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B Cell Linker Protein (BLNK) Is a Selective Target of Repression by PAX5-PML Protein in the Differentiation Block That Leads to the Development of Acute Lymphoblastic Leukemia*

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PAX5 is a transcription factor that is required for the development and maintenance of B cells. Promyelocytic leukemia (PML) is a tumor suppressor and proapoptotic factor. The fusion gene *PAX5-PML* has been identified in acute lymphoblastic leukemia with chromosomal translocation t(9;15)(p13;q24). We have reported previously that PAX5-PML dominant-negatively inhibited PAX5 transcriptional activity and impaired PML function by disrupting PML nuclear bodies (NBs). Here we demonstrated the leukemogenicity of PAX5-PML by introducing it into normal mouse pro-B cells. Arrest of differentiation was observed in PAX5-PML-introduced pro-B cells, resulting in the development of acute lymphoblastic leukemia after a long latency in mice. Among the transactivation targets of PAX5, B cell linker protein (BLNK) was repressed selectively in leukemia cells, and enforced BLNK expression abrogated the differentiation block and survival induced by PAX5-PML, indicating the importance of BLNK repression for the formation of preleukemic 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)[2] domain family of transcription factors. PAX5 is expressed exclusively from the pro-B to mature B cell stage and is down-regulated 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 has been shown to cause B lymphoid maturation arrest at the pro-B cell stage (6). Previous studies have 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 have been shown previously to have dominant-negative effects on PAX5 transcriptional activity and have been suggested to be mainly responsible for the differentiation disorder of ALL with these fusion genes (9–12). Consistently, a previous study has reported that PAX5 haploinsufficiency cooperated 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 proapoptotic factor (14, 15). In normal cells, the PML protein is localized in discrete subnuclear compartments called PML nuclear bodies (NBs) (16). In PML NBs, PML co-accumulates with more than 70 proteins that are involved in tumor suppression, apoptosis, regulation of gene expression, anti-viral responses, and DNA

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[2] The abbreviations used are: PAX, paired box; ALL, acute lymphoblastic leukemia; NB, nuclear body; APL, acute promyelocytic leukemia; RAR, retinoic acid receptor; ATO, arsenic trioxide; PML, promyelocytic leukemia; P-PAL, PAX5-PML-induced acute lymphoblastic leukemia; BLNK, B cell linker protein.

repair. PML has been suggested to exert its effects by regulating the functions of binding partners at the core of PML NBs (17). PML NBs have been found previously 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 used clinically in the treatment of APL, reportedly induced the restoration of disrupted PML NBs and apoptosis in APL cells, resulting in 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 two cases of B-progenitor ALL with chromosomal translocation t(9;15)(p13;q24) (25). We have demonstrated previously 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 have shown that the expression of PAX5-PML in a non-hematological tumor cell line induced the disruption of PML NBs and resistance to apoptosis and that 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 and the potential of ATO therapy for this type of ALL (11).

In this 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—The anti-PML antibody (H-238); anti-PAX5 N antibody (N-19); anti-CD19 antibody (4G7), phycoerythrin-conjugated; and arsenic trioxide have been described previously (11). The anti-CD43 antibody, phycoerythrin-conjugated; anti-B220 antibody, allophycocyanin-Cy7-conjugated; anti-IgM antibody, allophycocyanin-conjugated; and anti-human CD8 antibody, V450-conjugated were purchased from BD Biosciences, BioLegend (San Diego, CA), and Beckman Coulter (Miami, FL), respectively. The anti-mouse PML antibody for immunostaining was from LSbio (Seattle, WA).

Plasmids—PAX5-PML/pCDNA has been described previously (11). PAX5-PML/MigRI was constructed by subcloning PAX5-PML cDNA fragments into MigRI. MigRI is a bicistronic retroviral vector using GFP as a transfection marker and was a 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, has been 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, has been described previously (11). pBGJR was provided by Dr. Stefano Rivella (Memorial Sloan-Kettering Cancer Center).

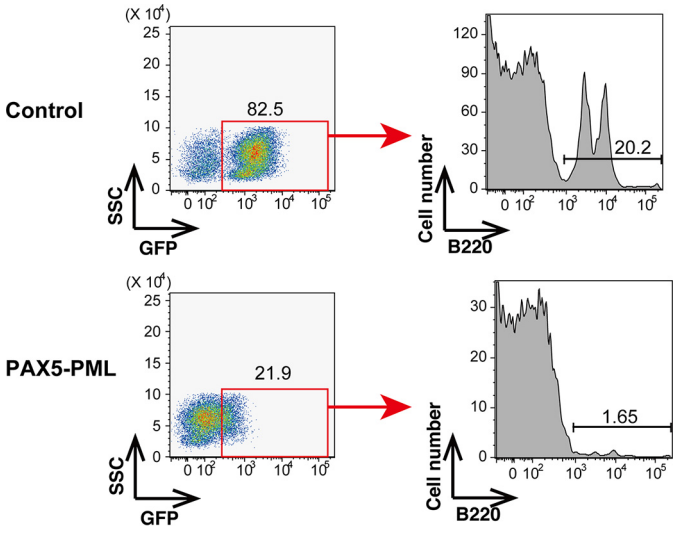

FIGURE 1. **PAX5-PML prevented B cell development *in vivo*.** Bone marrow cells transfected with an empty vector and a PAX5-PML expression vector were transplanted into sublethally irradiated syngenic mice. Mice were designated control/BL6 and PAX5-PML/BL6, respectively. Three months later, bone marrow 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 (percent) is shown in the *left panel*. The expression of B220 was plotted on histograms, and the ratio (percent) of B220-positive cells is shown in the *right panel*. These experiments were performed using two mice for each group, and similar results were observed. Representative data are shown. *SSC*, side scatter.

Cell Culture—OP9 cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 15% FBS. S17 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS. P-PAL cells were cultured in 10% FBS and 2 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) containing RPMI 1640 (Invitrogen).

Lentiviral Infection and Transplantation of Cells—Bone marrow cells of C57BL/6 mice were transfected with an empty vector and PAX5-PML expression vector by a lentivirus system described previously (27) and then transplanted into sublethally irradiated (4.5 Gy, 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 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-mercaptoethanol (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 (a gift from Dr. R. A. Van Etten, Tufts-New England Medical Center, Boston, MA), into 293T cells using Lipofectamine 2000 (Invitrogen). The retrovirus was transfected into pro-B cells 48 h after collection with 5 μg/ml protamine (Wako, Osaka, Japan). GFP-positive cells were collected by FACS 48 h after transfection. These cells (5×10^6/mouse) were then transplanted into half-lethally irradiated (2 Gy) NOD/SCID mice. All animal experi-

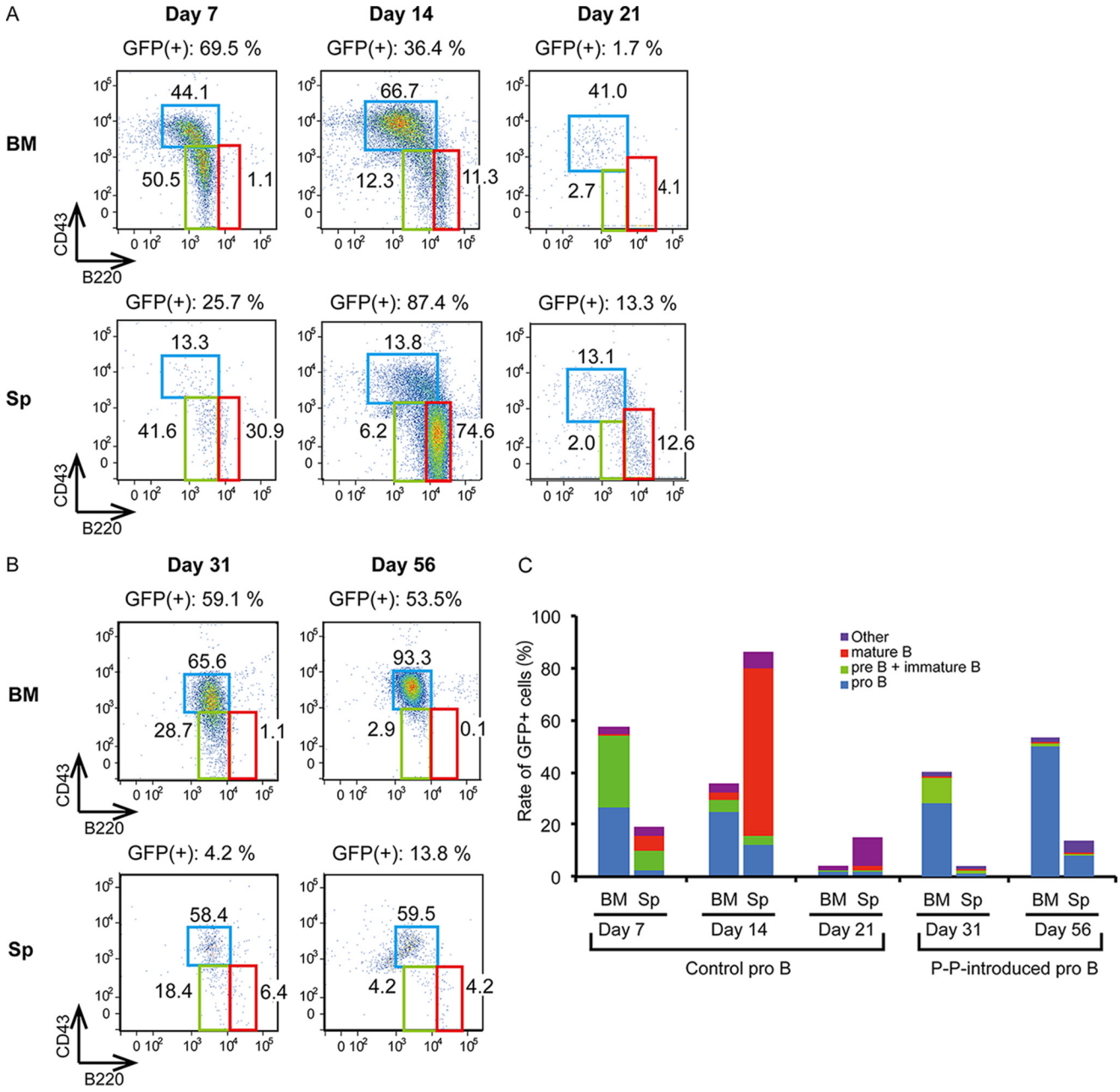

FIGURE 2. **Introduction of PAX5-PML into pro-B cells caused differentiation arrest in mice.** *A*, a B cell differentiation analysis of control cells. Bone marrow (*BM*) and spleen (*Sp*) cells were collected on the indicated days after the transplantation of pro-B cells transfected with an empty vector 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 is presented at the *top*. *Blue*, *green*, and *red squares* indicated the pro-B, pre-B and immature B, and mature B cell fractions, respectively. Numbers are the rate (percent) 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*, the rate of GFP-positive cells. The averages of the GFP^+ cell rates in *A* and *B* are plotted. Two mice per 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 cells. Control pro-B cells differentiated into mature B cells, moved to the spleen (*SP*) on day 14, and disappeared until day 21, whereas PAX5-PML-introduced (*P-P-introduced*) pro-B cells maintained the pro-B cell phenotype and remained in the bone marrow (*BM*) 56 days after transplantation.

ments 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 Luciferase Assay—These methods were performed as described previously (11, 29, 30). The probes of 5′-GAATGGGGCACTGAGGCGTGACCACCGC-3′ and 5′-AACTTGGCGATGCGCTCCAGCGAGTTTT-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 has been described previously (11). BLNK-luc/pGL4 was constructed by inserting a nucleotide fragment containing two 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—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, whereas ~20% of GFP-positive cells expressed B220 in control mice (Fig. 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 because of cell death, by day 21 (Fig. 2, *A* and *C*). 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 because of the introduction of PAX5-PML (Fig. 2, *B* and *C*).

PAX5-PML-introduced Pro-B cells Caused ALL in Mice—All eight mice transplanted with PAX5-PML-introduced pro-B cells died between days 63 and 158. A pathological examination revealed severe splenomegaly and a marked infiltration of leukocytes in the bone marrow and spleen (Fig. 3*A*). A flow cytometry analysis showed that these infiltrated leukocytes were GFP-positive and mainly had the pro-B cell phenotype (Fig. 3*B*), suggesting that these mice died because of ALL that developed from PAX5-PML-introduced cells. The leukemia cells obtained were transplanted serially into BALB/c mice. The 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 (Fig. 3*C*). 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 a fourth transplantation and then cultured with S17, a bone marrow stromal cell line, in 10% FBS and 2 μM 2-mercaptoethanol-containing DMEM. After a few repetitions to sort GFP-positive cells, the cells showed stable and exponential growth without the S17 co-culture (Fig. 4*A*). Cells were GFP-positive and had the pro-B cell phenotype (Fig. 4*B*). We designated this cell line 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 have

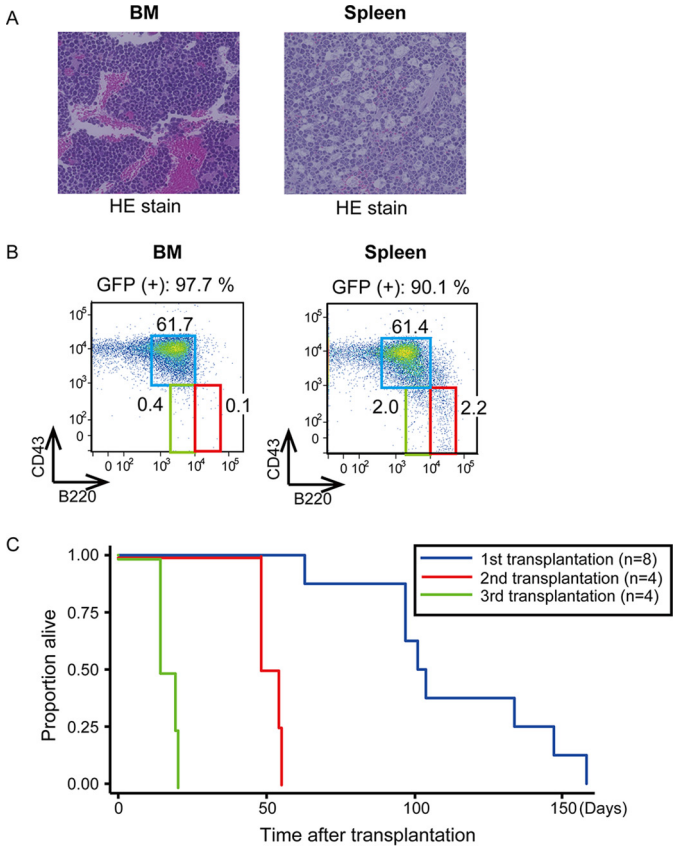

FIGURE 3. **ALL development from PAX5-PML-induced cells in mice.** *A*, infiltration of immature hematopoietic cells. Sections of the indicated organs from a dying mouse transplanted with PAX5-PML-introduced pro-B cells were stained with H&E (*HE*). *BM*, bone marrow. *B*, flow cytometric analysis of the infiltrated cells. Bone marrow and spleen cells from the same mouse as in *A* were analyzed as in Fig. 2. *C*, 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, 5×10^6 cells were transplanted into irradiated (2 Gy) BALB/c mice.

previously demonstrated the disruption of PML NBs because of the overexpression of PAX5-PML and the reconstruction of the disrupted PML NBs by ATO in HeLa cells (11). Therefore, here we 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 (Fig. 5*A*) or P-PAL cells (Fig. 5*B*). Furthermore, ATO treatment did not significantly alter PML NBs, reduce the number of tumor cells, or improve the survival of leukemic mice (Fig. 5, *A* and *B*, and 6, *A* and *B*). These results indicated that PAX5-PML did not exert its dominant-negative effect on PML function in this system and 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 (Fig. 5*C*), its protein expression was so weak that it could not be detected by immunoblotting using whole-cell lysates of P-PAL cells. The protein expression of PAX5-PML was confirmed by immunoblotting immunoprecipitates of the whole-cell lysate with an

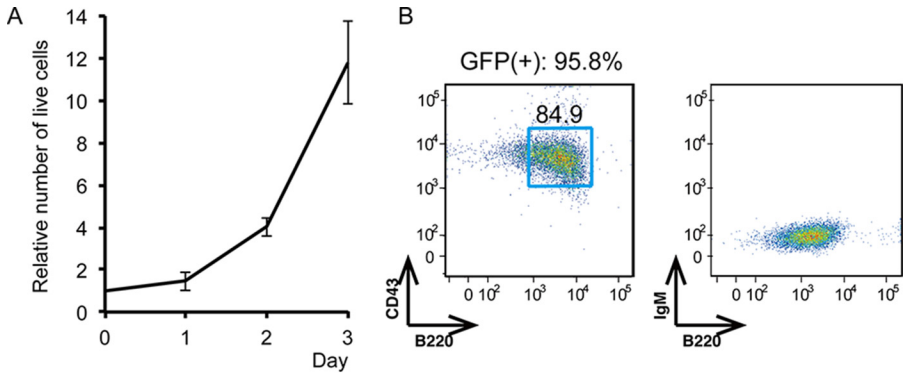

FIGURE 4. **Establishment of P-PAL.** *A*, growth curve of P-PAL. P-PAL (1×10^5/ml) was co-cultured with S17 (2×10^3/ml). Live cell numbers were counted using the trypan blue exclusion method at the indicated time and plotted as relative values to that on day 0. *B*, flow cytometric analysis of P-PAL.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

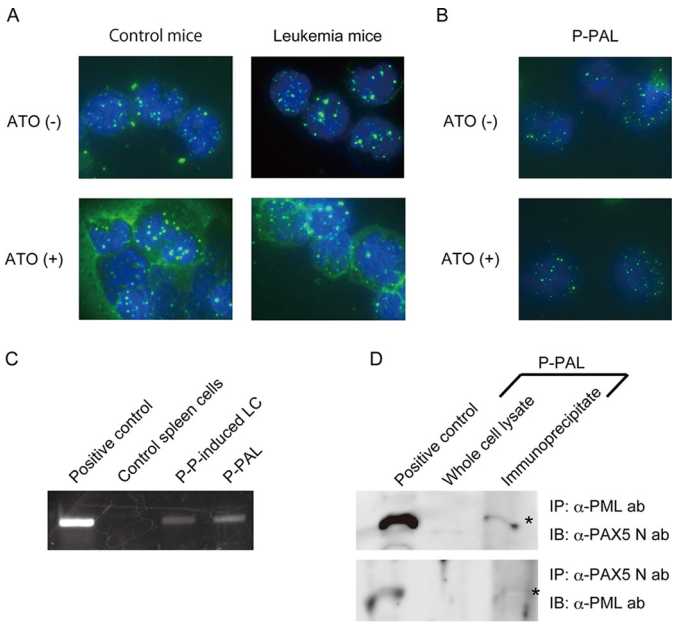

FIGURE 5. **Disruption of PML NBs did not occur in PAX5-PML-induced leukemia cells.** *A*, intact PML NBs in PAX5-PML-induced leukemia cells. Bone marrow (*BM*) cells were collected from control and leukemic mice with or without ATO treatment (intraperitoneal injections of 5 μg/g/day for 3 days) and immunostained with an anti-mouse PML antibody. *B*, 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 (*IP*) with the indicated antibodies was subjected to immunoblotting (*IB*) with the indicated antibodies. The whole-cell lysate of 293T cells transfected with the PAX5-PML expression vector was used as a positive control.

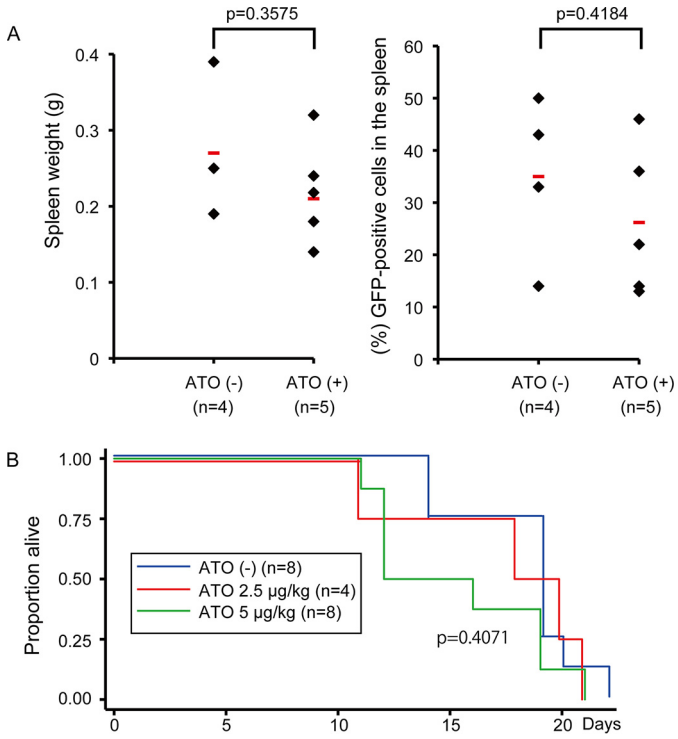

FIGURE 6. **ATO treatment caused neither a significant reduction in the number of tumor cells nor improvement in the survival of leukemic mice.** *A*, no significant reduction was observed in the number of leukemia cells after ATO treatment. Irradiated (2 Gy) BALB/c mice were transplanted with 5×10^6 leukemia cells. ATO (5 μg/g) was administered intraperitoneally daily from days 2–16 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 *t* test, and *p* values are shown. *B*, the survival of leukemia mice was not prolonged by ATO treatment. Leukemia mice were generated as in *A*, and the indicated amount of ATO was administered daily from days 7–16. Survival curves were plotted according to the Kaplan-Meier method. Differences in survival were analyzed by log-rank test, and the *p* value is shown.

anti-PAX5 antibody or anti-PML antibody (Fig. 5*D*), 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 have demonstrated previously 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. 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 repressed significantly in leukemia cells, whereas the repression of *CD19*, *CD72*, and *CD79A* was mild, suggesting the

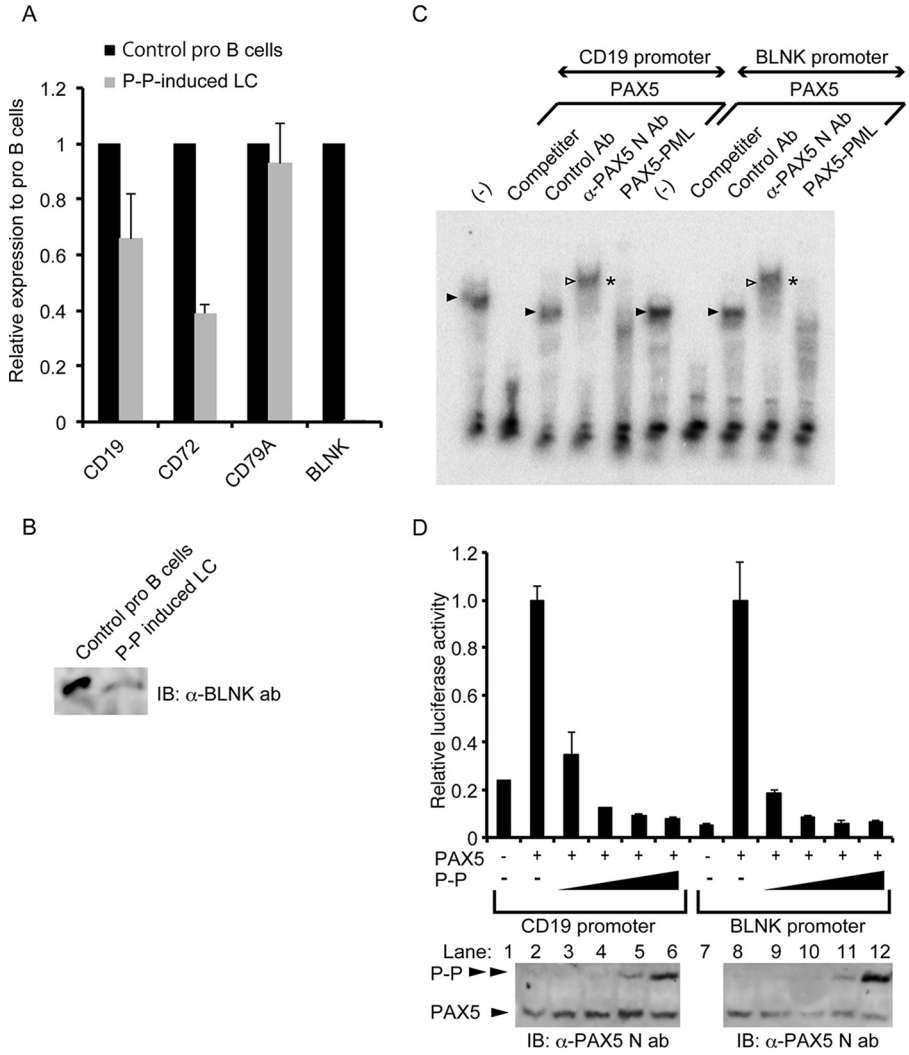

FIGURE 7. **Selective repression of BLNK in PAX5-PML-induced leukemia cells.** *A*, quantification of mRNA expression of PAX5 transcriptional target genes. The expression of the indicated genes was quantified by quantitative RT-PCR using mRNA from pro-B cells employed in the transplantation assay (*control pro-B cells*) and PAX5-PML-induced leukemia cells (*P-P-induced LC*) 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 mean ± S.D.). *B*, reduced expression of the BLNK protein in PAX5-PML-induced leukemia cells. Lysates of the indicated cells were subjected to immunoblotting (*IB*) with an anti-BLNK antibody (*ab*). *C*, PAX5-PML bound very weakly 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 Ab*), or an anti-PAX5 N antibody as indicated. *Black* and *white arrowheads* 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 are shown. *D*, the dominant-negative transcriptional repression by PAX5-PML was similar in both promoters. The luciferase assay was performed by transfecting 125 ng of PAX5/pCDNA, increasing the amounts of PAX5-PML/pCDNA (31.25–125 ng), and the reporter genes containing the PAX5 binding sites of the indicated promoters into 293T cells. Luciferase activities in three independent transfection experiments are shown as average values relative to the basal activation of each reporter gene by PAX5 (results are mean ± S.D.). Another set of cells transfected with the same plasmids as in the luciferase assay described above were lysed for immunoblotting. The lysates were subjected to immunoblotting with an anti-PAX5 N antibody to determine the protein expression levels of PAX5 and PAX5-PML (*bottom panel*). PAX5 and PAX5-PML (*P-P*) are indicated by *single* and *double arrowheads*, respectively.

importance of *BLNK* repression for the differentiation block by PAX5-PML (Fig. 7*A*). We confirmed that BLNK protein expression was reduced in PAX5-PML-induced leukemia cells (Fig. 7*B*). 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 (Fig. 7*C*). 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 previous findings and those of others (11, 31) and suggested that PAX5-PML had no preference of DNA binding between the two promoters. Although PAX5-PML exhibited a weak DNA binding ability, PAX5 transactivity on the *BLNK* promoter was inhibited dominant-negatively 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 (Fig. 7*D*). 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

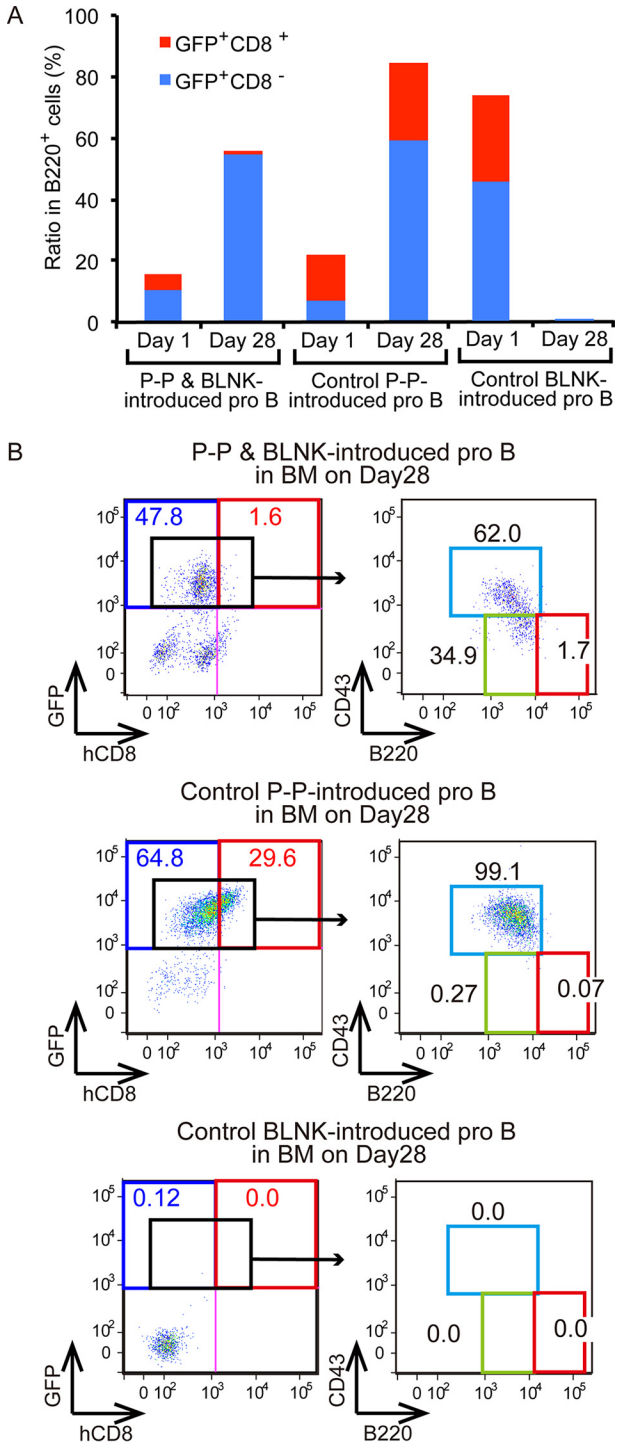

FIGURE 8. **Enforced expression of BLNK abrogated the PAX5-PML-induced preleukemic state in mice.** A bicistronic expression vector for PAX5-PML and GFP was transduced into mouse pro-B cells together with that for BLNK and hCD8 or an expression vector for hCD8 alone, and the pro-B cells were designated *P-P & BLNK-introduced pro-B* and *Control P-P-introduced pro-B*, respectively. Other control pro-B cells transduced with a GFP expression vector and a bicistronic expression vector for BLNK and hCD8 were also prepared and designated *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 expression 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. Two mice per group were analyzed. The hCD8 (BLNK)$^+$ fraction of P-P & BLNK-introduced pro-B could not survive, even when they expressed GFP (PAX5-PML). *B*, the remaining 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. The differentiation status of the fraction indicated by the *black square* was analyzed as in Fig. 2*A*. Representative data are shown. GFP-positive cells remained in mice 28 days after transplantation caused differentiation block.

was markedly weaker than that of PAX5 (Fig. 7*D*, *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 to further establish the importance of BLNK repression for 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 (Fig. 8*A*). 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, when they expressed GFP (Fig. 8*A*). In addition, other control pro-B cells expressing GFP, hCD8, and BLNK but not PAX5-PML could not survive in the recipient mice (Fig. 8*A*). The remaining GFP^+ cells in the bone marrow were mainly pro-B cells in both mouse groups transplanted with PAX5-PML-introduced pro-B cells (Fig. 8*B*). 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 that 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 the PAX5-PML-induced differentiation block and initial survival by enforced BLNK expression further established its involvement in the formation of the preleukemic state induced by PAX5-PML (Fig. 8). BLNK is an adaptor protein that bridges B cell receptor-associated kinases with a multitude of signaling components and is essential for B cell differentiation but not proliferation. The ablation of *Blnk* in mice has been shown previously 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 B cell receptor, the signal of which is required for the survival and differentiation of B cells. CD19 is a co-stimulatory molecule

that amplifies B cell receptor signaling. Gene ablation 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 gave advantage to the development of leukemia by PAX5-PML and may be the reason for the selective repression of *BLNK*. A previous study has reported that 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 in the CD19 promoter and BLNK promoter, and PAX5-PML bound very weakly to both promoters (Fig. 7*B*). It currently remains unknown how PAX5-PML acts as dominant-negative inhibitor of PAX5 without DNA binding ability. We have demonstrated previously 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 (Fig. 7*D*, *lanes 4* and *10*) and that PAX5-PML may suppress the expression of BLNK despite its very weak expression in PAX5-PML-induced leukemia cells (Figs. 5*D* and 7*A*). No significant difference was observed in the dominant-negative effects of PAX5-PML on PAX5 transactivity between the two promoters (Fig. 7*D*). 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 Fig. 2*A*. 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 succeed in selectively suppressing BLNK may be able to pass through this selection and survive in recipient mice as preleukemia 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 the preleukemic state, it is not the only 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 (Figs. 4D and 5*A*). Although we showed that PAX5-PML did not require the disruption of PML NBs for the development of leukemia in mice (Fig. 5*A*) and that ATO was not effective in the treatment of PAX5-PML-induced ALL (Fig. 6, *A* and *B*), 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, here we 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 multistep development of B-ALL and sheds new light on this field.

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