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Leukemogenesis of b2a2-type p210 BCR/ABL in a Bone Marrow Transplantation Mouse Model Using a Lentiviral Vector

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Abstract

The BCR/ABL fusion oncogene found in Philadelphia-positive leukemia exists in three principle forms: p190, p210 and p230. P210 BCR/ABL is commonly found in patients with chronic myelogenous leukemia (CML) and is further categorized into b3a2 or b2a2 subtypes on the basis of the BCR breakpoint. Although these 2 subtypes may be clinically heterogeneous, only the b3a2 BCR/ABL gene has been extensively studied at the molecular and cellular levels. In the present study, we compared the in vivo leukemogenic activity of the b3a2 and b2 a2 BCR/ABL genes by using lentiviral transduction/transplantation mouse models. Lineage-depleted bone marrow cells of BALB/c mice were transduced with a lentiviral vector including either b2a2 or b3a2 BCR/ABL cDNA and then transplanted into lethally irradiated mice. In this model, p210 BCR/ABL subtype developed only $B220^{+}$, $CD3e^{-}$, $Gr1^{-}$, and $Mac1^{-}$ B-cell acute lymphoblastic leukemia but not myeloid leukemia. There were no differences in the incidence of leukemogenesis, the white blood cell count, the percentage of blast cells, or the survival rates between the b2a2 and b3a2 groups. We have demonstrated that b2a2-type BCR/ABL has leukemogenic activity similar to that of b3a2-type BCR/ABL.
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Key words: BCR/ABL, lentiviral vector, mouse leukemia model, integration site, leukemogenesis

Introduction

The BCR/ABL gene was originally detected in chronic myelogenous leukemia (CML) at the translocation junction of the Philadelphia chromosome[1], which is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22, t (9 ; 22) (q34 ; q11)[2]. Because the BCR/ABL gene is present in almost all patients with CML, it is considered a hallmark of CML. The fusion gene can also be found in some patients with acute lymphoblastic leukemia (ALL) but is rarely found in other hematological malignancies[3]. The 3 principal forms of the BCR/ABL gene (p190, p210 and p230) are found in distinct types of leukemia. The p210 BCR/ABL gene is divided into the b3a2 and b2a2 subtypes on the basis of the BCR

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breakpoint location, either between exon b3 and b4 or between exon b2 and b3, respectively[4,5]. In most cases, CML cells have either b3a2 or b2a2 transcripts, but in 5% of cases alternative splicing events allow the simultaneous expression of both fusion products[6].

Whether the subtypes of the p210 BCR/ABL gene influence the clinical and hematological features of CML has been disputed. Clinical studies have suggested that the duration of the chronic phase is 4-fold longer in patients with b2a2-subtype CML than in patients with b3a2-subtype CML[7]. However, this finding was not confirmed in subsequent studies with large numbers of patients[8,9]. We have previously reported that patients with b3a2 transcripts had significantly greater platelet counts than did patients with b2a2 transcripts[10]. Shepherd et al. have found that in a subgroup of patients with white blood cell (WBC) counts less than 100×10^9/L, platelet count were significantly higher in patients with the b3a2 transcripts. However, there was no correlation between BCR/ABL subtypes and clinical features, cytogenetic response, duration of chronic phase, or survival among all 119 patients[9].

The leukemogenesis of BCR/ABL genes has been studied with transgenic mouse models[11] and retroviral transduction / transplantation mouse models[12]. Li et al. have demonstrated that oncoretrovirus-mediated ex vivo transfer of the BCR/ABL gene into mouse bone marrow (BM) cells induces a CML-like myeloproliferative syndrome in recipient mice. However, because this disease is aggressive, this model system is not appropriate for studying the chronic phase of CML. In addition, the in vivo leukemogenic activity was studied only for the b3a2-subtype but not for the b2a2-subtype. In the present study, we directly compared the in vivo leukemogenic activity of the b3a2 and b2a2 BCR/ABL genes by using lentiviral transduction/transplantation model mice, because the lentiviral vector has many advantages over the oncoretroviral vector in the transduction of slowly dividing hematopoietic stem cells (HSCs)[13]. We found that the lentiviral-mediated transfer of the p210 BCR/ABL gene into lineage-negative BM cells causes acute B-cell lymphoblastic leukemia but not CML in recipient mice. Further improvements in the experimental system are required to examine the leukemogenesis of the BCR/ABL oncogene.

Materials and Methods

B2a2-type BCR/ABL cDNA Construct

The pGD210 retroviral construct containing b3a2-type BCR/ABL cDNA was kindly provided by Dr. D. Baltimore (Massachusetts Institute of Technology, Boston, MA). The BCR/ABL cDNA was digested with SalI and KpnI, and the SalI-KpnI fragment was then inserted into the pBluescript II plasmid (Stratagene, Santa Clara, CA) (**Fig. 1a**). Exon b3 of the BCR gene was removed with the following procedures. Two types of polymerase chain reaction (PCR) were performed with 4 primers. The BCR-HindIII primer (5′-TGC AGA GTG GAG GGA GAA CAT CC-3′) and the HindIII-BsaI primer (5′-CCG GAG AC**G GTC TCT CTT** CCT TAT TGA TGG TCA G-3′) generated a PCR product between the HindIII site and the end of exon b2 in the BCR cDNA (**Fig. 1b**). The BsaI-KpnI primer (5′-GGA TTT AA**G GTC TCT GAA G**AA GCC CTT CAG CGG CCA GTA-3′) and the ABL-KpnI primer (5′-ATT GAT CCC GCT GCT CAG CAG AT-3′) generated a PCR product between the start of ABL and KpnI in the ABL cDNA (**Fig. 1c**). The first PCR fragment was digested with HindIII and BsaI. The second PCR fragment was digested with BsaI and KpnI. After being cut, both PCR fragments were ligated at the BsaI breakpoint(HindIII-KpnI PCR fragment) (**Fig. 1d**). As a result of this ligation, exon b3 and the BsaI site were completely removed to form the HindIII-KpnI PCR fragment. The HindIII-KpnI PCR fragment was inserted into pBluescript II, which was cut with HindIII and KpnI (**Fig. 1e**). The SalI-KpnI fragment of pBluescript II, which lost the b3 exon, was then changed into the SalI-KpnI site of the b3a2-type BCR/ABL cDNA (**Fig. 1f**). Finally, the b2a2-type BCR/ABL cDNA was generated from the b3a2-type BCR/ABL of pGD210. All sequences were confirmed in both orientations.

Plasmid Construction

The b3a2-type BCR/ABL expression lentiviral

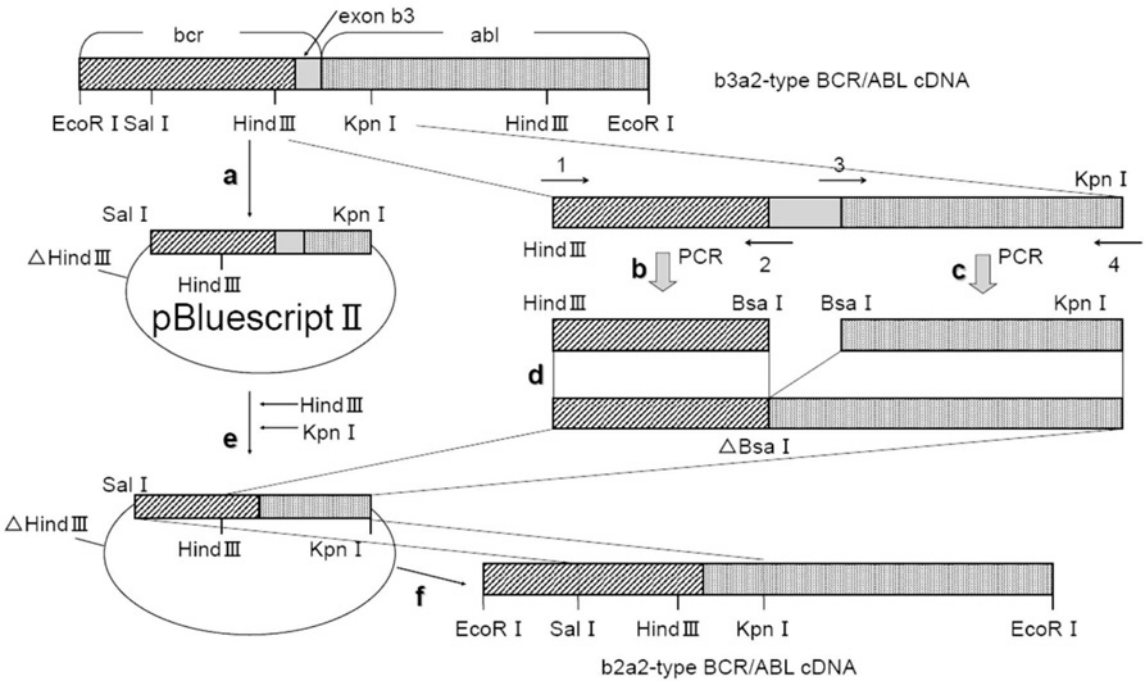

Fig. 1 Strategy of b2a2 BCR/ABL cDNA construction. The junction site of b3a2 BCR/ABL cDNA was inserted into the plasmid. The sequences upstream (1－2) and downstream (3－4) of exon b3 were amplified with PCR using primers (2 and 3) containing a BsaI site in the tail. The PCR fragments were digested and ligated to remove exon b3. The ligated PCR product was inserted into the original junction digested by HindIII and KpnI to produce the b2a2 BCR/ABL cDNA. The b2a2 DNA fragment was exchanged for that of b3a2.

vector plasmid pCL20c Mp-b3a2 was produced by inserting b3a2 p210 BCR/ABL cDNA into an SJ1 HIV1 vector[14]. In this construct, the transgene was expressed under the control of the murine stem cell virus (MSCV) long terminal repeat (LTR)-U3 promoter. The b2a2-type BCR/ABL expression vector pCL20c Mp-b2a2 was produced by inserting b2a2-type cDNA, as described above. The BCR/ABL and GFP co-expression lentiviral vector, pCL20c Mp-b3a2-sEF1-GFP or pCL20c Mp-b2a2-sEF1-GFP, was produced by inserting the EF1α promoter lacking an intron and carrying GFP cDNA between BCR/ABL cDNA and HIV1 3′LTR. A GFP-only expression vector, pCL20c Mp-GFP, was used as a control vector[15].

Lentiviral Vector Preparation

Self-inactivating (SIN) lentiviral vectors pseudotyped with retroviral ecotropic envelopes were prepared and titrated as described previously[10,14–17]. Briefly, vectors were produced by 4-way co-transfection of 293T cells (gag-pol, rev-tat, retroviral ecotropic envelope, and transfer vector plasmid), and the amount of the infectious vector particle (titer) was determined on 3T3 cells. The titer was expressed as transducing units per mL (TU/mL).

Transduction and Transplantation

All procedures were performed in accordance with the animal experimentation guidelines of Nippon Medical School. The BM cells were obtained from BALB/c mice (Saitama Experimental Animals Supply, Saitama, Japan) without fluorouracil pretreatment, and then the differentiated cells were depleted using the Mouse Hematopoietic Progenitor Cell Enrichment Set-DM (Pharmingen, San Diego, CA). The lineage-depleted (Lin^-) BM cells were prestimulated at a concentration of 1×10^6 cells/mL in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum with 20 ng/mL murine interleukin-3 (mIL-3) (Kirin Brewery, Tokyo, Japan), 50 ng/mL murine stem cell factor (mSCF) (Kirin Brewery), 50 ng/mL human thrombopoietin (hTPO) (Kirin Brewery), and 50 ng/mL human interleukin-6 (hIL-6) (Wako Pure Chemicals Industries, Osaka,

Japan). After 48 hours of prestimulation, these cells were transduced for 12 hours with the lentiviral vectors at a multiplicity of infection (MOI) of 3 (e.g., concentration of the vector in the transduction medium is 3×10^6 TU/mL where cell density is 1×10^6 cells/mL) in the first set of experiments and at an MOI of 20 in the second set of experiments. The transduction was performed in the presence of cytokines (mIL3, mSCF, hTPO, hIL6) and 8 μg/mL polybrene on a RetroNectin (TaKaRa, Otsu, Japan)-coated 6-well plate. One day after transduction, the cells were collected, washed with phosphate-buffered saline (PBS), and then resuspended in PBS containing 2% fetal bovine serum. The transduced marrow cells (2×10^5 cells per mouse) were injected into the tail vein of lethally irradiated (10 Gy) BALB/c mice. In the first set of experiments, we used the b3a2 or b2a2 expression vector without the GFP reporter (b3a2, n=10; b2a2, n=10; GFP control, n=9). In the second set of experiments, we used the b3a2 expression vector with a GFP reporter (b3a2, n=10; b2a2, n=10; GFP control, n=10) to easily identify gene-transduced cells. Blood samples from the transplanted mice were collected from the retro-orbital plexus 4, 6, 8, 12, 16, 20, and 24 weeks after transplantation, and the complete blood count with differential was routinely examined. In the first set of experiments, we examined the vector copy number in WBCs using real-time PCR. In the second set of experiments, we used flow cytometry to examine the expression of lineage-differentiation markers (Gr1, Mac1, CD3e, and B220) and GFP. The blood differentiation counts were determined independently by 2 hematologists. Leukemia was diagnosed by the presence of leukemic blasts in the peripheral blood.

Flow Cytometry Analysis

Whole blood cells were analyzed to evaluate GFP expression in WBC, red blood cell, and platelet fractions. WBCs were obtained from peripheral blood by hemolysis and then incubated with combinations of monoclonal antibodies (B220-phycoerythrin [PE], Mac1-Allophycocyanin [APC], CD3e-PE, and Gr1-APC) (BD Biosciences, Franklin Lakes, NJ) for 15 minutes on ice. The GFP/PE/APC fluorescence was analyzed with FACSCalibur (BD Biosciences).

Vector Copy Number Calculation with Real-time PCR

Genomic DNA extracted from 3T3 cells and peripheral blood leukocytes of mice receiving transplants was subjected to real-time PCR to estimate the transduction rate or the vector titer. The primer/probe sets FPLV2 (modified in one base to 5′-AC**T** TGA AAG CGA AAG GGA AAC-3′ due to a difference in the HIV1 strain), RPLV2, and LV2[18] were used to detect the lentiviral vector provirus. TaqMan Ribosomal RNA control reagents (Applied Biosystems, Foster City, CA) were used to determine the amount of genomic DNA. To estimate the transduction rate, genomic DNA extracted from known percentages of GFP^+ cells or genomic DNA spiked with plasmid DNA were used as a standard, and values were obtained as percentages or average copy number per diploid[16].

Southern Blot Analysis

Genomic DNA was extracted from spleen cells and BM cells of leukemic mice. After digestion by the restriction enzyme XbaI, genomic DNA (5 μg) was separated with 0.8% agarose gel electrophoresis, transferred onto a Hybond-N + membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), and hybridized with a radiolabeled RRE fragment DNA probe (761bp MfeI-BstBI fragment from pCL20c Mp-GFP). The hybridized bands were visualized using BAStation ver. 2.2 (Fuji Photo Film, Tokyo, Japan).

Northern Blot Analysis

Total RNA was extracted after the cells had been transduced and cultured for 5 days. The RNA was subjected to electrophoresis, transferred, and hybridized with an ABL probe (1,061 bp DraIII fragment from pCL20c Mp-b3a2) labeled with an Alkphos Direct Labeling Kit (Amersham Biosciences). The band intensity was normalized against the intensity of 28S rRNA and the average integrated vector copy number determined with real-time PCR.

Western Blot Analysis

After 4 to 5 days of transduction with lentiviral vectors at MOIs of 1.5 to 2.0, the transduced 3T3 cells were harvested. The cells were resuspended in 200 μL of PBS containing a protease inhibitor cocktail (Roche, Penzberg, Germany) to which 200 μL of 2×sample buffer (containing 10% 2-mercaptethanol)preheated to 98℃ was added[19]. After electrophoresis of cell lysates, proteins were transferred and then incubated with 1 μg/mL anti-abl antibody clone 8E9 (Pharmingen) or 0.5–1 μg/mL anti-phosphotyrosine antibody clone 4G10 (Upstate Biotechnologies, Lake Placid, NY), followed by incubation with peroxidase-conjugated anti-mouse IgG antibody (Amersham Biosciences).

Linear Amplification-mediated PCR

Linear amplification-mediated (LAM)-PCR was performed as described previously, with modifications[17,20,21]. To identify the 3′-end junction, linear amplification was performed with the primer 5′-biotin-TTT TGC CTG TAC TGG GTC TCT CTG-3′ (HIV3-I). Biotinylated linear amplification products were immobilized on Dynabeads (Dynabeads kilobaseBINDER Kit, Dynal Biotech ASA, Oslo, Norway), and the second strand was synthesized with random hexamers. Synthesized DNA was digested with the restriction enzyme ApoI or Tsp 509I (New England Bio Labs, Beverly, MA) and then ligated with an asymmetric DNA linker (LC-Tsp-a: 5′-ACT GAC AGC GGA GAT AAT CGG TGC GAG TAG CAT ACT AGA G-3′, LC-Tsp-b: 5′-AAT TCT CTA GTA TGC TAC TCG CAC CGA TTA TCT CCG CTG TCA GT-3′). The ligated Dynabead/DNA complex was denatured with 0.1 N NaOH, and the supernatant was transferred to a clean tube and neutralized with 0.1 N HCl. This material served as a template for nested PCR. The primers for outside PCR were 5′-TCT CTG GCT AAC TAG GGA AC-3′ (HIV3-II) and 5′-ACT GAC AGC GGA GAT AAT CG-3′ (LC-1). The primers for inside PCR were 5′-GCC TTG AGT GCT TCA AGT AGT G-3′ (HIV3-III) and 5′-GTG CGA GTA GCA TAC TAG AG-3′ (LC-2). The PCR products were separated with electrophoresis on Spreadex EL 800 (Elchrom Scientific, Cham, Switzerland). Bands were extracted from the gel using BandPic (Elchrom Scientific). Additional PCR was performed directly against the extracted gel fragments using the HIV3-III and LC-2 primers, and products were then sequenced directly by using the HIV3-III or LC-2 primer. Integration site sequences were validated with the intact LTR-genomic junction, and the sequence was compared with the National Center for Biotechnology Information (NCBI) mouse genomic database (Build 35.1) using the Blast algorithm. The 5′-end junction sequence was determined using a similar method with the following primers: 5′-biotin-AGG GTC TGA GGG ATC TCT AGT TAC-3′ (HIV5-I), 5′-CAG TGG GTT CCC TAG TTA GC-3′ (HIV5-II) and 5′-GCA AAA AGC AGA TCT TGT CTT C-3′ (HIV5-III).

Statistical Analyses

A 2-sample *t*-test for equal variances was used to assess differences in the mean values of variables between the 2 vectors. Kaplan-Meier curves were produced and analyzed with SPSS for Windows ver. 14.0J (SPSS Japan, Tokyo, Japan).

Results

Evaluation of the p210 BCR/ABL Lentiviral Vectors

The lentiviral vectors used in these experiments are shown in **Figure 2a**. In these constructs, the b3a2- and b2a2-type p210 BCR/ABL oncogenes were expressed under the control of the oncoretrovirus MSCV LTR-U3 promoter. In a subset of the experiments, EF1α promoter-driven GFP was used as a co-expressing marker gene. The vectors were pseudotyped with a retrovirus ecotropic envelope for safety, as ecotropic pseudotyped vectors can infect mouse cells but not human cells. We used BCR/ABL-expressing vectors and the control vector of similar titers in each subset (b3a2: 1.2×10^7 TU/mL; b2a2: 6.7×10^6 TU/mL; GFP: 6.3×10^6 TU/mL in the first subset; b3a2-GFP: 1.7×10^8 TU/mL; b2a2-GFP: 7.8×10^7 TU/mL; GFP: 8.4×10^7 TU/mL in the second subset). Gene expression of both types of p210 BCR/ABL cDNAs was confirmed by both Northern blot and Western blot analysis (**Fig. 2b, c** upper panel),

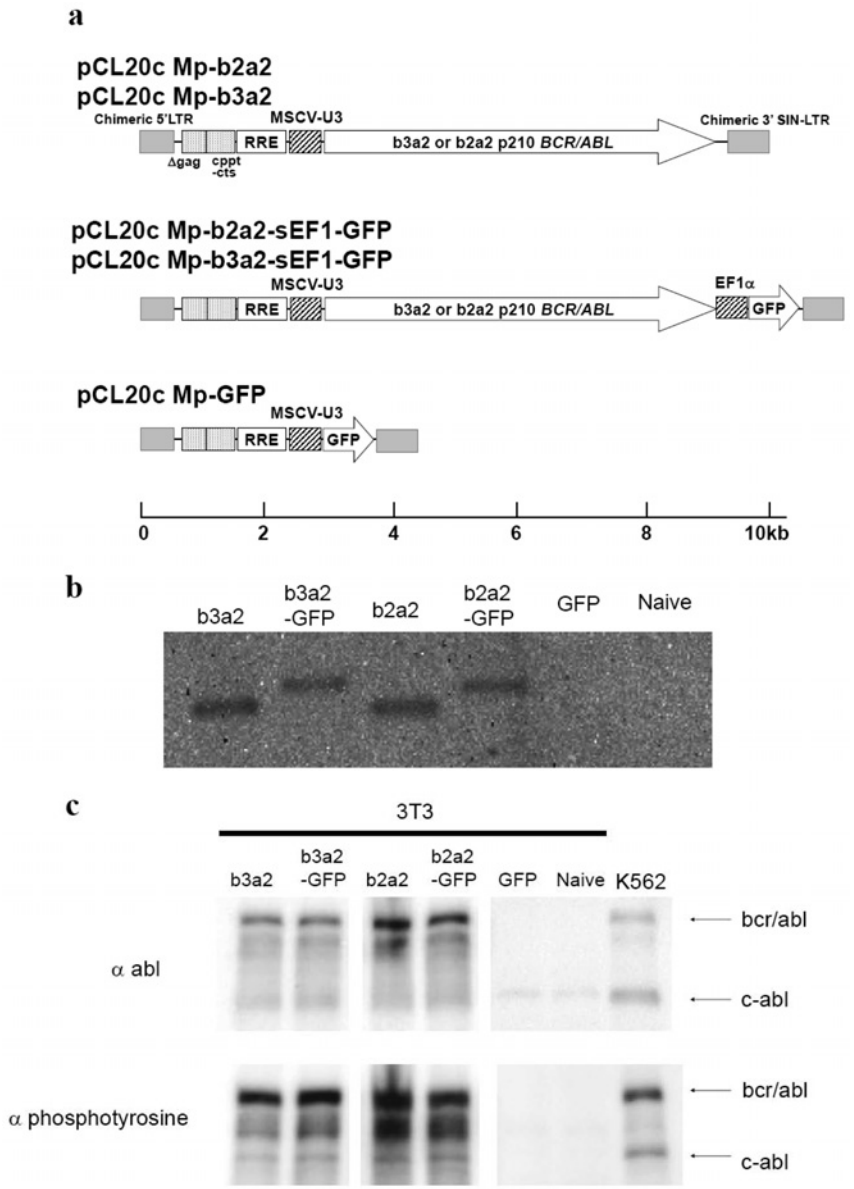

Fig. 2 Construction of BCR/ABL expressing lentiviral vectors. (a) The schematic construction of the vectors is shown to scale. The b2a2- or b3a2-type p210 BCR/ABL or GFP is expressed under the control of the MSCV LTR-U3 promoter. In the double gene-expression vector, GFP is expressed under the control of the EF1 α promoter, and the EF1 α-GFP cassette was inserted between BCR/ABL cDNA and HIV1 LTR. (b) Expression levels in the 3T3 cells were evaluated with Northern blot analysis. The expression levels of each vector were similar. Lane b3a2, 3T3 cells transduced with pCL20c Mp-b3a2; Lane b3a2-GFP, 3T3 cells transduced with pCL20c Mp-b3a2-sEF1-GFP; Lane b2a2, 3T3 cells transduced with vCL20c Mp-b3a2; Lane b2a2-GFP, 3T3 cells transduced with vCL20c Mp-b2a2-sEF1-GFP; Lane GFP, 3T3 cells transduced with vCL20c Mp-GFP; Lane Naive, nontransduced 3T3 cells. (c) Kinase activities of the p210 BCR/ABL cDNA products of the 3T3 cells. The 3T3 cells were transduced with each vector, and cell lysates were subjected to Western blot analysis. Protein expression and tyrosine kinase activity were confirmed using anti-abl or anti-phosphotyrosine antibodies. Lane b3a2, 3T3 cells transduced with vCL20c Mp-b3a2; Lane b3a2-GFP, 3T3 cells transduced with vCL20c Mp-b3a2-sEF1-GFP; Lane b2a2, 3T3 cells transduced with vCL20c Mp-b3a2; Lane b2a2-GFP, 3T3 cells transduced with vCL20c Mp-b2a2-sEF1-GFP; Lane GFP, 3T3 cells transduced with vCL20c Mp-GFP; Lane Naïve, nontransduced 3T3 cells; Lane K562, K562 cells as positive controls for p210 BCR/ABL fusion protein. RRE, rev responsive element; cPPT-CTS, central polypurine tract- central termination sequence; SIN-LTR, self-inactivating long terminal repeat.

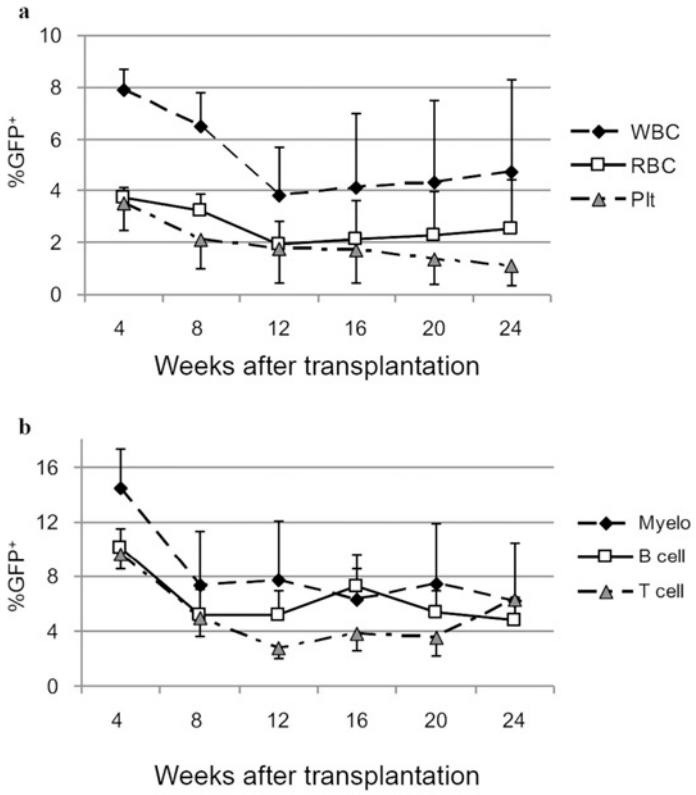

Fig. 3 Follow-up of GFP expression in peripheral blood of control mice. GFP expression in peripheral blood was followed for 24 weeks in control mice, which received transplants of BM cells transduced with GFP vector. GFP expression rates decreased slowly after transplantation and arrived at plateau levels 12 weeks later. All lineage cells (a) and myeloid cells, B cells and T cells (b) showed detectable GFP expression 24 weeks after transplantation. RBC, red blood cell; Plt, platelet; Myelo, myeloid cell.

and self-phosphorylation of the p210 BCR/ABL proteins was also confirmed **(Fig. 2c** lower panel).

Development of B-cell ALL after Transplantation of the Two Types of p210 BCR/ABL Genes into Lin⁻ Mouse BM Cells

Lin⁻ BM cells were transduced with b3a2- or b2a2-type p210 BCR/ABL vectors or with a GFP-only vector (vCL20c Mp-GFP) as a control. The transduction rates of b3a2, b2a2, and GFP vectors in Lin⁻ BM cells were evaluated 6 days after transplantation, and the resulting transduction efficiencies were 0.4% to 0.7%, 0.2% to 0.4%, and 3.5% to 7.1%, respectively.

The transduced cells were transplanted into lethally irradiated mice. In the control group, GFP expression could be detected 24 weeks after transplantation in all lineage cells **(Fig. 3a, b)**. None of the GFP-transduced mice (n=19) developed leukemia during a 6-month observation period. In the 2 types of BCR/ABL-transduced mice, 6 of 20 mice in the b3a2 group and 9 of 20 mice in the b2a2 group developed acute leukemia. At the time of diagnosis, the WBC count of leukemic mice was 34,000 ± 7,000/mm^3 (n=6) in the b3a2 group and 61,000 ± 18,000/mm^3 (n=9) in the b2a2 group **(Table 1)**. There were no statistically significant differences in the WBC, hemoglobin, or platelet counts of the 2 groups ($p>0.05$). The percentage of blast cells in Wright-Giemsa-stained blood smears was 45% ± 7% (n=4) in the b3a2 group and 50% ± 11% (n=9) in the b2a2 group **(Fig. 4a)**. There was no difference in percentages of blast cells between the 2 groups ($p>0.05$). The average copy number of peripheral

Table 1 Complete blood counts of leukemic mice

	b3a2 (n=6)	b2a2 (n=9)	GFP (n=18)	P value**
WBC (10^3/mm^3)	34.0	61.1	11.3	0.25
Hb (g/dL)	13.5	13.3	14.6	0.80
Plt (10^3/mm^3)	336.8	542.0	580.4	0.13
Marking level	0.57 ± 0.18*	0.39 ± 0.06*	N.D.	0.29

*average copy number: b3a2, n=3: b2a2, n=5. **b3a2 vs. b2a2. N.D.: no data

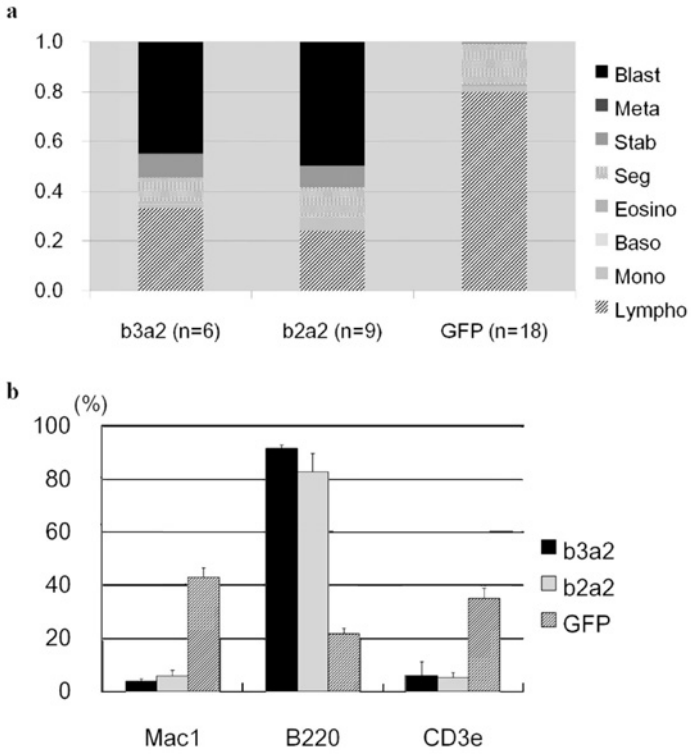

Fig. 4 Analysis of peripheral blood differentiation in leukemic mice. We evaluated differentiation of peripheral blood in leukemic mice. (a) Blast cells appeared in BCR/ABL-positive leukemic mice, whereas the peripheral blood of control mice did not contain blast cells. (b) We analyzed the surface markers Mac1 (myelocytes), B220 (B cells), and CD3e (T cells) in GFP$^+$ peripheral blood cells. In leukemic mice, almost all GFP$^+$ cells were B220$^+$, Mac1$^-$, and CD3e$^-$. Blast, blast cells; Meta, metamyelocyte; Myelo, myelocyte; Stab, stab cell; Seg, segmented cell; Eosino, eosinophil; Baso, basophil; Mono, monocyte; Lympho, lymphocyte.

leukocytes in the first set of experiments showed no significant difference between the b3a2 group (0.57± 0.18 [n=3])and the b2a2 group (0.39 ± 0.06 [n=5]) ($p< 0.05$) (**Table 1**).

In the first set of experiments, the gene marking level in peripheral leukocytes after transplantation was measured with real-time PCR for the b3a2 and b2a2 groups and with GFP expression for the GFP group. In the second set of experiments, the gene marking level was measured with GFP expression for the b3a2, b2a2, and GFP groups. In the b3a2 and b2a2 groups, the vector copy number in the peripheral blood was significantly higher in mice with leukemia than in mice without leukemia 4 weeks after transplantation (0.46 ± 0.07 [n=9] vs. 0.02 ± 0.01 [n=9], $p<0.001$). In the second set of experiments, b3a2-GFP- or b2a2-GFP-transduced mice had low GFP levels 4 weeks after

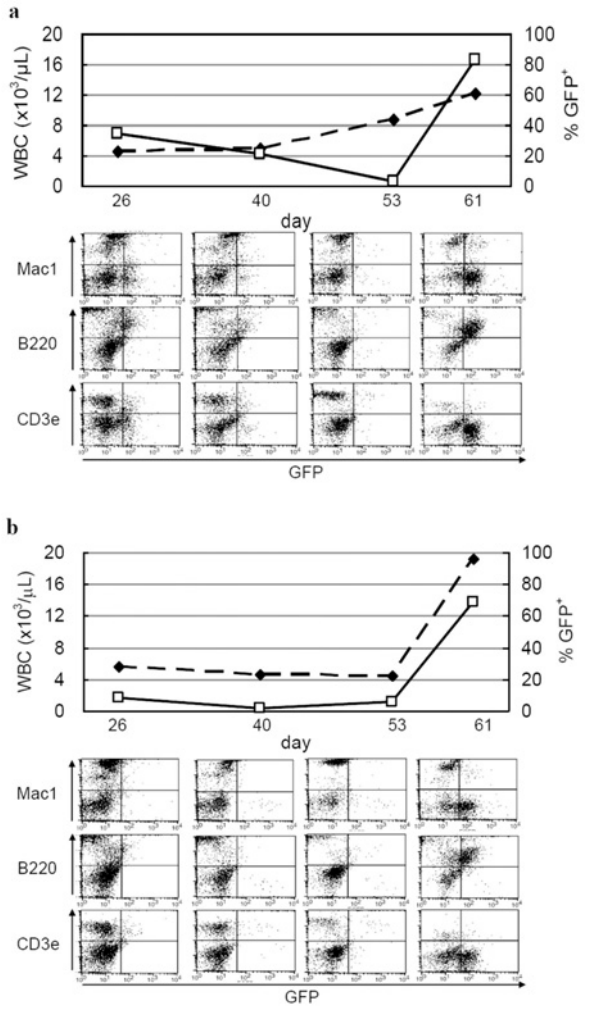

Fig. 5 Time course of WBC count and GFP$^+$ levels in WBC, and FACS analysis of b2a2-transplanted mice (a) and b3a2-transplanted mice (b). Total WBC count (broken line with closed diamonds) and percentage of GFP$^+$ WBCs (solid line with open squares) are plotted (upper panels of A and B). Lineage analyses of peripheral leukocytes performed at the indicated times are shown below. Although the initial marking levels were low, and the complete blood counts, including WBC counts, were normal, GFP$^+$, B220$^+$, CD3e$^-$, Mac1$^-$ B-cell populations began to expand around day 61 in the peripheral blood of b2a2-transplanted mice (ID 2-5) and b3a2-transplanted mice (ID 1-1).

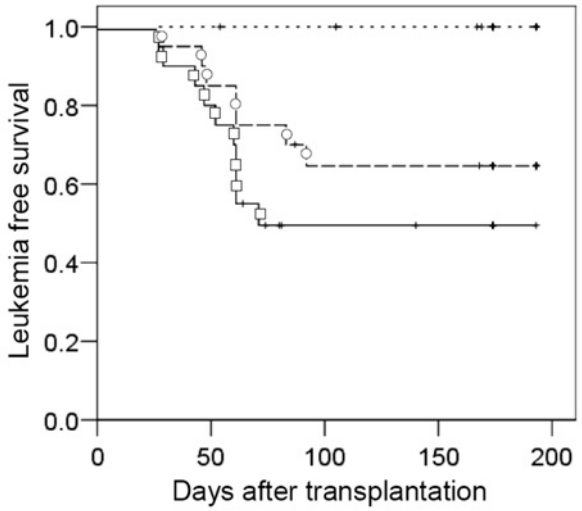

Fig. 6 Development of leukemia after transplantation of Lin$^-$ BM cells transduced with two subtypes of p210 BCR/ABL. Kaplan-Meier analysis of recipient mice. Lethal leukemia developed within 3 months after transplantation. Mice that did not develop leukemia during the initial 3 months remained disease-free during the 6-month observation period. Broken line with open circles, b3a2 (n = 20); solid line with open squares, b2a2 (n = 20); dotted line, GFP (n = 19).

transplantation, except for some mice in which a higher GFP marking level led to leukemia (0.72 ± 0.32 [n=8] vs. 0.10 ± 0.02 [n=9], $p<0.05$). Although the MOI was identical between the groups, the short-term and long-term GFP marker levels were higher in the GFP control group.

The phenotype of all leukemia cells examined with flow cytometry was B220$^+$ CD3e$^-$ Gr1$^-$ Mac1$^-$ GFP$^+$, and the type of leukemia was identified **(Fig. 4b)** as B-cell ALL (B-ALL). The B220$^+$ abnormal cell population accounted for 91.6% ± 1.3% (n=3) and 82.8% ± 6.9%(n=4) of GFP$^+$ peripheral blood leukocytes in leukemic mice from the b3a2 group and the b2a2 group, respectively ($p>0.05$), and the leukemia appeared 26 to 82 days after transplantation **(Fig. 6)**. In leukemic mice, GFP$^+$ leukocytes were suddenly increased as a homogeneous cell population in flow cytometry, suggesting monoclonal growth of transduced cells **(Fig. 5)**. There was no statistically significant difference in leukemia-free survival (82 ± 5 days vs. 56 ± 3 days, $p>0.05$) between the b3a2- and b2a2-subtypes **(Fig. 6)**. The pathological analysis of B-ALL mice from both groups revealed similar disease phenotypes **(Fig. 7)**. Large blasts with a large and pleomorphic immature nucleus were apparent in peripheral blood smears. The nucleoli were also visible. Both the b2a2 and b3a2 types of mice with leukemia had hypercellular BM due to B-ALL cell invasion **(Fig. 7)**. The B-ALL cells of both types also

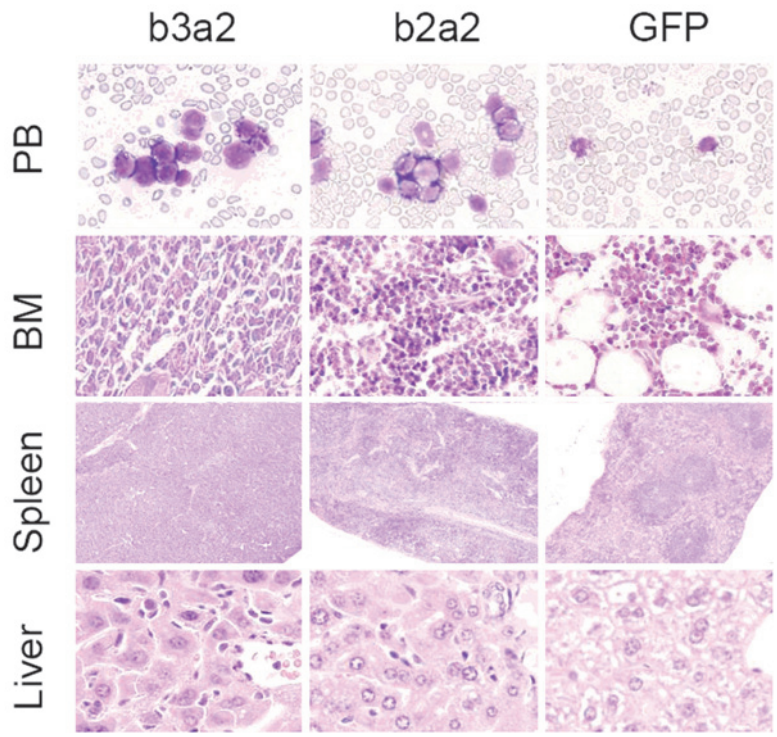

Fig. 7 Histopathologic analysis of BCR/ABL cDNA-transduced mice that developed leukemia. Wright-Giemsa staining of peripheral blood films and hematoxylin-eosin staining of organ sections were performed. Immature blast cells appeared in the peripheral blood of p210 BCR/ABL-transplanted mice. The BM and spleens were massively infiltrated by leukemic cells. The normal structure of the spleen was disrupted. Mononuclear cell infiltration of the liver was evident. b3a2, leukemic mice that expressed b3a2-type BCR/ABL; b2a2, leukemic mice that expressed b2a2-type BCR/ABL; GFP, GFP-transduced mice.

had massively infiltrated the spleen and destroyed its normal follicle structure (**Fig. 7**). The average mass of the spleens in the b3a2 leukemia group was 196 ± 23 mg (n=3) and that in the b2a2 leukemia group was 221 ± 63 mg (n=4) ($p>0.05$). B-ALL cells had also infiltrated the spaces between hepatocytes.

Identification of Vector Insertion Sites by Using Southern Blot and LAM-PCR

To examine the contribution of insertional mutagenesis on the development of B-ALL, we performed integration site analysis of DNA obtained from leukemic mice. The number of vector insertion sites was evaluated with Southern blot analysis. One or two dominant bands were detected in DNA extracted from the spleen or BM cells of leukemic mice (**Fig. 8a, b**), suggesting monoclonal or oligoclonal expansion of leukemia cells in the recipient mice.

Vector integration sites were identified with LAM-PCR, and 14 integration sites were mapped unequivocally to the mouse genome for the 15 leukemic mice that were examined (**Table 2**). Most integration sites were mapped within the transcription units of RefSeq genes (64%). All leukemic mice except 1 (experiment 2, b2a2 #2-2) showed forward integration (direction of the vector genome and the cellular gene are identical), and all integration sites were mapped within introns. No integration into cancer-associated common integration sites of gamma-retrovirus or integration into known oncogenes was observed, suggesting that insertional mutagenesis played no role in this model.

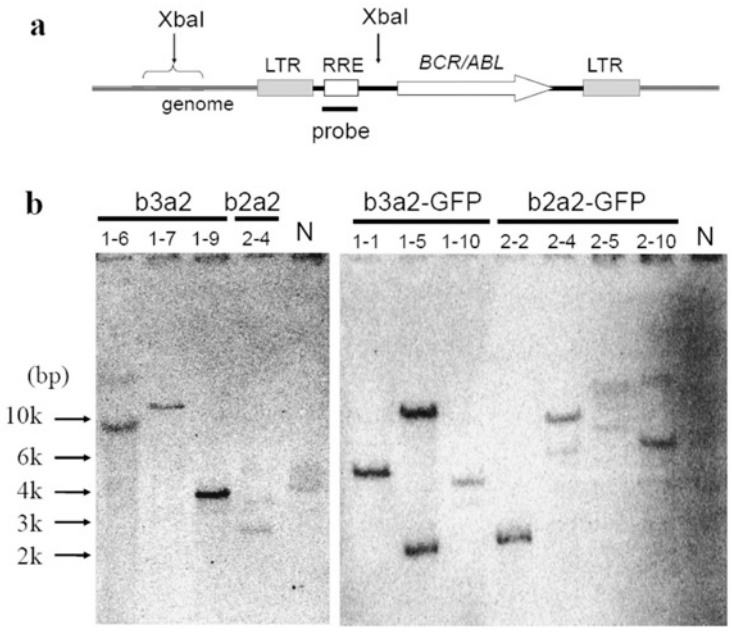

Fig. 8 Integration site analysis of the expression vectors in leukemic cells. (a) Genomic DNA obtained from spleen cells and BM cells of B-ALL mice was digested with XbaI to visualize integrated clones with Southern blot analysis. The position of the probe is indicated. (b) Integration site analysis with Southern blotting. Each band represents a unique integration site. Numbers above the blot are unique mouse IDs. N, negative control.

Discussion

For the first time, we have demonstrated leukemogenesis induced by b2a2 p210 BCR/ABL cDNA. All previous papers have shown that only b3 a2 p210 BCR/ABL cDNA induced acute leukemia or myeloproliferative disease by means of retroviral vectors or transgenic mice[5,19,20]. In most patients with CML, leukemic cells have either b3a2 or b2a2 transcripts, but in 5% of cases both fusion products can be expressed in alternative splicing events[8]. In this mouse model, b2a2 and b3a2 BCR/ABL cDNA allowed us to compare these functions without the effect of RNA splicing. The similarity of leukemogenesis caused by the b2a2 and b3a2 BCR/ABL variants was suggested by the rates of leukemia development, WBC counts, and leukemia-free survival times in this mouse model. Previous clinical papers have reported that the platelet counts in patients with CML and b3a2 mRNA or both types were significantly higher than in patients with b2a2 mRNA alone[9–11]. However, in the present study platelet counts did not differ significantly between the 2 types of B-ALL mice. In the second transplantation experiment, we evaluated the GFP expression in platelets of the leukemic mice but detected only a low rate and a low level of GFP expression with flow cytometry (% GFP of platelet: 0.20 ± 0.09%). We found the transduced gene expression in platelets was insufficient to evaluate differences in the platelet count.

In our transduction/transplantation mouse model, B-ALL developed 4 to 12 weeks after transplantation. Four weeks after transplantation, mice with relatively higher marking levels indicated significantly higher rates of leukemia development. On the other hand, only 1 or 2 vectors were integrated into leukemic cells, as detected with Southern blot analysis of extracted DNA. This finding suggests that a greater number of transduced B-cell progenitors might result in the higher rates of B-ALL development.

We have, for the first time, used the lentiviral vectors rather than a retroviral vector to induce BCR/ABL-positive leukemia in a mouse model.

Table 2 Integration site characteristics obtained from leukemic mice.

Type	Experiment	ID	Gene	Function	Distance from transcription start site	Relative direction
b3a2	1	1-6	*Sepp1*	Selenium binding	+13 kb	Forward
		1-7	Unknown			
		1-9	*Zcchc7*	Unknown	Inside (48 kb)	Forward
	2	1-1	*hypothetical LOC545194*	Unknown	+19 kb	Forward
			A930001N09Rik	Unknown	Inside (32 kb)	Forward
		1-5	*Txnl2*	Electron transporter	+109 kb	Forward
		1-10	*Matr3*	Metal ion binding Nucleic acid binding	Inside (1.7 kb)	Forward
b2a2	1	2-1	*Zfp207*	Nucleic acid binding Transcription factor activity	Inside (10 kb)	Forward
				Unknown	SINE	
		2-2	*Nucks1*	Kinase activity	Inside (9.4 kb)	Forward
		2-4	Unknown			
		2-5	*Ythdf3*	Unknown	Inside (6.4 kb)	Forward
			Utx	Binding	Inside (90 kb)	Forward
		2-8	*Sirt 2*	DNA binding Hydrolase activity	Inside (17 kb)	Forward
		2-9	*Mnat1*	Metal ion binding Protein binding	Inside (72 kb)	Forward
			LOC381358	Unknown	− 1.1 kb	Forward
	2	2-2	*Hist1h4m*	DNA binding	+2.6 kb	Reverse
		2-4	Unknown			
		2-5	Unknown			
		2-10	*Cntnap2*	Protein binding	Inside (160 kb)	Forward

Lentiviral vectors are integrated inside or around the coding region of active genes, whereas retroviral vectors tend to be inserted around the head of the coding region, which includes the promoter region[22,23]. This difference suggests lentiviral vectors are less likely to cause insertional mutagenesis than are retroviral vectors. In the conventional BCR/ABL mouse model using retroviral vectors, ALL development might be affected by insertional mutagenesis as secondary events, which lead to blastic crisis in patients with CML.

The lack of CML-like disease appears to be due to inefficient transduction of HSCs. The BCR/ABL mutation is found in all hematopoietic lineages in almost all patients CML, indicating that the primary BCR/ABL translocation originates in HSCs to create CML stem cells that lead to myeloproliferative syndrome[24]. Accordingly, the BCR/ABL gene must be transferred and expressed in HSCs of donor mice for CML-like polyclonal disease to develop in recipient mice. It has been reported that any of the 3 principle forms of the BCR/ABL gene can induce a CML-like disease when a gamma retroviral vector and fluorouracil-treated donor cells are used[12]. In contrast, ALL could be generated by primary mutation and subsequent transformation in committed B-cell progenitors. Castor et al. have demonstrated that ALL with P210 BCR/ABL originates from lymphomyeloid HSCs, whereas ALL with P190 BCR/ABL originates from B-cell progenitors, although the transformed leukemia-initiating stem cells in both ALLs had a committed

B-cell progenitor phenotype[25]. It is likely that our lentiviral vector can transduce committed B-cell progenitors but not HSCs. Because an additional event is required for ALL transformation of BCR/ABL-positive committed cells, B-ALL should develop after a longer latency than that required for CML development and be monoclonal or oligoclonal[25]. This model is consistent with our observations.

The transduction efficiency of our lentiviral vectors carrying the BCR/ABL genes was not sufficient to transduce mouse HSCs, although the GFP-only control vector achieved multilineage gene marking. Improvement of the vector system is essential to study the leukemogenesis of the BCR/ABL genes. The inefficient transduction might be due to low titers of BCR/ABL-expressing lentiviral vectors, which were caused by large sizes of vector RNAs **(Fig. 2a)**[26] and the envelope type. However, it is hard to say that vector sizes are essential factors because conventional retroviral vectors have the same disadvantage regarding vector sizes as do lentiviral vectors. We used a lentiviral vector pseudotyped with ecotropic envelope for safety reasons, because the ecotropic vector cannot infect human cells. However, VSV-G pseudotyped particles are highly stable and easily concentrated with ultracentrifugation. For efficient vector preparation, VSV-G pseudotyped lentiviral vectors are superior to ecotropic vectors, although VSV-G pseudotyped vectors expressing the BCR/ABL oncogene are oncogenic in human cells. Therefore, the handling of such vectors must be strictly regulated for public safety. Another possible improvement is a change of the internal promoter. It has been reported that promoter activity influences the phenotype of transformed cells. In transgenic mice with p210 BCR/ABL under the control of the metallothionein-1 gene promoter, mice developed B-ALL and T-cell ALL[27,28], whereas a CML-like disease was seen in transgenic mice expressing BCR/ABL controlled by the stronger tec promoter[29]. This finding suggests that higher transgene expression in immature hematopoietic progenitors contributes to the development of CML-like disease[27–29]. Our lentiviral vector had MSCV LTR-U3 as an internal promoter and exhibited less transgene expression in vitro and in vivo compared with the MSCV oncoretrovirus vector (data not shown).

In conclusion, lentivirus-mediated transfer of the p210 BCR/ABL gene, either the b3a2 or b2a2 subtype, into lineage-negative BM cells caused B-ALL but not CML in mice under our experimental conditions. There was no difference in the ALL phenotypes between the two subtypes. Possible modifications of the protocol for evaluation of myeloid leukemogenic activities of BCR/ABL variants include the use of the VSV-G envelope, a strong internal promoter, and fluorouracil-treated donor cells.

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