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response to As_2O_3 therapy. Further investigation is required to confirm the clinical significance.

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Authorship

Contribution: E.G., A.T., and F.H. designed the experiments, performed the research, and the analyzed data; E.G. and A.T. wrote the manuscript; A.A. prepared the clinical samples and performed the research; and A.T., H.K., and T.N. interpreted the data and supervised the experiments.

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Correspondence: Akihiro Tomita, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan; e-mail: atomita@med.nagoya-u.ac.jp.

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ORIGINAL ARTICLE

PAX5–PML acts as a dual dominant-negative form of both PAX5 and PML

S Kurahashi[1], F Hayakawa[1], Y Miyata[1], T Yasuda[1], Y Minami[1], S Tsuzuki[2], A Abe[1] and T Naoe[1]

[1]*Department of Hematology and Oncology, Nagoya University, Graduate School of Medicine, Nagoya, Japan and* [2]*Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan*

PAX5 is a transcription factor required for B-cell development and maintenance. PML is a tumor suppressor and a pro-apoptotic factor. A fusion gene, *PAX5–PML*, was found in acute lymphoblastic leukemia (ALL) with chromosomal translocation t(9;15)(p13;q24), but no functional analysis has been reported. Here, we demonstrate that PAX5–PML had a dominant-negative effect on both PAX5 and PML. PAX5–PML dominant negatively inhibited PAX5 transcriptional activity in the luciferase reporter assay and suppressed the expression of the PAX5 transcriptional targets in B-lymphoid cell line. Surprisingly, PAX5–PML hardly showed DNA-binding activity *in vitro* although it retained the DNA-binding domain of PAX5. Additional experiments, including chromatin immunoprecipitation (ChIP) assay, suggested that PAX5–PML bound to the promoter through the association with PAX5 on the promoter. On the other hand, coexpression of PAX5–PML inhibited PML sumoylation, disrupted PML nuclear bodies (NBs), and conferred apoptosis resistance on HeLa cells. Furthermore, treatment with arsenic trioxide (ATO) induced PML sumoylation and reconstitution of PML NBs, and overcame the anti-apoptotic effect of PAX5–PML in HeLa cells. These data suggest the possible involvement of this fusion protein in the leukemogenesis of B-ALL in a dual dominant-negative manner and the possibility that ALL with PAX5–PML can be treated with ATO.
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Introduction

PAX5 is a member of the highly conserved paired-box (PAX) domain family of transcription factors. As is the B-cell specific activator protein, PAX5 is exclusively expressed from the pro-B to the mature B-cell stage and downregulated during terminal differentiation into plasma cells (Nutt *et al.*, 1998). Pax5 is not only indispensable for B-lineage commitment but its continuous expression is also essential to maintain this fate (Nutt *et al.*, 2001; Mikkola *et al.*, 2002; Busslinger, 2004). PAX5 functions as both a transcriptional activator of B-lineage specific genes and a repressor of B-lineage inappropriate genes (Nutt *et al.*, 1998); that is, it activates *CD19* (Kozmik *et al.*, 1992), *MB-1* (Maier *et al.*, 2003) and *BLNK* (Schebesta *et al.*, 2002), and it represses *MCSFR* (Morrison *et al.*, 1998), *Notch1* (Souabni *et al.*, 2002) and *FLT3* (Holmes *et al.*, 2006). Among them, *CD19* is the best investigated target, and PAX5 directly controls its expression. It has been shown that the *CD19* promoter region has a PAX5-binding consensus sequence (Kozmik *et al.*, 1992).

To further emphasize the essential role of PAX5 for proper B-cell development, *PAX5* gene aberrations are frequently found in B-cell neoplasia. The t(9;14)(p13;q32) translocation found in a subset of B-cell non-Hodgkin's lymphoma brings the potent enhancer of the *IGH* gene close to the *PAX5* promoter, leading to the aberrant expression of normal PAX5 protein (Busslinger *et al.*, 1996; Iida *et al.*, 1996). Furthermore, the *PAX5* gene is 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 (Mullighan *et al.*, 2007; Familiades *et al.*, 2009). These mutations consist of partial or complete hemizygous deletions, homozygous deletions, partial or complete amplifications, point mutations or fusion genes (Mullighan *et al.*, 2007). These aberrations of the *PAX5* gene will impair PAX5 function more or less, and will be involved in blocking B-cell differentiation.

PML is a potent growth suppressor that, when overexpressed, can block cell cycle progression in a variety of tumor cell lines (Dyck *et al.*, 1994); conversely, $PML^{-/-}$ mouse embryo fibroblasts replicate significantly faster than their $PML^{+/+}$ mouse embryo fibroblasts (Wang *et al.*, 1998a). PML also has an essential role in DNA damage or stress-induced apoptosis, and $PML^{-/-}$ cells are resistant to a variety of apoptotic signals (Wang *et al.*, 1998b). In normal cells, PML protein is localized in, and is essential for the biogenesis of, discrete subnuclear compartments designated as PML nuclear bodies (NBs) (Ishov *et al.*, 1999). In PML NBs, PML coaccumulates with >70 proteins that are involved in tumor suppression, apoptosis, regulation of gene expression, anti-viral response and DNA repair. PML is

Correspondence: Dr F Hayakawa, Department of Hematology and Oncology, Nagoya University, Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.
E-mail: bun-hy@med.nagoya-u.ac.jp
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thought to exert its function by regulating binding partners' functions as a core of PML NBs (Dellaire and Bazett-Jones, 2004). Intriguingly, PML NBs are disrupted in human acute promyelocytic leukemias (APL) by PML–retinoic acid receptor (RAR)α, an oncogenic fusion protein of PML and RARα, which is thought to be the mechanism of the anti-apoptotic effect of PML–RARα (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994).

It has been reported that PML NB formation requires PML to be conjugated to small ubiquitin-related modifier-1 (Ishov *et al.*, 1999; Zhong *et al.*, 2000a). Arsenic trioxide (ATO), a chemotherapeutic agent clinically used in the treatment of APL, induces PML sumoylation. Increased PML sumoylation induced by ATO treatment leads to the restoration of PML NBs disrupted by PML–RARα, and then is followed by apoptosis of APL cells, which results in prolonged remission of the disease (Chen *et al.*, 1997; Zhu *et al.*, 1997; Muller *et al.*, 1998; Niu *et al.*, 1999). These findings underscore the importance of PML sumoylation and the integrity of PML NBs in tumor suppression.

A fusion gene, *PAX5–PML*, was found in two cases of B-progenitor ALL with chromosomal translocation t(9;15)(p13;q24) (Nebral *et al.*, 2007). PAX5 moiety of PAX5–PML retains its DNA-binding domain but loses its transactivation domain, suggesting that PAX5–PML is a dominant-negative form of PAX5. In addition, PAX5–PML will be able to associate with PML because it retains almost the entire PML, which might lead to disruption of PML NBs and impaired PML function; however, no functional analysis of PAX5–PML has been performed. Here, we demonstrate that PAX5–PML had a dominant-negative effect on both PAX5 and PML. PAX5–PML dominant negatively inhibited PAX5 transcriptional activity in the luciferase reporter assay and suppressed the expression of PAX5 transcriptional targets in cells. On the other hand, coexpression of PAX5–PML inhibited PML sumoylation, disrupted PML NBs, and conferred apoptosis resistance on HeLa cells. Furthermore, treatment with ATO induced PML sumoylation and reconstitution of PML NBs, and overcame apoptosis resistance. These data suggest 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.

Results

PAX5–PML suppressed PAX5 transcriptional activity in a DNA-binding-independent manner

According to DNA sequence information of the breakpoint reported previously (Nebral *et al.*, 2007), we first made the PAX5–PML expression vector by overlapping PCR using complementary DNA of PAX5 and PML as templates. PAX5–PML retained almost the entire PML and DNA-binding domain of PAX5 but lost its transactivation domain (Figure 1a). This raised the possibility that PAX5–PML acted as a dominant-negative form of PAX5; therefore, we investigated the effect of PAX5–PML on PAX5 transcriptional activity. In 293T cells, PAX5 increased luciferase expression from the reporter gene containing the high-affinity PAX5-binding site of *CD19* promoter. Coexpression of PAX5–PML decreased it in a dose-dependent manner, suggesting its dominant-negative effect on PAX5 transcriptional activity (Figure 1b). Of note, PAX5–PML coexpression also decreased basal luciferase expression (lane 1 vs lane 2), although it did not affect the basal luciferase expression from the reporter genes containing the GATA-1 regulatory domain of the promoter of EKLF and erythropoietin receptor, kind gifts from Dr Masayuki Yamamoto (Tohoku University, data not shown). These data suggested that observed repression was not nonspecific suppression by PAX5–PML. To reveal the mechanism of the inhibition of PAX5 transactivation by PAX5–PML, we examined the DNA-binding ability of PAX5–PML in an electromobility shift assay (EMSA). The single band obtained from the PAX5-containing sample was challenged by the presence of non-radiolabeled competitors and was supershifted by anti-PAX5 N antibody but not by control goat immunoglobulin (Ig) G and was thought to be the PAX5–DNA complex (Figure 1c, left panel). Unexpectedly, PAX5–PML showed little DNA-binding ability although it had an intact DNA-binding domain of PAX5 (Figure 1c, right panel). To reveal the mechanism of the observed suppression of PAX5 transactivation in a luciferase assay without direct DNA binding, we first confirmed whether PAX5–PML binding on the promoter was impaired under the conditions of our luciferase assay. We performed a ChIP assay using 293T cells similarly transfected with plasmids, as in Figure 1b, showing impaired promoter binding of Flag–PAX5–PML (Figure 1d, left panel, lane 2 vs lane 3). Comparable expression levels between Flag–PAX5 and Flag–PAX5–PML were confirmed by immunoblotting (Figure 1d, right panel). Notably, impaired promoter binding of PAX5–PML was restored by the coexpression of non-tagged PAX5, which was not immunoprecipitated directly with anti-Flag antibody (Figure 1d, lane 3 vs lane 4). We also demonstrated that PAX5–PML could associate with PAX5 (Supplementary Figures S1A and B). These results suggested that PAX5–PML exerted its dominant-negative effect on PAX5 transcriptional activity by binding to the promoter by the association with PAX5 on the promoter. To further confirm that transcriptional repression by PAX5–PML was independent of its DNA-binding ability, we introduced a DNA-binding dead mutation into PAX5 and PAX5–PML (designated as PAX5 M and PAX5–PML M, respectively), and performed luciferase assays. DNA-binding dead mutation is a combination of mutations of P80R, V26G and 126INS/DEL, which have been reported to impair PAX5 DNA-binding ability (Mullighan *et al.*, 2007). Impaired DNA-binding ability of PAX5 M and PAX5–PML M was confirmed by EMSA, although the effect of the mutation was not significant in PAX5–PML M because

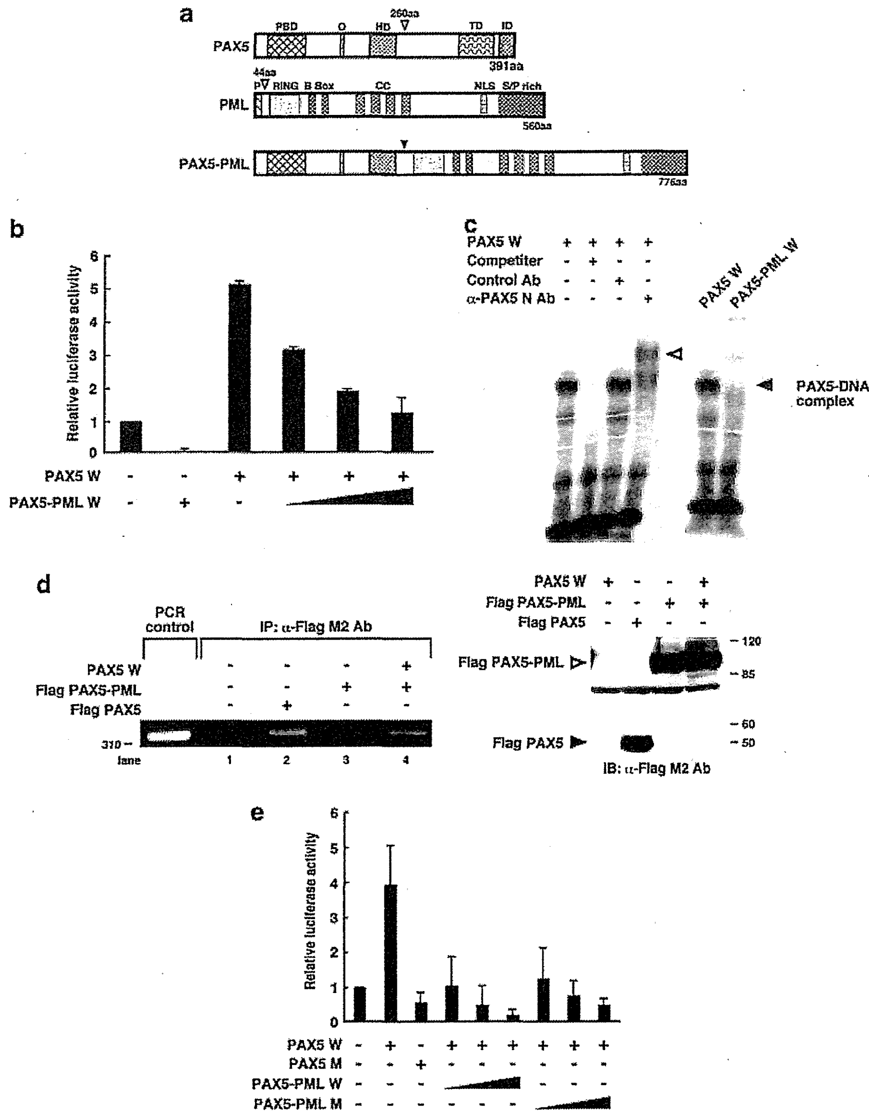

Figure 1 Dominant-negative effect of PAX5–PML on the transcriptional activity of PAX5. (**a**) Schematic presentation of PAX5, PML and PAX5–PML. PBD, paired box domain; O, conserved octapeptide; HD, homeobox domain; TD, transactivation domain; ID, inhibitory domain; P, proline-rich sequence; RING, 'really interesting new gene' finger; B box, B box zinc fingers; CC, coiled-coil domain; NLS, nuclear localization signal; S/P-rich, serine/proline-rich region. (**b**) Dominant-negative transcriptional repression by PAX5–PML. Luciferase assay was performed as described in Materials and methods section by transfecting with 125 ng PAX5 W/pCDNA and increasing amounts of PAX5–PML W/pCDNA (31.25–125 ng). Luciferase activities in three independent transfection experiments are shown as average values relative to the basal activity observed in control cells (results are the mean ± s.d.). (**c**) PAX5–PML had impaired DNA-binding ability. Equal amounts of PAX5 were incubated with a radiolabeled oligonucleotide probe containing PAX5-binding site of *CD19* promoter in the presence of a 200-fold molar excess of unlabeled oligonucleotide (competitor), normal goat IgG (control antibody) or anti-PAX5 N antibody as indicated (left panel). PAX5 DNA complex (indicated by a black arrow) was visualized with an imaging analyzer. Supershifted band is indicated by a white arrow. Equal amounts of PAX5 and PAX5–PML were subjected to the same EMSA (right panel). Similar results were obtained from three independent experiments. Representative data are shown. (**d**) PAX5–PML binding to the promoter was mediated by PAX5. 293T cells were transfected with CD19–luc/PGL4 and the indicated expression vector. Cells were subjected to ChIP assay with anti-Flag antibody. Coexpression of PAX5 improved PAX5–PML binding to the promoter, suggesting that PAX5–PML bound to the promoter by the association with PAX5 on the promoter (left panel). 293 T cells transfected with plasmids as in ChIP assay were subjected to immunoblotting (IB) with anti-Flag antibody, and similar expression levels between Flag–PAX5 and Flag–PAX5–PML were confirmed (right panel). Similar results were obtained from two independent experiments. Representative data are shown. (**e**) Transcriptional repression by PAX5–PML did not depend on its DNA-binding ability. Luciferase assay using DNA-binding dead mutant of PAX5 and PAX5–PML was performed as in **b**. PAX5 lost its transcriptional activity by the mutation, while transcriptional repression by PAX5–PML was hardly affected.

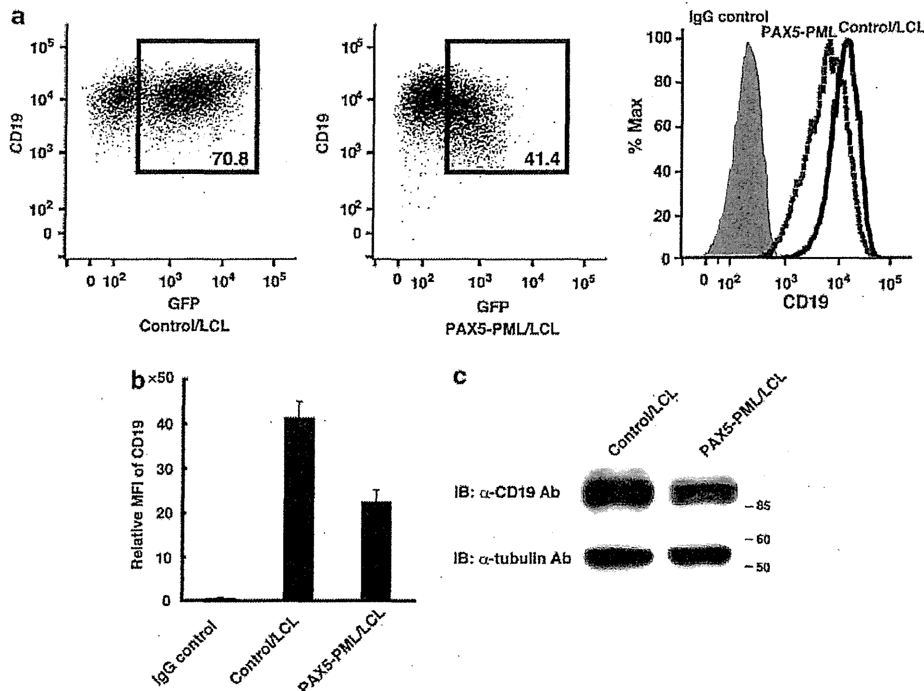

Figure 2 Suppression of CD19 expression by PAX5–PML in LCL. (**a**) The correlation between CD19 and GFP expression was analyzed with a flow cytometer (left and middle panel) in the indicated LCL 7 days after lentivirus infection. GFP-positive cells were gated as indicated and their CD19 expressions were compared (right panel). Solid line and broken line indicate the histograms of Control/LCL and PAX5–PML/LCL using anti-CD19 antibody, respectively. Gray shading indicates the histogram of Control/LCL using control IgG. Similar results were obtained from two independent experiments. Representative data are shown. (**b**) Mean fluorescence intensity (MFI) of CD19 relative to control IgG in (**a**) was measured and is plotted on bar charts. The average with standard deviation is shown. CD19 expression in GFP-positive cells of PAX5–PML/LCL was reduced by 55% compared with that in Control/LCL. (**c**) The same LCL as in (**a**) was lysed on the same day as flow cytometer analysis and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and IB with anti-CD19 antibody and anti-tubulin antibody.

of its originally impaired DNA-binding ability (Supplementary Figure S2). In the luciferase assay, PAX5 M showed severely impaired transcriptional activity in correspondence with its impaired DNA-binding ability, while the dominant-negative effect of PAX5–PML was hardly affected by the mutation (Figure 1e). These results indicated that transcriptional repression by PAX5–PML was independent of its DNA-binding ability.

PAX5–PML suppressed CD19 expression in LCL
Next, we examined if PAX5–PML actually suppressed the expression of the PAX5 target gene. LCL, a cell line of mature B cells expressing PAX5 and CD19 endogenously, was transfected with the empty vector (Control/LCL) or PAX5–PML expression vector (PAX5–PML/LCL) using a lentivirus system. Both vectors expressed green fluorescent protein (GFP) as a gene induction marker. Seven days after lentivirus infection, the correlation between GFP and CD19 expressions was investigated with a flow cytometer, and CD19 expressions of GFP-positive cells in both cells were compared. The correlation between GFP and CD45, whose expression was not regulated by PAX5, was also investigated. CD19 expression in Control/LCL and CD45 expression in PAX5–PML/LCL were constant regardless of GFP expression (Figure 2a, left panel and Supplementary Figure S3), whereas CD19 expression in PAX5–PML/LCL was low in the GFP-positive fraction (Figure 2a, middle panel). CD19 expression in PAX5–PML/LCL measured by mean fluorescence intensity was reduced by 55% compared with that in Control/LCL (Figure 2a, right panel and Figure 2b). Immunoblot analysis also showed lower CD19 expression level in PAX5–PML/LCL (Figure 2c). These results indicated that PAX5–PML suppressed CD19 expression in human B-lymphoid lineage cells.

PAX5–PML repressed PML sumoylation and disrupted PML NBs
An alternative PML fusion protein, PML–RARα, associates with PML through its coiled-coil domain and disrupts PML NBs, which is thought to confer apoptosis resistance on APL cells. As shown in Figure 1a, PAX5–PML retained almost the entire PML, including the coiled-coil domain, suggesting the possibility that PAX5–PML also associated with PML and disrupted PML NBs. These findings prompted us

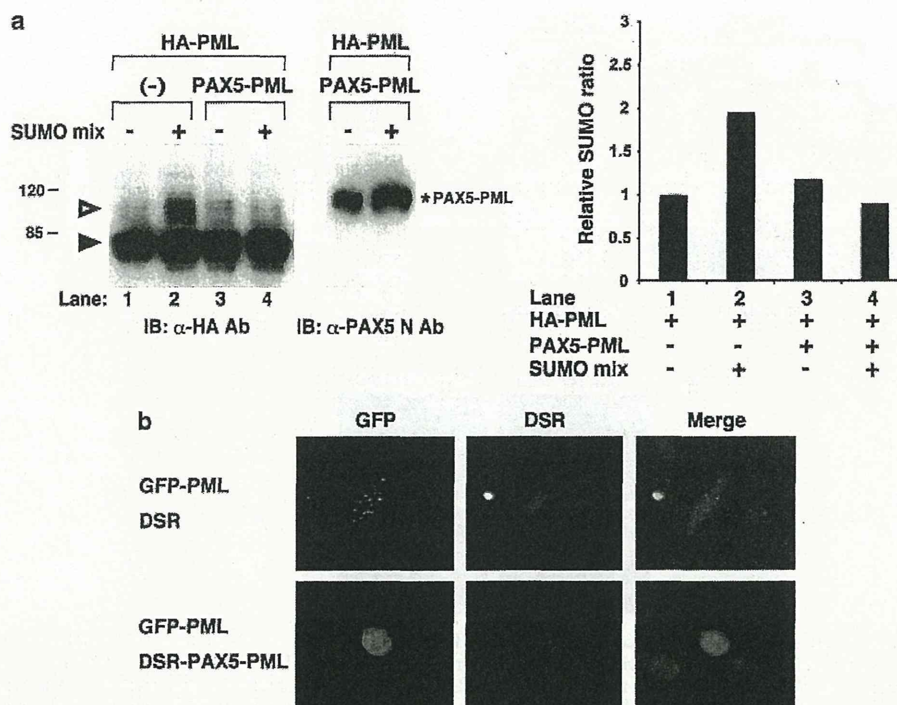

Figure 3 PAX5–PML repressed PML sumoylation and disrupted PML NBs. (**a**) Repression of PML sumoylation by PAX5–PML. HeLa cells were transfected with the expression vectors of small ubiquitin-related modifier (SUMO) and Ubc9 (SUMO mix), HA-tagged PML and PAX5–PML as indicated and subjected to IB with anti-HA antibody (left panel) and anti-PAX5 N antibody (center panel). Sumoylated and unsumoylated PML are indicated by white and black arrows, respectively. Ratio of sumoylated PML to total (sumoylated and unsumoylated PML) was calculated (SUMO ratio). The values relative to SUMO ratio of lane 1 were presented as bar charts (right panel). (**b**) Disruption of PML NBs by PAX5–PML. HeLa cells were transfected with the expression vectors for the indicated proteins and PML localization was observed with fluorescence microscopy in living cells. PML NBs were observed under coexpression of DS red (DSR) alone, while PML showed diffuse nuclear localization under DSR–PAX5–PML coexpression.

to investigate the effect of PAX5–PML on PML NB formation. First, we examined whether PAX5–PML affected PML sumoylation, which is an essential modification of PML to form PML NBs. In HeLa cells, PML sumoylation was enhanced by the coexpression of small ubiquitin-related modifier and its E2 ligase, Ubc9, whereas under PAX5–PML coexpression, PML was hardly sumoylated, even with small ubiquitin-related modifier and Ubc9 coexpression (Figure 3a). It was suggested that PAX5–PML repressed PML sumoylation. Next, PML localization was investigated by fluorescence microscopy of living HeLa cells expressing GFP-fused PML with or without coexpression of DS red-fused PAX5–PML. PML NBs were observed under the coexpression of DS red alone, while PML showed diffuse nuclear localization under DS red–PAX5–PML coexpression, indicating the disruption of PML NBs by PAX5–PML coexpression (Figure 3b and Supplementary Figure S5A). Similar results were obtained in LCL (Supplementary Figure S4).

ATO-induced PML sumoylation and reconstructed PML NBs in PAX5–PML transfected cells

ATO induces PML sumoylation and the reconstruction of PML NBs disrupted by PML–RARα in APL cells, which is thought to lead to apoptosis of APL cells. We investigated whether ATO could induce PML sumoylation and PML NB reconstruction also in PAX5–PML-positive cells. Treatment with ATO enhanced PML sumoylation in HeLa cells and derepression of PML sumoylation was observed in PAX5–PML co-transfected cells (Figure 4a). To reveal the effect of ATO on PML localization, HeLa cells stably expressing PAX5–PML (PAX5–PML/HeLa) were established. PAX5–PML/HeLa did not clearly show PML NBs, although PML localization did not have a distinct diffuse nuclear pattern similar to that of PAX5–PML overexpressing cells in Figure 3b, probably because of its lower expression level. Notably, clear PML NBs were observed after ATO treatment, indicating the reconstruction of PML NBs (Figure 4b and Supplementary Figure S5B).

ATO overcame the apoptosis resistance conferred by PAX5–PML

Finally, we examined whether ATO could induce apoptosis in PAX5–PML/HeLa. In our previous study, we demonstrated that apoptosis induced by trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, was PML dependent, using PML kd/HeLa, whose PML was stably knocked down by short hairpin RNA (Hayakawa *et al.*, 2008). It is also reported that

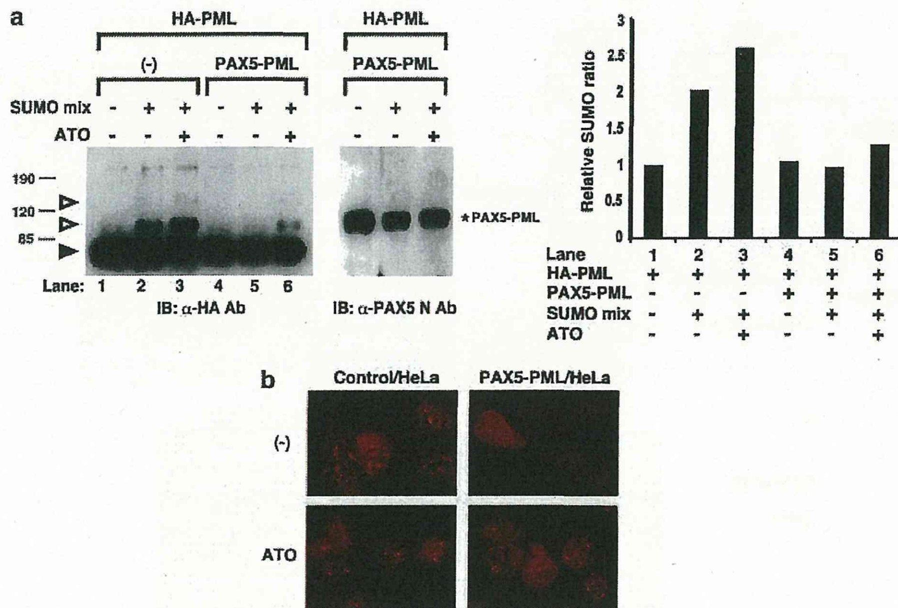

Figure 4 ATO induced PML sumoylation and reconstructed PML NBs in PAX5–PML transfected cells. (**a**) Derepression of PML sumoylation by ATO. HeLa cells were transfected with expression vectors and subjected to IB as in Figure 3a, except that the indicated cells were treated with 10 μM ATO for 6 h before cell lysis. Relative SUMO ratio were calculated and presented as in Figure 3a. (**b**) Reconstruction of PML NBs by ATO. Control HeLa and HeLa cells stably expressing PAX5–PML (PAX5–PML/HeLa) were treated with ATO (10 μM 6 h) and subjected to immunostaining with anti-PML antibody.

irradiation causes PML-dependent apoptosis (Yang *et al.*, 2002). We used TSA and irradiation to induce PML-dependent apoptosis and PML kd/HeLa as a control of apoptosis-resistant cells. TSA treatment and irradiation caused the appearance of a sub-G1 peak in cell cycle analysis, a marker of apoptosis, in all cells; however, the ratio of apoptotic cells was significantly reduced in PML kd/HeLa ($P = 0.020$ in TSA treatment and $P = 0.002$ in irradiation) compared with Control/HeLa. And PAX5–PML/HeLa showed significant reduction of apoptosis in irradiation ($P = 0.001$) and a tendency of apoptosis resistance in TSA treatment ($P = 0.088$) (Figure 5 and Supplementary Figure S6). These results suggested that disruption of PML NBs by PAX5–PML conferred apoptosis resistance on HeLa cells. Furthermore, PML kd/HeLa showed resistance also to ATO-induced apoptosis ($P = 0.021$), while ATO treatment induced apoptosis in PAX5–PML/HeLa similarly to Control/HeLa ($P = 0.645$). These results indicated that ATO could overcome the apoptosis resistance conferred by PAX5–PML.

Discussion

The data presented here demonstrate that PAX5–PML has a dominant-negative effect on both PAX5 and PML. PAX5–PML repressed PAX5 transcriptional activity in the luciferase assay and suppressed the expression of the PAX5 target gene. Given the critical role of PAX5 in B-cell differentiation, it is possible that this dominant-negative effect is involved in the differentiation block occurring in PAX5–PML-positive ALL. On the other hand, PAX5–PML repressed PML sumoylation, disrupted PML NBs, and conferred apoptosis resistance on HeLa cells. Considering that disruption of PML NBs by PML–RARα is thought to confer a survival advantage on APL cells and that PML was reported to have PML NBs-dependent pathway for apoptosis in lymphocytes (Zhong *et al.*, 2000b), this dominant-negative effect on PML will act favorably for the survival of PAX5–PML-positive ALL cells. Thus, this dual dominant-negative effect of PAX5–PML may participate in the leukemogenesis of PAX5–PML-positive ALL. This putative mechanism is illustrated in Figure 6.

It was surprising that PAX5–PML demonstrated impaired DNA-binding ability in EMSA (Figure 1c). All PAX5 fusion proteins previously reported contain the DNA-binding domain and thus were predicted to have equal DNA-binding ability to wild-type PAX5 (Nebral *et al.*, 2009). Indeed, some of those fusion proteins showed similar DNA-binding ability to wild-type PAX5 in EMSA (Kawamata *et al.*, 2008); however, the DNA-binding ability of PAX5 fusion proteins and the way they bind to the PAX5 target promoter may vary and need to be examined in each case. As for fusion proteins with impaired DNA binding, including PAX5–PML, their binding to the PAX5 target promoter will depend on their heterodimerization with PAX5, as shown in Figure 1d. An agent designed to disrupt the interaction between PAX5 and PAX5–PML could be of therapeutic benefit.

It is yet to be revealed how PAX5–PML represses PAX5 transactivity in heterodimer complex on the

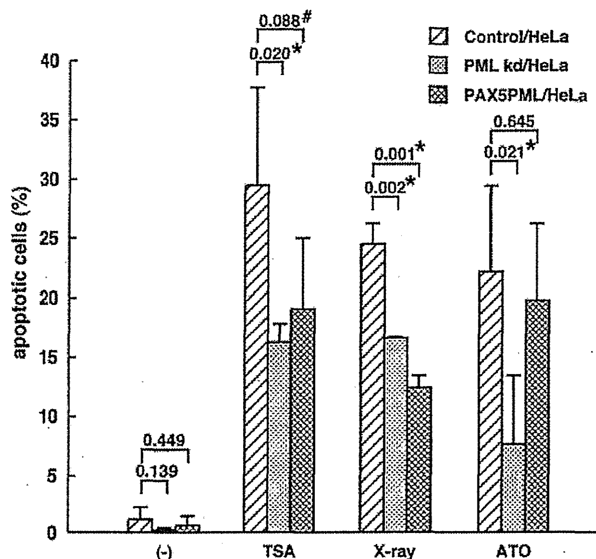

Figure 5 ATO overcame the apoptosis resistance conferred by PAX5–PML. Indicated cells were treated with 1 μM TSA for 48 h, 8 Gry irradiation 72 h before analysis, or 5 μM ATO for 48 h, or left untreated. Ratio of apoptotic cells was measured by analyzing sub G1 peak by cell cycle analysis and is plotted on bar charts. The average of two independent analyses and standard deviations are shown. Numbers indicate *P*-values of statistical comparison. Asterisks and a sharp indicate statistical significant difference and a tendency of statistical significant difference, respectively. Both PML kd/HeLa and PAX5–PML/HeLa showed resistance to TSA- and irradiation-induced apoptosis, suggesting their resistance to PML-induced apoptosis. ATO treatment overcame the resistance and PAX5–PML/HeLa showed apoptosis similarly to control/HeLa.

promoter. PAX5–TEL was shown to associate with mSin3A, a component of the corepressor complex, through the TEL moiety, and the recruitment of the corepressor complex was expected to be the mechanism of transcriptional repression by PAX5–TEL (Fazio *et al.*, 2008). It was reported that PML also associated with the corepressor complex of mSin3A, N-CoR and HDAC1 (Khan *et al.*, 2001; Wu *et al.*, 2001). Thus, recruitment of the corepressor complex might also be the mechanism for PAX5–PML. HDAC inhibitors might be able to release the differentiation block in these cases.

The importance of PML NB reconstruction in the action mechanism of ATO has not been fully established since several other mechanisms are proposed, such as increased reactive oxygen species in cells and c-Jun N-terminal kinase (JNK) activation (Miller *et al.*, 2002). In this report, we demonstrated for the first time that ATO could reconstruct PML NBs disrupted by PML fusion protein other than PML–RARα and overcome the induced apoptosis resistance. These findings further support the importance of PML NB reconstruction in ATO-induced apoptosis and suggest that the point of action of ATO is the PML moiety of PML fusion protein. These speculations correspond to the very recent report that ATO may directly bind to the B2 box domain of PML and PML–RARα, and enhance

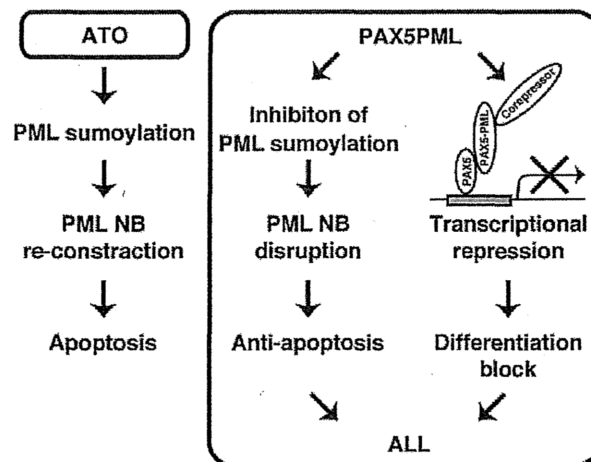

Figure 6 Schematic presentation of putative molecular pathology of PAX5–PML-positive ALL and its molecular targeting therapy.

their association with Ubc9 (Jeanne *et al.*, 2010; Zhang *et al.*, 2010).

Our results suggest the possibility that ATO is an effective drug for PAX5–PML-positive ALL. ATO is approved as a drug for APL in many countries and its safety has been confirmed. It is worth administering ATO to these patients when the disease shows resistance to conventional chemotherapy. Such trials offer important clues regarding the action mechanism of ATO. In summary, our data suggest the involvement of this fusion protein in the leukemogenesis of B-ALL in a dual dominant-negative manner and provide a rationale for ATO therapy for PAX5–PML-positive ALL.

Materials and methods

Antibodies, plasmids and cell culture

The sources of antibodies and plasmids, and the cell culture conditions are detailed in the Supplementary Data on the journal's website.

Transient transfection, immunoprecipitation, immunoblotting and immunofluorescence

These were performed as described previously (Hayakawa and Privalsky, 2004; Hayakawa *et al.*, 2004) except that transient transfections into LCL were performed by nucleofection using the Nucleofector system (Amaxa Biosystems, Koln, Germany) according to the manufacturer's instructions.

Luciferase assay

In all, 293T cells (1×10^5) were transfected with CD19–luc/PGL4 (112.5 ng), the reference *Renilla* luciferase expression vector phRG–TK (12.5 ng; transfection efficiency control), and 125 ng of the indicated expression vectors. The CD19–luc/PGL4 plasmid contains two copies of the high-affinity PAX5-binding site of *CD19* promoter (Czerny *et al.*, 1993) and the firefly luciferase gene. Total amounts of transfected plasmids were adjusted to 375 ng with an empty vector. Firefly luciferase activity normalized for *Renilla* luciferase activity was analyzed using the dual-luciferase reporter assay system (Promega,

Heidelberg, Germany). Luciferase activities in three independent transfection experiments are shown as average values relative to the basal activity observed in control cells (results are the mean ± s.d.).

EMSA
PAX5 and PAX5–PML were synthesized *in vitro* using the TNT transcription/translation system (Promega) and concentrated four times with Centricon (Millipore, Billerica, MA, USA). The amounts of proteins were measured with immunoblotting using anti-PAX5 N antibody. Equal amounts of PAX5 and PAX5–PML were incubated with the probe of 5′-GAATGGGGCACTGAGGCGTGACCACCGC-3′, high-affinity PAX5-binding site sequence in *CD19* promoter, at room temperature in binding buffer: 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol and 0.5 μg of poly (dI–dC), and then subjected to electrophoresis as described previously (Hayakawa *et al.*, 2004).

ChIP assay
ChIP assay was performed as described previously (Atsumi *et al.*, 2006).

Lentivirus infection
This was performed as described previously (Rivella *et al.*, 2003).

Detection and quantification of apoptosis
This assay was also performed as described previously (Hayakawa *et al.*, 2008). Statistical comparisons were performed by using *t*-test.

Conflict of interest

Dr Naoe received research funding from Kyowa Hakko Kirin Co., Ltd, Wyeth, and Chugai Pharmaceutical Co., Ltd. The remaining authors declare no conflict of interest.

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