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BLNK is a selective target of repression by PAX5-PML in the differentiation block that leads to the development of acute lymphoblastic leukemia*

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ABSTRACT

PAX5 is a transcription factor that is required for the development and maintenance of B cells. PML is a tumor suppressor and pro-apoptotic factor. The fusion gene, *PAX5-PML,* has been identified in acute lymphoblastic leukemia (ALL) with chromosomal translocation t(9;15)(p13;q24). We previously reported that PAX5-PML dominant-negatively inhibited PAX5 transcriptional activity and impaired PML function by disrupting PML nuclear bodies (NBs). We herein demonstrated the leukemogenicity of PAX5-PML by introducing it into normal mouse pro B cells. The arrest of differentiation was observed in PAX5-PML-introduced pro B cells, resulting in the development of ALL after a long latency in mice. Among the transactivation targets of PAX5, BLNK was selectively repressed in leukemia cells and enforced BLNK expression abrogated differentiation block and survival induced by PAX5-PML, indicating the importance of BLNK repression for the formation of pre-leukemic state. We also showed that PML NBs were intact in leukemia cells and attributed this to the low expression of PAX5-PML, indicating that the disruption of PML NBs was not required for the PAX5-PML-induced onset of leukemia. These results provide novel insights into the molecular mechanisms underlying the onset of leukemia by PAX5 mutations.

PAX5 is a member of the highly conserved paired-box (PAX) domain family of transcription factors. PAX5 is exclusively expressed from the pro B to mature B cell stage and is downregulated during terminal differentiation into plasma cells (1). PAX5 is indispensable for B-lineage commitment by the transcriptional activation of B-lineage-specific genes (2) such as *CD19* (3), *CD79A* (4), and *B cell linker protein (BLNK)* (5), and its target disruption was shown to cause B lymphoid maturation arrest at the pro B cell stage (6). Previous studies identified the *PAX5* gene as the most frequent target of somatic mutations in childhood and adult B-progenitor acute lymphoblastic leukemia (ALL), being altered in 38.9% and 34% of cases, respectively (7,8), and these findings further emphasized the essential role of PAX5 in the proper development of B cells Somatic mutations consist of partial or complete hemizygous deletions, homozygous deletions, partial or complete amplifications, point mutations, or fusion genes (7). These aberrations in the *PAX5* gene are considered to impair PAX5 function and play a role in blocking B cell differentiation. PAX5 fusion proteins such as PAX5-TEL, PAX5-ENL, PAX5-PML, and PAX5-C20S were previously shown to have dominant-negative effects on PAX5 transcriptional activity and were suggested to be mainly responsible for the differentiation disorder of ALL with these fusion genes (9-12). Consistently, a previous study reported that PAX5 haploinsufficiency co-operated with the constitutive activation of STAT5 to initiate ALL in mice (13); however, the oncogenicity of PAX5 mutations including fusion genes has yet to be demonstrated.

PML is a potent growth suppressor and pro-apoptotic factor (14,15). In normal cells, the PML protein is localized in discrete subnuclear compartments designated as PML nuclear bodies (NBs) (16). In PML NBs, PML co-accumulates

with more than 70 proteins that are involved in tumor suppression, apoptosis, the regulation of gene expression, anti-viral responses, and DNA repair. PML has been suggested to exert its effects by regulating the functions of binding partners as the core of PML NBs (17). PML NBs were previously found to be disrupted in human acute promyelocytic leukemia (APL) by PML-RARα, an oncogenic fusion protein of PML and retinoic acid receptor (RAR) α, which is considered to be the underlying mechanism responsible for the anti-apoptotic effects of PML-RARα (18-20). Arsenic trioxide (ATO), a chemotherapeutic agent clinically used in the treatment of APL, reportedly induced the restoration of disrupted PML NBs and apoptosis in APL cells, resulting in the prolonged remission of this disease (21-24). These findings emphasize the importance of the integrity of PML NBs in tumor suppression.

The fusion gene, *PAX5-PML,* has been detected in 2 cases of B-progenitor ALL with chromosomal translocation t(9;15)(p13;q24) (25). We previously demonstrated that PAX5-PML dominant-negatively inhibited PAX5 transcriptional activity in a luciferase reporter assay and suppressed the expression of PAX5 transactivation targets when expressed in a B-lymphoid cell line. Furthermore, we showed that the expression of PAX5-PML in a non-hematological tumor cell line induced the disruption of PML NBs and resistance to apoptosis, and also that an ATO treatment induced the reconstitution of PML NBs and abrogation of apoptosis resistance. These findings suggested the possible involvement of this fusion protein in the leukemogenesis of B-ALL in a dual dominant-negative manner as well as the potential of ATO therapy for this type of ALL (11).

In the present study, we demonstrated the leukemogenicity of PAX5-PML by introducing it into normal mouse pro B cells, and showed selective BLNK repression among the transactivation targets of PAX5 in leukemia cells. We also showed that PML NBs were intact in leukemia cells, indicating that the disruption of PML NBs was not required for the PAX5-PML-induced onset of leukemia. These results provide novel insights into the molecular mechanisms underlying the onset of leukemia by PAX5 mutations.

EXPERIMENTAL PROCEDURES

Antibodies and reagents

An anti-PML antibody (H-238), anti-PAX5 N antibody (N-19), anti-CD19 antibody (4G7) PE-conjugated, and arsenic trioxide were described previously (11). An anti-CD43 antibody PE-conjugated, anti-B220 antibody APC-Cy7-conjugated, anti-IgM antibody APC-conjugated, and anti-human CD8 antibody V450-conjugated were purchased from BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), and Beckman Coulter (Miami, FL), respectively. An anti-mouse PML antibody for immunostaining was from LSbio (Seattle, WA).

Plasmids

PAX5-PML/pCDNA was described previously (11). PAX5-PML/MigRI was constructed by subcloning PAX5-PML cDNA fragments into MigRI, respectively. MigRI is a bicistronic retroviral vector using green fluorescent protein (GFP) as a transfection

marker and was a kind gift from Dr. W. S. Pear (University of Pennsylvania, Philadelphia, PA). Another bicistronic retroviral vector using the extracellular domain of human CD8 (hCD8), MSCV-hCD8, was described previously (26). BLNK/MSCV-hCD8 was constructed by subcloning mouse BLNK cDNA obtained from Addgene (Cambridge, MA). PAX5-PML/pBGJR, a lentivirus expression vector, was described previously.(11) pBGJR was kindly provided by Dr. Stefano Rivella (Memorial Sloan-Kettering Cancer Center).

Cell culture

OP9 cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS). S17 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS. P-PAL cells were cultured in 10% FBS and 2 μM 2-mercaptoethanol (2-ME; Sigma-Aldrich, St. Louis, MO) containing RPMI 1640 (Invitrogen).

Lentiviral Infection and Transplantation of Cells

The bone marrow cells of C57BL/6 mice were transfected with an empty vector and PAX5-PML expression vector by a previously described lentivirus system (27), and then transplanted into sub-lethally irradiated (4.5 Gry, two times) syngenic mice.

Retroviral Infection and Transplantation of Cells

These methods have been described previously (28). Briefly, B220+c-kit+ pro B cells were collected from the fetal livers of 10-week-old BALB/c mice by fluorescence-activated cell sorting (FACS), and then cultured on OP9 cells in OP9 culture medium supplemented with stem cell factor (10 ng /ml, Peprotech, Rocky Hill, NJ), flt3-ligand (10 ng /ml, Peprotech), interleukin-7 (10 ng /ml, Peprotech), and 2-ME (2 μM). PAX5-PML/MigRI and the empty MigRI vector were introduced into these pro B cells by a retrovirus transfection method. Viral stocks were generated by transfecting retroviral vectors and EcoPak, a packaging vector, which was a kind gift from Dr. Van Etten RA (Tufts-NEMC, Boston, MA), into 293T cells using Lipofectamine 2000 (Invitrogen). The retrovirus was transfected into pro B cells 48h after collection with 5 μg /ml protamine (Wako, Osaka, Japan). GFP-positive cells were collected by FACS 48h after transfection. These cells (5 X 10^6 /mouse) were then transplanted into half lethally irradiated (2 Gry) NOD/SCID mice. All animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee at Nagoya University.

Transient transfection, immunoprecipitation, immunoblotting, immunofluorescence staining, EMSA, and the luciferase assay

These methods were performed as described previously (11,29,30). The probes of 5'-GAATGGGGCACTGAGGCGTGACCACC GC-3' and 5'-AACTTGGCGATGCGCTCCAGCGAGTTT T-3', high-affinity PAX5-binding site sequences in the CD19 promoter and BLNK promoter (5), respectively, were used for EMSA. CD19-luc/pGL4 and BLNK-luc/pGL4 were used as the reporter genes for the luciferase assay. CD19-luc/pGL4 was described previously (11). BLNK-luc/pGL4 was constructed by inserting the nucleotide fragment containing 2

copies of the high-affinity PAX5-binding site sequence in the BLNK promoter into the pGL4.20 vector (Promega, Madison, WI). The *Renilla* luciferase expression vector, phRG-TK, was from Promega.

RESULTS

Introduction of PAX5-PML into pro B cells caused the arrest of differentiation in mice.

In order to investigate the leukemogenicity of PAX5-PML, we first attempted to transfect it into the hematopoietic progenitors of mice. Bone marrow cells were collected from BALB/c mice, transfected with a lentivirus vector expressing PAX5-PML and GFP, and transplanted into lethally irradiated BALB/c mice. B cells were not generated from GFP-positive cells in these mice, while approximately 20% of GFP-positive cells expressed B220 in control mice (Figure 1). Although the expression of PAX5-PML was expected to start at the pro B cell stage, similar to PAX5, it appeared to begin in earlier hematopoietic progenitors in this system. We speculated that PAX5-PML prevented the development of or was toxic to lymphoid progenitors; therefore, we established a new system to introduce PAX5-PML into pro B cells. We transfected the bicistronic retroviral vector expressing PAX5-PML and GFP into $B220^{+}/kit^{+}$ pro B cells sorted from the fetal livers of BALB/c mice and transplanted these cells into half-lethally irradiated NOD/SCID mice. Control pro B cells expressing GFP alone existed predominantly in the bone marrow until day 7, and differentiated into mature B cells, moved to the spleen until day 14, and then almost disappeared, possibly due to cell death, by day 21 (Figure 2A and 2C). On the other hand, PAX5-PML-introduced pro B cells remained in the pro B cell stage and stayed in the bone marrow for over 56 days, indicating the arrest of differentiation due to the introduction of PAX5-PML (Figure 2B and 2C).

PAX5-PML-introduced pro B cells caused ALL in mice

All 8 mice transplanted with PAX5-PML-introduced pro B cells died between days 63 and 158. A pathological examination revealed severe splenomegaly and the marked infiltration of leukocytes in the bone marrow and spleen (Figure 3A). A FCM analysis showed that these infiltrated leukocytes were GFP-positive and mainly had the pro B cell phenotype (Figure 3B), suggesting that these mice died due to ALL, which developed from PAX5-PML-introduced cells. The leukemia cells obtained were serially transplanted into BALB/c mice. Mice died more rapidly with repetitions of transplantation, that is, mice that received second and third transplantations died between days 48 and 55 and between days 14 and 20, respectively (Figure 3C). These results indicated that the introduction of PAX5-PML into pro B cells caused ALL in mice.

Establishment of PAX5-PML-induced leukemia cell lines

GFP-positive cells were collected from mice that received fourth transplantation and then cultured with S17, a bone marrow stromal cell line, in 10% FBS and 2 μM 2-ME-containing DMEM. After a few repetitions to sort GFP-positive cells, cells showed stable and exponential growth without

the S17 co-culture (Figure 4A). Cells were GFP-positive and had the pro B cell phenotype (Figure 4B). We designated this cell line as P-PAL (PAX5-PML-induced ALL).

The PML nuclear body was not disrupted in PAX5-PML-induced leukemia cells

The disruption of PML NBs by PML-RARα is known to be involved in the leukemogenesis of APL. ATO has been shown to induce apoptosis in APL cells through the reconstruction of PML NBs (see introduction). We previously demonstrated the disruption of PML NBs due to the overexpression of PAX5-PML and the reconstruction of the disrupted PML NBs by ATO in HeLa cells (11). Therefore, we herein determined whether PML NBs were disrupted in PAX5-PML-induced leukemia cells. The disruption of PML NBs was not detected in PAX5-PML-induced leukemia cells (Figure 5A) or P-PAL (Figure 5B). Furthermore, the ATO treatment did not significantly alter PML NBs, reduce the number of tumor cells, or improve the survival of leukemic mice (Figure 5A, 5B, 6A, and 6B). These results indicated that PAX5-PML did not exert its dominant-negative effect on PML function in this system and also that the inhibition of PML function was not necessary for the PAX5-PML-induced onset of leukemia.

We examined the expression of PAX5-PML in P-PAL in an attempt to clarify why PAX5-PML did not disrupt PML NBs in PAX5-PML-induced leukemia cells. Although the mRNA expression of PAX5-PML was confirmed by RT-PCR (Figure 5C), its protein expression was so weak that it could not be detected by immunoblotting using whole cell lysates of P-PAL. The protein expression of PAX5-PML was confirmed by immunoblotting immunoprecipitates of the whole cell lysate with an anti-PAX5 antibody or anti-PML antibody (Figure 5D), indicating that PAX5-PML did not significantly disrupt PML NBs because of its weak expression in leukemia cells.

Selective repression of BLNK may be important for the differentiation block by PAX5-PML

PAX5 is known to have many target genes for transcriptional activation and regulates B cell differentiation. We previously demonstrated that PAX5-PML had a dominant-negative effect on PAX5 transcriptional activity. PAX5-PML appeared to be involved in leukemogenesis through the differentiation block caused by the inhibition of PAX5 transactivity. In order to elucidate the mechanism underlying the differentiation block by PAX5-PML, we quantified the mRNA expression levels of PAX5 target genes such as *CD19, CD79A, BLNK, and CD72* at the pro B cell stage in PAX5-PML-induced leukemia cells. The expression of *BLNK* was significantly repressed in leukemia cells, whereas the repression of *CD19, CD72,* and *CD79A* was mild, suggesting the importance of *BLNK* repression for the differentiation block by PAX5-PML (Figure 7A). We confirmed that BLNK protein expression was reduced in PAX5-PML-induced leukemia cells (Figure 7B). In an attempt to determine whether this differential inhibition of PAX5 transactivity was intrinsic to PAX5-PML, we compared its DNA binding ability and inhibitory effects on transactivation by PAX5 between a *CD19* promoter and *BLNK* promoter. No significant

difference was observed in the DNA binding ability of PAX5-PML between the *CD19* promoter and *BLNK* promoter in EMSA (Figure 7C). Its DNA binding ability to both promoters was very weak; however, the DNA binding domain of PAX5 was maintained in PAX5-PML. These results were consistent with our and others previous findings (11,31). These results suggested that PAX5-PML had no preference of DNA binding between the two promoters. Although PAX5-PML exhibited weak DNA binding ability, PAX5 transactivity on the *BLNK* promoter was dominant-negatively inhibited by PAX5-PML in the reporter gene assay, similar to that on the *CD19* promoter. PAX5-PML did not show any preference of inhibition of PAX5 transactivity between the *CD19* promoter and *BLNK* promoter (Figure 7D). These results indicated that the selective suppression of BLNK was extrinsic to PAX5-PML. In these reporter gene assays, PAX5-PML almost completely inhibited PAX5 transactivity even when its expression level was markedly weaker than that of PAX5 (Figure 7D lanes 4 and 10). Taken together with the DNA binding ability of PAX5-PML being markedly weaker than that of PAX5, these results suggested that the mechanism underlying the dominant-negative inhibition of PAX5 transactivity was not the occupation of promoters by PAX5-PML.

We also determined whether the enforced expression of BLNK abrogated the differentiation block and survival in order to further establish the importance of BLNK repression for the PAX5-PML-induced leukemia development. We transfected the bicistronic retroviral vector expressing PAX5-PML and GFP together with that expressing BLNK and hCD8 into mouse pro B cells and transplanted them into mice. Only GFP^+hCD8^- cells but not GFP^+hCD8^+ cells could survive in the recipient mice on day 28 (Figure 8A). On the other hand, control pro B cells expressing PAX5-PML, GFP, and hCD8 but not BLNK could survive in the recipient mice regardless of hCD8 positivity if they expressed GFP (Figure 8A). In addition, another control pro B cells expressing GFP, hCD8, and BLNK but not PAX5-PML could not survive in the recipient mice (Figure 8A). Remained GFP^+ cells in bone marrow were mainly pro B cells in the both mouse groups transplanted with PAX5-PML-introduced pro B cells (Figure 8B). These results indicated that the enforced expression of BLNK abrogated the PAX5-PML-induced differentiation block and survival in mice.

DISCUSSION

Although numerous types of PAX5 fusion genes have been identified to date, their oncogenicities have not yet been confirmed. Our mouse leukemia model is the first to be induced by the PAX5-fusion gene. The introduction of PAX5-PML caused a differentiation block in pro B cells, which may have been the result of the suppression of PAX5 transactivity by PAX5-PML. Our results demonstrated that the differentiation block by PAX5-PML did not require the suppression of all PAX5 target genes. The repression of *CD19, CD72,* and *CD79A* was not required for the differentiation block. In other words, the repression of *BLNK* was sufficient for the differentiation block caused by PAX5-PML. Abrogation of PAX5-PML-induced differentiation block and initial survival by enforced BLNK expression further established its involvement in

the formation of pre-leukemic state induced by PAX5-PML (Figure 8). BLNK is an adaptor protein that bridges BCR-associated kinases with a multitude of signaling components and is essential for B cell differentiation, but not proliferation. The ablation of *Blnk* in mice was previously shown to cause a B cell differentiation block at the pro B cell stage without reducing the number of bone marrow cells (32). On the other hand, CD79A and CD19 are known to be essential for differentiation and proliferation. CD79A is the main component of BCR, the signal of which is required for the survival and differentiation of B cell. CD19 is a co-stimulatory molecule that amplifies BCR signaling. Both gene ablations of *Cd19* and *Cd79A* in mice not only caused a differentiation block, but also decreased the number of B cells (33,34). These findings suggested that the escape of *CD19* and *CD79A* from repression by PAX5-PML was reasonable for the development of leukemia by PAX5-PML and may be the reason for the selective repression of *BLNK*. A previous study reported that the leukemia cells of patients with PAX5-PML-positive ALL expressed CD19 and CD79A (25), implying that escape from repression by PAX5-PML also occurred in human leukemia cells.

The exact mechanism underlying selective *BLNK* repression has not yet been elucidated. No significant preference of the DNA binding of PAX5 was observed between the CD19 promoter and BLNK promoter, and PAX5-PML very weakly bound to both promoters (Figure 7B). It currently remains unknown how PAX5-PML acts as dominant-negative inhibitor of PAX5 without DNA binding ability; we previously demonstrated that PAX5-PML inhibited the transactivity of PAX5 by binding to PAX5 on the promoter (11). This model may explain the inhibition of PAX5 transactivity by PAX5-PML even when its expression was markedly lower than that of PAX5 (Figure 7D lanes 4 and 10) and also that PAX5-PML may suppress the expression of BLNK despite of its very weak expression in PAX5-PML-induced leukemia cells (Figure 5D and 7A). No significant difference was observed in the dominant-negative effects of PAX5-PML on PAX5 transactivity between the 2 promoters (Figure 7D). Therefore, the differential repression of PAX5 target genes appeared to be extrinsic to PAX5-PML. Transplanted pro B cells usually die soon after they differentiate into mature B cells, unless they are stimulated with a specific antigen, as shown in Figure 2A. Therefore, transplanted pro B cells remained in their recipient mice only when they succeeded in causing a differentiation block and resisted the growth-suppressive effects of PAX5-PML. Only cells that succeeded in selectively suppressing BLNK may be able to pass through this selection and survive in recipient mice as pre-leukemia cells. Some feedback signals and/or cellular compensatory mechanisms may be involved in maintaining the expression of CD19 and CD79A.

Although we demonstrated the involvement of BLNK repression in PAX5-PML-induced formation of pre-leukemic state, it is not only the mechanism underlying the development of leukemia. The long latency to develop leukemia after the differentiation block by the introduction of PAX5-PML implies the requirement of additional genetic and/or epigenetic events. BLNK repression and PAX5-PML expression appear to be insufficient to induce autonomous proliferation. Target(s) of a second hit for the development of leukemia have

not yet been identified. We performed microarray analyses to compare mRNA expression profiles between normal pro B cells and PAX5-PML-induced primary leukemia cells, but were unable to identify the gene expression change(s) responsible.

PML exerts its function by forming PML NBs and is involved in tumor suppression and stress-induced apoptosis. PML-RARα has been suggested to contribute to the development of APL through a dominant-negative effect on RARα and the disruption of PML NBs. Therefore, PAX5-PML may also have contributed to the development of leukemia through the disruption of PML NBs; however, our results clearly demonstrated that PML NBs were not disrupted in PAX5-PML-induced leukemia cells, and this may have been due to the insufficient expression of PAX5-PML (Figure 5A and 4D). Although we showed that PAX5-PML did not require the disruption of PML NBs for the development of leukemia in mice (Figure 5A) and also that ATO was not effective in the treatment of PAX5-PML-induced ALL (Figure 6A and 6B), these results denied neither the involvement of the disruption of PML NBs in the development of APL induced by PML-RARα nor the reconstruction of PML NBs in the mechanism of action of ATO in APL cells. It currently remains unclear whether ATO is effective for patients with PAX5-PML-positive ALL because the status of PML NBs in the leukemia cells of such patients has not yet been examined (25).

In summary, we herein demonstrated the leukemogenicity of PAX5-PML and partially elucidated the molecular mechanism underlying the development of leukemia. The genetic events leading to the development of leukemia during the long latency after a differentiation block need to be identified. Our model is suitable for analyzing the multi-step development of B-ALL and sheds new light on this field.

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Conflicts of Interest

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Author Contributions

F.H., N.I., S.K., and S.T. designed the research, performed experiments, and wrote the paper. T.M., Y.K., T.Y., and K.S. performed experiments. T.N. and H.K. designed the research. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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[7]The abbreviations used are: ALL, acute lymphoblastic leukemia; PAX, paired-box; BSAP, B cell-specific activator protein; BLNK, B cell linker protein; NBs, nuclear bodies; APL, acute promyelocytic leukemia; RARα, retinoic acid receptor α; ATO, arsenic trioxide; GFP, green fluorescent protein; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; 2-ME, 2-mercaptoethanol; FACS, fluorescence-activated cell sorting

FIGURE LEGENDS

FIGURE 1. PAX5-PML prevented B-cell development *in vivo*. Bone marrow cells transfected with an empty vector and PAX5-PML expression vector were transplanted into sub-lethally irradiated syngenic mice. Mice were designated as Control/BL6 and PAX5-PML/BL6, respectively. Three months later, bone marrow cells in the indicated mice were analyzed using a flow cytometer. GFP-positive cells in the DAPI-negative lymphoid fraction of bone marrow cells were gated and the expression of B220 was examined. GFP-positive cells were gated in the red square and their ratio (%) was shown in the left panel. The expression of B220 was plotted on histograms, and the ratio (%) of B220-positive cells was shown in the right panel. These experiments were performed using 2 mice for each group and similar results were observed. We

showed representative data.

FIGURE 2. Introduction of PAX5-PML into pro B cells caused differentiation arrest in mice. (A) A B cell differentiation analysis of control cells. Bone marrow (BM) and spleen (Sp) cells were collected on the indicated days after the transplantation of pro B cells transfected with the empty vector expressing only GFP. The expression of CD43 and B220 in cells was analyzed by FACS after gating for the GFP+, DAPI-, and lymphoid fraction (scatter). The rate of GFP+ cells in the DAPI-negative lymphoid fraction was presented at the top. Blue, green, and red squares are indicated as the pro B, pre B and immature B, and mature B cell fractions, respectively. Numbers are the rate (%) of each fraction in GFP+ cells. (B) Differentiation arrest of PAX5-PML-introduced cells. Mice transplanted with pro B cells transfected with an expression vector for PAX5-PML and GFP were analyzed as in (A). (C) Bar graph of the rate of GFP-positive cells. The averages of the GFP+ cell rate in (A) and (B) are plotted on the bar chats. Two mice per each group were analyzed. The bar graphs are classified by color according to the rates of pro B, pre B and immature B, and mature B. Control pro B cells differentiated into mature B cells, and moved to Sp on Day 14, and disappeared until day 21, while PAX5-PML-introduced (P-P-introduced) pro B cells maintained the pro B cell phenotype and remained in BM 56 days after transplantation.

FIGURE 3. ALL development from PAX5-PML-induced cells in mice. (A) The infiltration of immature hematopoietic cells. Sections of the indicated organs from a dying mouse transplanted with PAX5-PML-introduced pro B cells were stained with Hematoxylin & Eosin (HE). (B) A flow cytometric analysis of the infiltrated cells. BM and spleen cells from the same mouse as in (A) were analyzed as in Figure 2. (C) The survival of mice with serial transplantations. Survival curves were plotted according to the Kaplan–Meier method. The curves of mice that received transplantations with PAX5-PML-introduced pro B cells, spleen cells from a primary transplanted mouse, and spleen cells from a secondary transplanted mouse are plotted as blue, red, and green lines, respectively. After the second transplantation, 5 X 10^6 cells were transplanted into irradiated (2Gry) BALB/c mice.

FIGURE 4. Establishment of P-PAL. (A) Growth curve of P-PAL. P-PAL (1 X 10^5 /ml) was co-cultured with S17 (2 X 10^3 /ml). Live cell numbers were counted using the tripanblue exclusion method at the indicated time and plotted as relative values to that on day 0. (B) A flow cytometric analysis of P-PAL. The surface marker of P-PAL was analyzed as in Figure 2, except for an additional analysis of IgM expression. The black square indicated the pro B cell fraction. P-PAL appeared to have the pro B cell phenotype.

FIGURE 5. The disruption of PML NBs did not occur in PAX5-PML-induced leukemia cells. (A) Intact PML NBs in PAX5-PML-induced leukemia cells. BM cells collected from control and leukemic mice with or without the ATO treatment (intraperitoneal injections of 5 μg /g /day for 3 days), and immunostained with an anti-mouse PML antibody. (B) The disruption of PML NBs was not detected in P-PAL. P-PAL was treated with or without 10 μM ATO for 6 h and subjected to immunostaining with an anti-mouse PML antibody. (C) PAX5-PML mRNA expression in PAX5-PML-induced leukemia cells (P-P-induced LC) and P-PAL. PAX5-PML mRNA expression was confirmed by RT-PCR amplifying the region that contained the junction of PAX5 and PML. PAX5-PML/pCDNA was used as a positive control for PCR. (D) PAX5-PML protein expression in P-PAL. The whole cell lysate of P-PAL and its immunoprecipitates with the indicated antibodies were subjected to immunoblotting with the indicated antibodies. The whole cell lysate of 293T cells transfected with the PAX5-PML expression vector was used as a positive control.

FIGURE 6. The ATO treatment caused neither a significant reduction in the number of tumor cells nor improvement in the survival of leukemic mice. (A) No significant reduction was observed in the number of leukemia cells after the ATO treatment. Irradiated (2Gry) BALB/c mice were transplanted with 5 X 10^6 leukemia cells. ATO (5 μg/g) was administered intraperitoneally daily from days 2 to 16 after transplantation. Mice were sacrificed on day 16. The spleen weight and ratio of GFP-positive cells in spleen cells were measured and plotted on scatter diagrams (left and right panels, respectively). The red bars indicate the average values. Statistical comparisons were performed using the *t*-test, and *p*-values are shown. (B) The survival of leukemia mice was not prolonged by the ATO treatment. Leukemia mice were generated as in (A), and the indicated amount of ATO was administered daily from days 7 to 16.

Survival curves were plotted according to the Kaplan–Meier method. Differences in survival were analyzed by the log-rank test, and a *p*-value is shown.

FIGURE 7. Selective repression of BLNK in PAX5-PML-induced leukemia cells. (A) Quantification of mRNA expression of PAX5 transcriptional target genes. The expression of the indicated genes was quantified by quantitative RT-PCR using mRNA from pro B cells employed in the transplantation assay (Control pro B), P-P-induced LC, and P-PAL, and plotted on bar charts. The average values relative to basal expression in control pro B cells in two independent analyses are shown (results are the mean ± SD). (B) Reduced expression of the BLNK protein in P-P induced LC. Lysates of the indicated cells were subjected to immunoblotting with an anti-BLNK antibody. (C) PAX5-PML very weakly bound to both PAX5 binding sites in the *CD19* and *BLNK* promoters. Equal amounts of PAX5 and PAX5-PML were incubated with radiolabeled oligonucleotides containing the PAX5 binding sites of the indicated promoters in the presence of a 200-fold molar excess of unlabeled oligonucleotides (competitor), normal goat IgG (control antibody), or an anti-PAX5 N antibody as indicated. Black and white arrows indicate PAX5 DNA complexes and supershifted bands, respectively. PAX5-PML DNA complexes were hardly observed and are indicated with asterisks. Similar results were obtained from two independent experiments. Representative data is shown. (D) The dominant-negative transcriptional repression by PAX5-PML was similar between both promoters. The luciferase assay was performed by transfecting 125 ng PAX5/pCDNA, increasing the amounts of PAX5-PML/pCDNA (31.25 ng–125 ng), and the reporter genes containing the PAX5 binding sites of the indicated promoters to 293T cells. Luciferase activities in 3 independent transfection experiments are shown as average values relative to the basal activation of each reporter gene by PAX5 (results are the mean ± SD). Another set of cells transfected with the same plasmids as the above described luciferase assay were lysed for IB. The lysates were subjected to IB with an anti-PAX5 N antibody in order to determine the protein expression levels of PAX5 and PAX5-PML (bottom panel). PAX5 and PAX5-PML (P-P) were indicated by a single and double arrow, respectively.

FIGURE 8. Enforced expression of BLNK abrogated the PAX5-PML-induced pre-leukemic state in mice. A bicistronic expression vector for PAX5-PML and GFP was transduced into mouse pro B cells together with that for BLNK and hCD8 or an expression vector for hCD8 alone, and the pro B cells were designated as P-P & BLNK-introduced pro B and Control P-P-introduced pro B, respectively. Another control pro B cells transduced with a GFP expression vector and a bicistronic expression vector for BLNK and hCD8 was also prepared and designated as Control BLNK-introduced pro B. These cells were transplanted into mice without the selection of GFP or hCD8 positive cells and the mice were analyzed after 28 days. (A) BLNK expression inhibited the survival of PAX5-PML-induced pro B cells. The expressions of GFP and hCD8 of the transplanted pro B cells (Day 1) and the bone marrow cells of the recipient mice (Day 28) were analyzed by FACS after gating for the lymphoid fraction and $B220^+$. The average rates of GFP^+hCD8^- cells and GFP^+hCD8^+ cells are plotted on the bar chats. Two mice per each group were analyzed. hCD8 $(BLNK)^+$ fraction of P-P & BLNK-introduced pro B could not survive, even when they expressed GFP (PAX5-PML). (B) The remained GFP-positive B cells caused differentiation block. The blue and red numbers in the left panels indicate the rates of GFP^+hCD8^- cells and GFP^+hCD8^+ cells, respectively. Differentiation status of the fraction indicated by black square was analyzed as in Figure 2A. A representative data is shown. GFP-positive cells remained in mice 28 days after transplantation caused differentiation block.

Figure 1

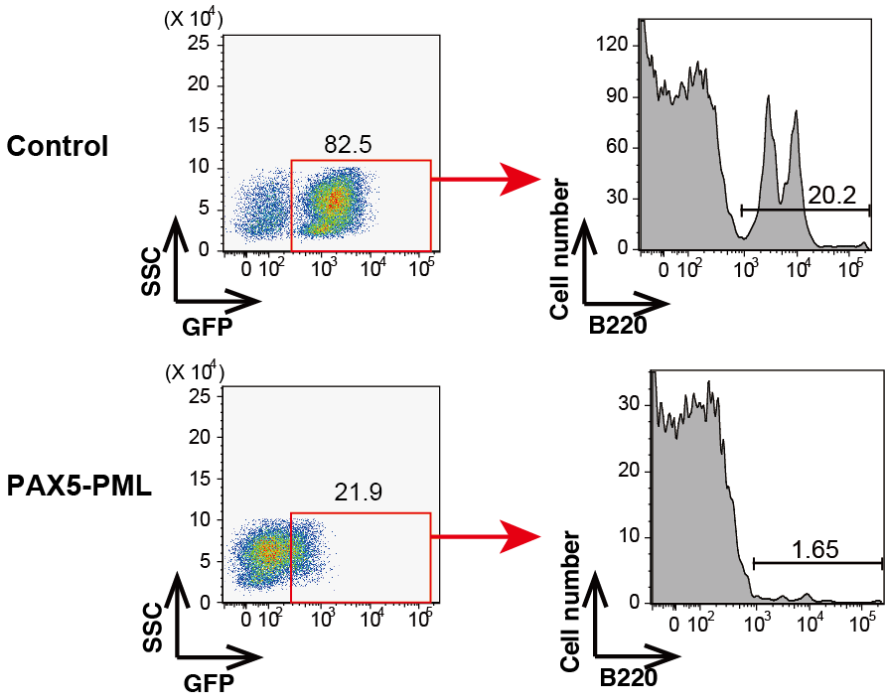
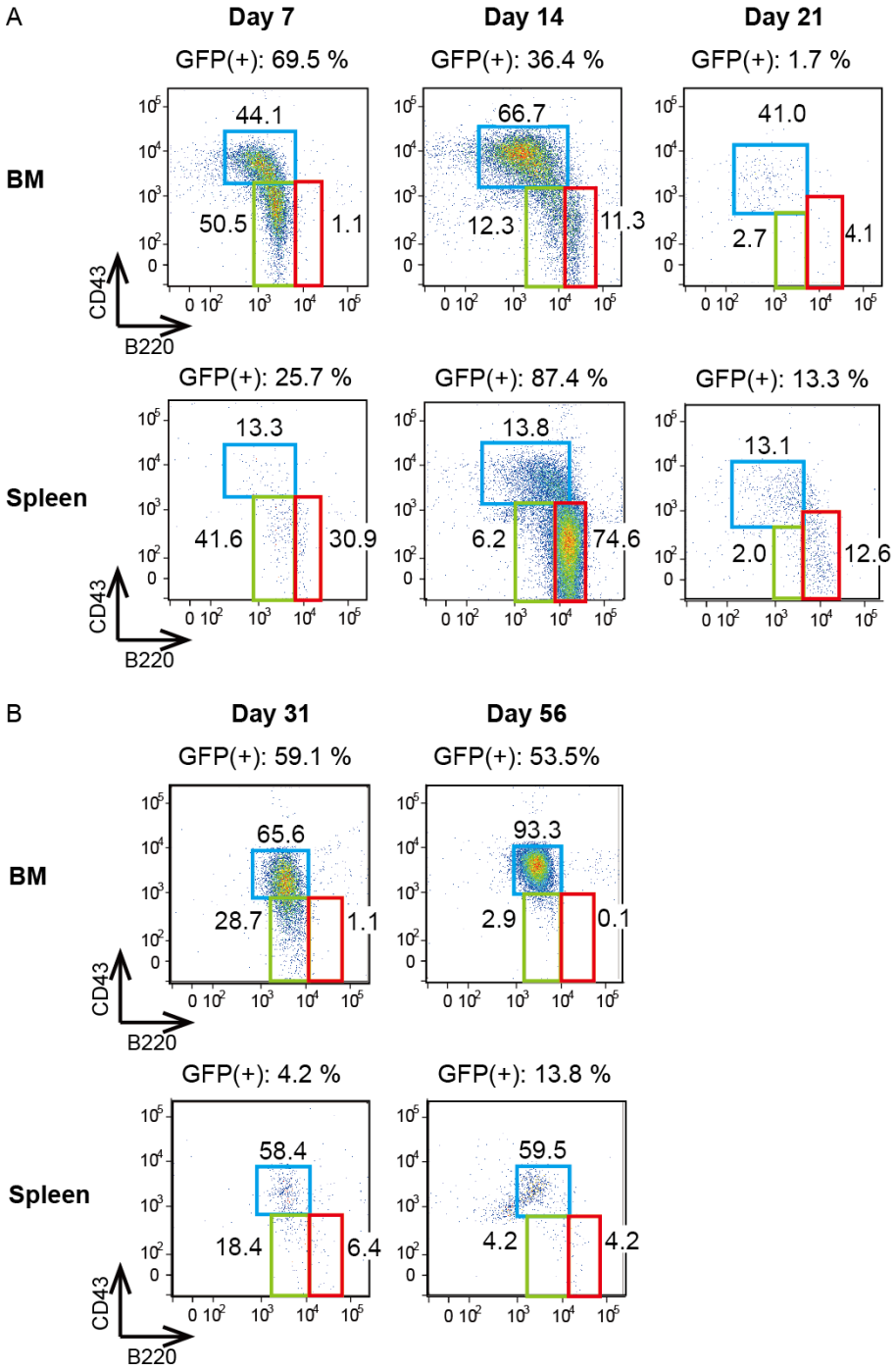


Figure 2



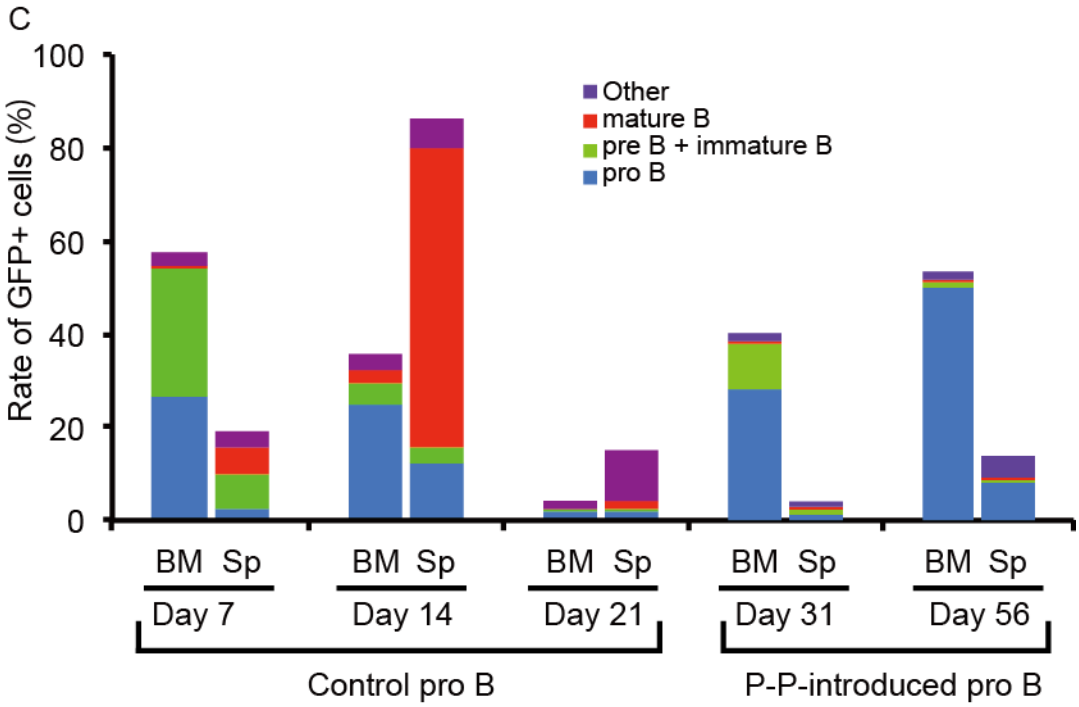


Figure 3

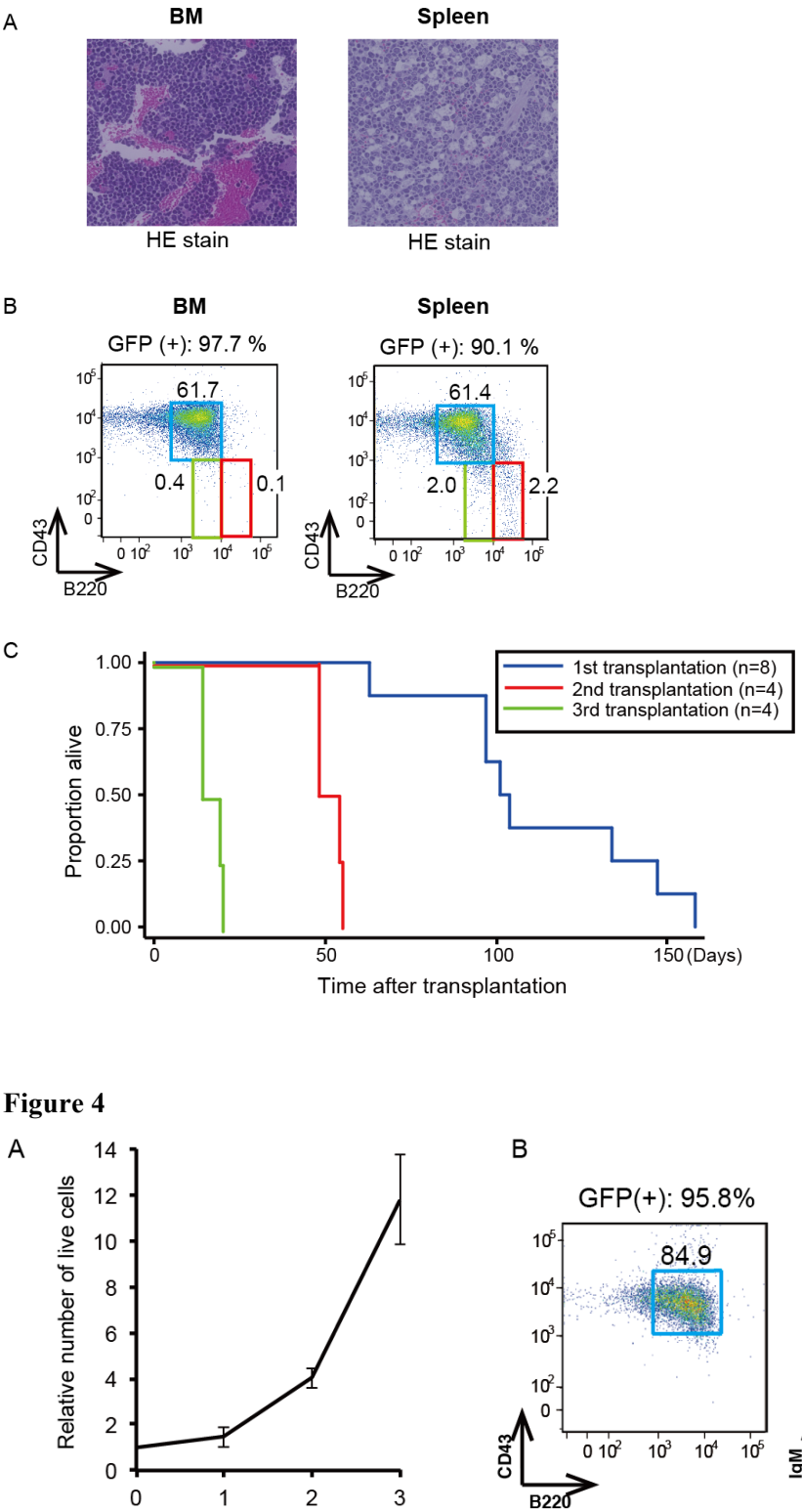


Figure 4

Figure 5

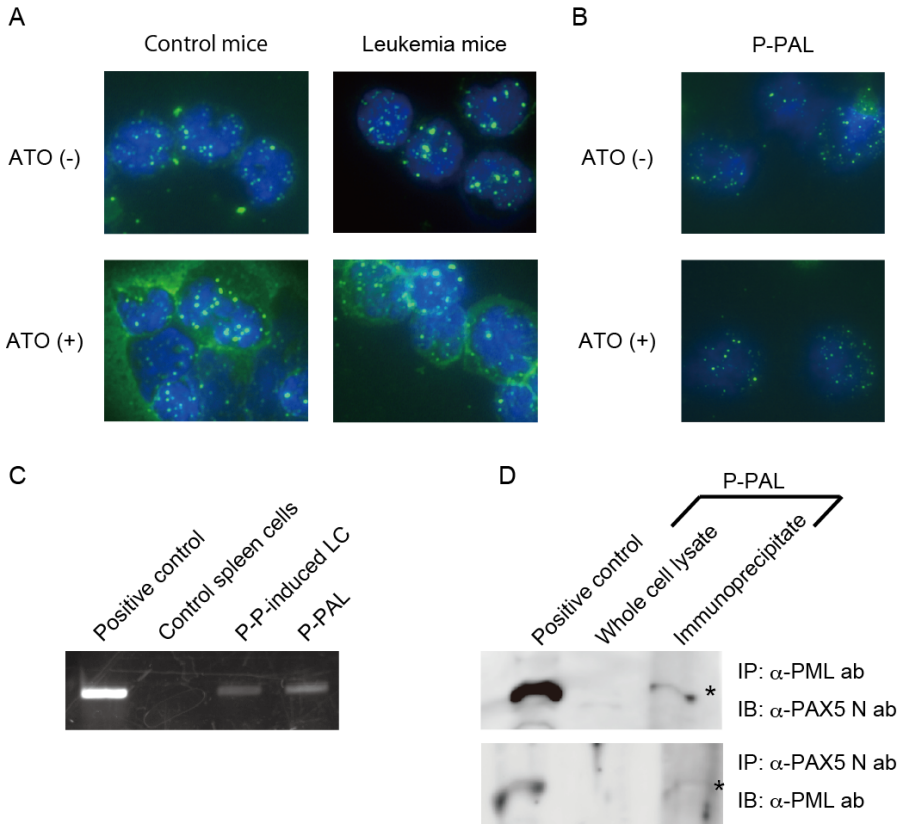


Figure 6

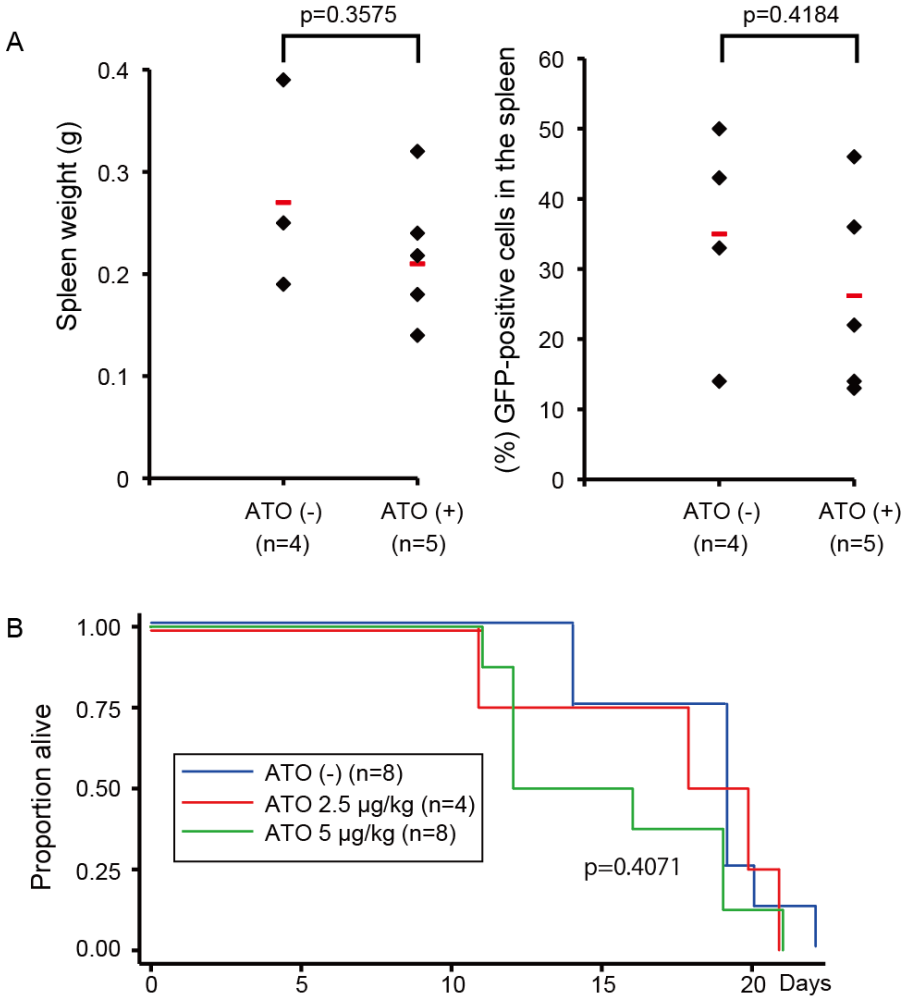


Figure 7

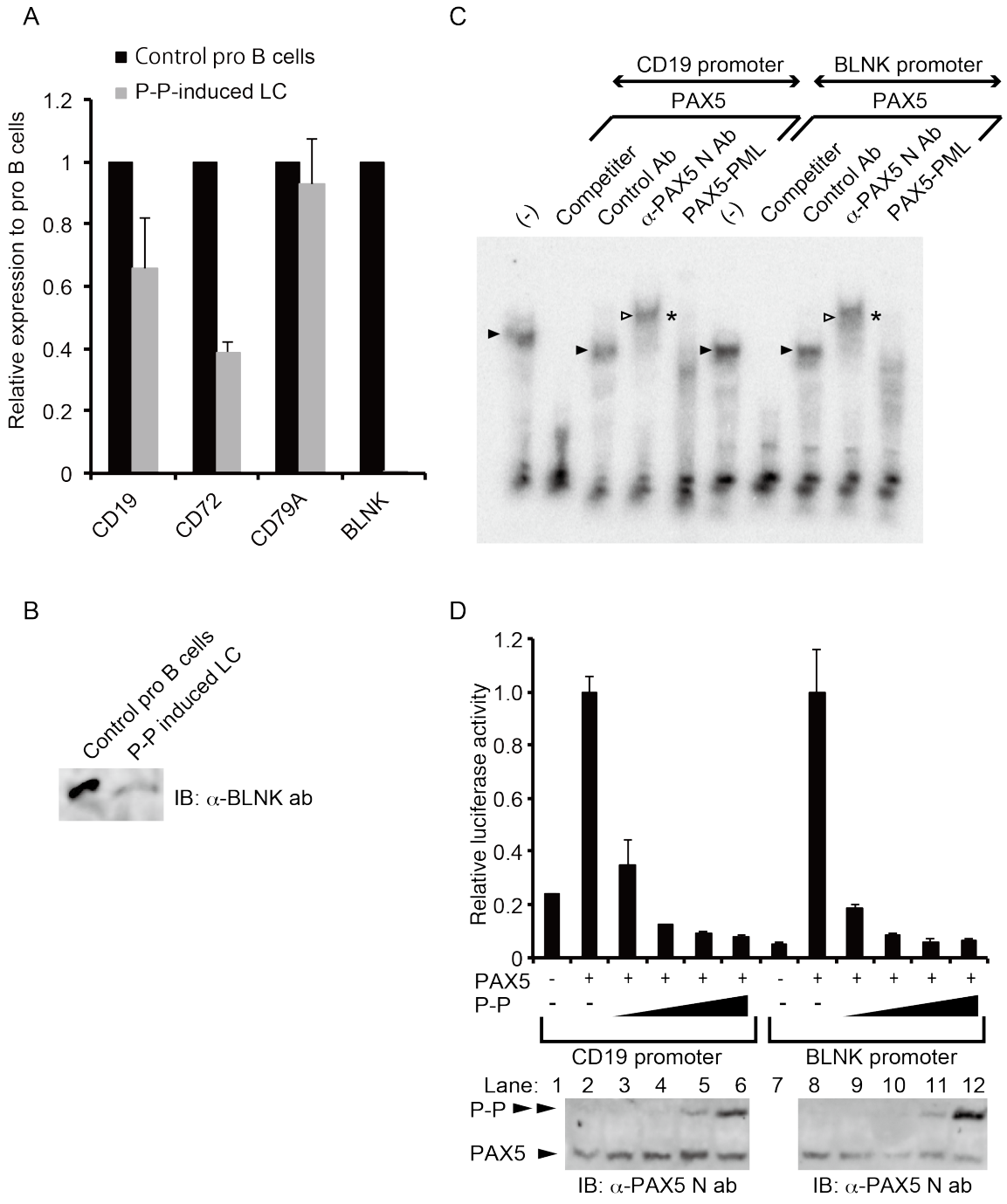
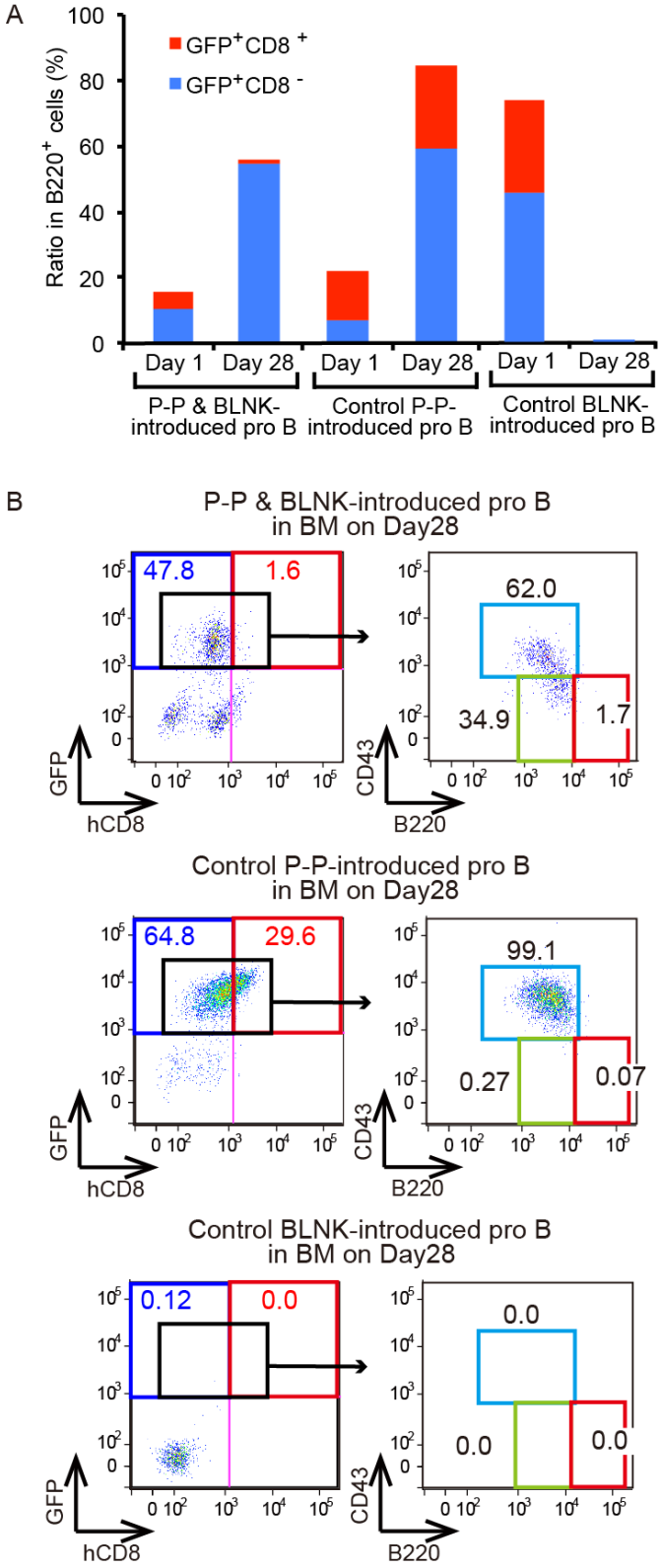


Figure 8



BLNK is a selective target of repression by PAX5-PML in the differentiation block that leads to the development of acute lymphoblastic leukemia

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