumors, *EHKI*-2, and *EHKI*-4 relative to that in a control spleen (C Spl) are shown. *Tumors with viral integrations. (d) Cooperative oncogenicity of overexpressed *Zfp521* with *E2A-PBX1*. Survival curves of *EPKI*, *Zfp521* transgenic (Tg), and *EPKI*/*Zfp521* Tg compound mice are shown with thin continuous, thin dotted, and thick continuous lines, respectively. The time point of polyinosinic–polycytidylic acid injection is indicated by an arrow and the diseased *EPKI*/Zfp521 Tg mice are numbered. (e) Flow cytometric and gene rearrangement analyses of leukemias developed in *EPKI*/Zfp521 Tg mice. The representative result of flow cytometry and the results of gene rearrangement are shown in the upper and lower panels, respectively. The germline (GL) and rearranged bands are indicated by arrows and arrowheads, respectively.

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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[9] 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 in addition to T-lineage ALL, as reported in previous studies.[7,8]

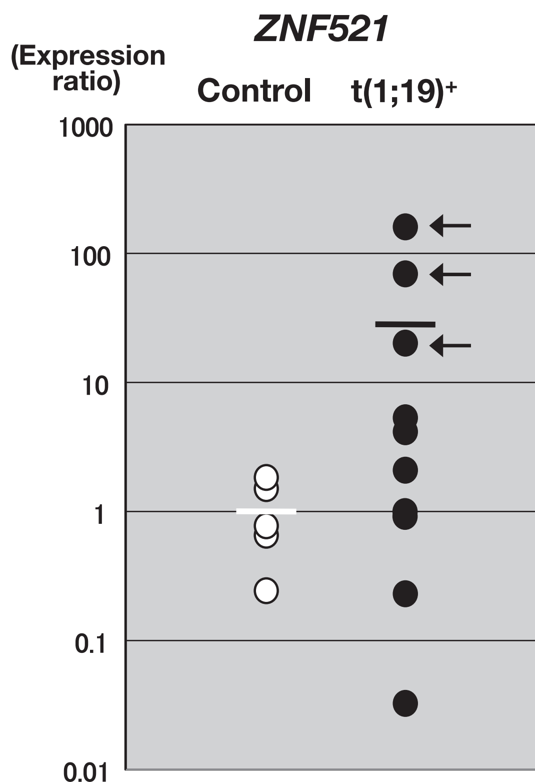

Fig. 5. Quantitative real-time PCR analysis of *ZNF521* in human leukemic cell lines with or without t(1;19). The mRNA expression levels in five t(1;19)-negative B-progenitor cell lines (control) and 10 t(1;19)-positive cell lines (t(1;19)$^+$) relative to the mean of the control cell lines (white bar) are indicated by white and black circles, respectively. The mean of t(1;19)$^+$ cell lines is indicated by a black bar. The relative expression ratio (vertical bar) is shown on a logarithmic scale. High expression lines with t(1;19) are indicated by arrows.

Using iPCR, we detected eight retroviral common integration sites, *Evi5*, *Ccnd3*, *Cbfa2t3 h*, *Gfi1*, *Ikzf1*, *Pecam1*, *Mycn*, 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/rtcgd/). Quantitative real-time PCR showed that three of them, *Gfi1*, *Mycn*, 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.

Mycn is a member of the MYC family of transcription factors. *Mycn* 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*

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as a frequent target of retrovirus insertions in mice with accelerated diseases.[36] 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[37] and *E2A-PBX1*-positive hematopoietic cells tend to undergo apoptosis, especially under cytokine-depleted conditions,[7] 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.[12] Recent studies have reported the overexpression of *ZNF521*, the human counterpart of *Zfp521* (also known as *EHZF*), in human B-cell lymphoblastic lymphoma samples[38] and the detection of the *Pax5/ZNF521* fusion gene in pre-B ALL samples.[39,40] 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,[9] 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,[41,42] a transcription factor required for B-cell development.[43] In addition, *Zfp521* was shown to enhance pre-B-cell receptor signaling and interfere with the IL-7/IL-7 receptor-mediated maturation pathway.[38] 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.[44] 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.[44] By crossing the knock-in mice with various types of *Cre* (*MxCre*$^+$, *Mb1Cre*$^+$, and *CD19Cre*$^+$) Tg mice, the compound mice (named as conditional *E2A-PBX1* Tg mice) expressed *E2A-PBX1*, as in our study. However, unlike our *EPKI* mice, the conditional *E2A-PBX1* Tg mice spontaneously developed leukemias with B-cell lineage.[44] The phenotypical 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 all the exon/intron structure of the *E2A* gene and knocked-in *PBX1* cDNA downstream of the *E2A* gene.[44] 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,[44] 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,[8,44,45] 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.

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Disclosure Statement

The authors have no conflict of interest.

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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

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