Disruption of *Sept6*, a Fusion Partner Gene of *MLL*, Does Not Affect Ontogeny, Leukemogenesis Induced by *MLL-SEPT6*, or Phenotype Induced by the Loss of *Sept4*

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Septins are evolutionarily conserved GTP-binding proteins that can heteropolymerize into filaments. Recent studies have revealed that septins are involved in not only diverse normal cellular processes but also the pathogenesis of various diseases, including cancer. SEPT6 is ubiquitously expressed in tissues and one of the fusion partner genes of *MLL* in the 11q23 translocations implicated in acute leukemia. However, the roles of this septin in vivo remain elusive. We have developed *Sept6*-deficient mice that exhibited neither gross abnormalities, changes in cytokinesis, nor spontaneous malignancy. *Sept6* deficiency did not cause any quantitative changes in any of the septins evaluated in this study, nor did it cause any additional changes in the *Sept4*-deficient mice. Even the depletion of Sept11, a close homolog of Sept6, did not affect the *Sept6*-null cells in vitro, thus implying a high degree of redundancy in the septin system. Furthermore, a loss of *Sept6* did not alter the phenotype of myeloproliferative disease induced by *MLL-SEPT6*, thus suggesting that Sept6 does not function as a tumor suppressor. To our knowledge, this is the first report demonstrating that a disruption of the translocation partner gene of *MLL* in 11q23 translocation does not contribute to leukemogenesis by the *MLL* fusion gene.

Septins, a family of conserved GTP-binding proteins, are characteristically found in the heteropolymeric filaments involved in diverse aspects of cell biology (10). The originally identified septins (Cdc3p, Cdc10p, Cdc11p, and Cdc12p) were found to be required for cytokinesis, such as budding in yeast, and the identification of septin homologs in higher eukaryotes that localize to the cleavage furrow in dividing cells also supported the idea that septins play an orthologous role in cytokinesis (14, 21). Mammalian septins localize in the cytoplasm and assemble into heteromeric complexes composed of three or more septin subunits (23). These heteromeric complexes tend to polymerize into filaments and then interact with actin stress fibers and/or microtubules (14, 23).

Recent advances in the field revealed that mammalian septins are not only required for cell cycle control, vesicle trafficking, and compartmentalization of the plasma membrane in nondividing cells but also associated with cancer and neurodegenerative disease, although their physiological significance remains largely unknown (14, 21). Indeed, *Sept4*-deficient mice exhibit male infertility due to impaired morphology and motility of the sperm flagellum (18). Notably, 4 of 13 septin genes identified so far in humans, *SEPT5*, *SEPT9*, *SEPT6*, and *SEPT11*, have all been cloned as fusion partner genes of the *MLL* (also called *ALL1* or *HRX*) gene in hematological malignancies by both our group and others (2, 26, 29, 39, 42, 52). SEPT5, which is exclusively expressed in the brain, heart, and megakaryocytes, belongs to the tissue-specific expressed subgroup of septins (58), while the other three septins belong to the ubiquitously expressed subgroup (15, 39, 42, 52). Of these four septins, Sept5 has been investigated most intensively. In the brain, Sept5 is associated with synaptic vesicles (22), while in platelets, Sept5 is a part of the macromolecular complex involved in platelet secretion (7). *Sept5*-deficient mice exhibited normal development, including synaptic properties presumably due to the functional compensation by other proteins

(43), except for functional abnormalities in the platelets (7). On the other hand, Sept9 assembles into a septin complex with Sept7 and Sept11 (34), and it is associated with the cytoskeleton, including microtubule network and actin filaments (33, 44, 50). Its depletion using RNA interference (RNAi) causes incomplete cell division with accumulation of binucleated cells (33, 50). Interestingly, an overexpression of *Sept9*, presumably due to genomic gain, has been observed in a variety of tumors (32, 47), thus suggesting that *Sept9* is involved in oncogenesis. However, the genomic loss or down-regulation of *Sept9* also has been observed in ovarian and breast cancers (20, 46), thus implying that *Sept9* behaves as a tumor suppressor. In contrast to *Sept5* and *Sept9*, little has been elucidated in *Sept6* and its closest homolog, *Sept11*, which was identified most recently (15). Sept6 is associated with synaptic vesicles like Sept5 in the brain (22) and assembles into a septin complex with Sept2 and Sept7 which interacts with actin stress fibers through adaptor proteins (23). Furthermore, we previously demonstrated that the fusion of *MLL* with *SEPT6* in males is always accompanied with a complete genomic loss of *SEPT6*, which is located on Xq24 (39), and that the overexpression of SEPT6 itself does not lead to the myeloid immortalization of murine hematopoietic progenitors in vitro, whereas the overexpression of *MLL-SEPT6* does (40). These findings suggest that SEPT6 plays a role in leukemogenesis as well as in normal functions, including neurotransmission.

The *MLL* gene is a proto-oncogene involved in acute leukemia (13, 27, 45, 54) as well as definitive hematopoiesis (41). *MLL* is fused with each partner gene to express in-frame MLL fusion oncoprotein which leads to the aberrant activation of target genes, including *HOX* genes (1). The phenotype of *MLL*-mediated leukemia depends on the fusion partner (16), thus suggesting that each fusion partner is critical for the leukemogenesis by MLL fusion protein. We recently demonstrated that MLL fusion protein alone induced myeloproliferative disease (MPD) but induced acute leukemia in concert only with secondary genotoxic stress (40). It is possible that the secondary genotoxic stress may result from a disruption of the fusion partner (1, 12) as well as mutation of other genes (17); however, no evidence has hitherto been shown concerning the contribution of such a disruption to leukemogenesis.

To clarify the function of SEPT6 in vivo, we disrupted the *Sept6* gene in the mouse but found no distinct phenotypes, thus suggesting that this gene product is not essential for ontogeny and oncogenesis. We also probed the compensation of other septins both in vivo and in vitro, and our findings provide some important clues regarding the diverse aspects of the septin system. Furthermore, this is the first report to examine whether the loss of a translocation partner gene contributes to the *MLL*-mediated leukemogenesis in 11q23 translocation.

MATERIALS AND METHODS

Gene targeting of *Sept6*. A 9.1-kb genomic clone carrying the third exon of *Sept6* was obtained by screening the genomic library cloned in λphage (Stratagene), which was derived from mouse strain 129/SvJ as described previously (37, 56). To construct the targeting vector, a 1.1-kb XhoI-BamHI fragment of pMC1NeoPolyA (Stratagene) harboring the neomycin resistance gene (*Neo*) flanked in the same orientation by two fragments derived from the genomic clone, a 4.0-kb SacI-BstXI fragment at the 5′ end and a 1.5-kb BamHI-BamHI fragment at the 3′ end, was introduced into a region between the SacI and ClaI sites of the modified pBluescript vector lacking the XhoI site. The BstXI site of

the genomic clone and the ClaI site of the pBluescript were converted to the XhoI site and the BamHI site by using an XhoI linker and a BamHI linker (Stratagene), respectively. The herpes simplex virus thymidine kinase gene (*tk*) cassette was also inserted into the SalI site in the pBluescript within the targeting vector in the sense orientation (Fig. 1A).

The targeting vector was linearized by KpnI digestion at the 3′ end of the *tk* cassette and electroporated into E14-1 male embryonic stem (ES) cells, followed by selection with G418 and ganciclovir, as previously described (37). To detect the correct homologous recombination, screening by a Southern blot analysis of BamHI- or SphI-digested genomic DNA from the ES cells was done as previously described (35) by using a 5′ probe or a 3′ probe (Fig. 1A). The 5′ probe was an NheI-digested 340-bp fragment, and the 3′ probe was generated with PCR as described in detail later. The chimeric male mice were generated by the blastocyst injection of the targeted ES cell clone. The genotyping of the offspring was done by PCR of each genomic DNA obtained from the mouse tail (described in detail later).

Cell culture. Mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 13.5 embryos by using standard methods and then were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The primary culture of the fetal brain was performed as described previously (59) but with slight modifications. Briefly, the fetal brain was prepared from embryonic day 14.5 embryos in Hanks' balanced salt solution and cultured for 4 days in N2-supplemented Dulbecco's modified Eagle's medium–F-12 medium containing 10 ng/ml of basic fibroblast growth factor (R & D Systems) on a dish precoated with poly-L-ornithine (Sigma) and fibronectin (Wako Chemicals).

Western blot analysis. Cells or brain tissues were harvested in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholic acid, 1% Nonidet P-40) supplemented with protease inhibitor cocktail (Sigma) on ice. The lysates were homogenized, mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue), and boiled for 5 min. The concentration of each protein sample was determined using a standard bicinchoninic acid assay (Pierce). A Western blot analysis of 20 μg of each sample was performed using monoclonal anti-α-tubulin (Sigma), polyclonal anti-Sept2 (23), two kinds of anti-Sept6, anti-Sept7 (23), and anti-Sept11 (15) antibodies to probe membranes as previously described (36). In addition, 50 μg of lysates of the cerebellum derived from *Sept6/ Sept4*-deficient mice and their littermates was analyzed by Western blot analysis as described previously (18). The anti-Sept6 antibodies used were an affinity-purified polyclonal rabbit antibody (anti-Sept6-2) against amino acids 301 to 427 of SEPT6 and the antibody anti-Sept6-1 as described previously (22).

Immunostaining. Immunostaining of the cells in primary culture of fetal brain was performed and viewed using a Fluoview FV300 confocal microscope (Olympus) as described previously (30). The anti-Sept6-1 or the anti-α-tubulin antibody and fluorescein isothiocyanate-conjugated anti-guinea pig or anti-mouse immunoglobulin (Sigma) were used as the first and second antibody, respectively. F-actin was visualized with rhodamine-conjugated phalloidin (Sigma).

RNAi. MEFs (10^5 cells) were transfected with small interfering RNA (siRNA) duplexes as follows: Sept11-siRNA, 5′-GAGCUGCGAAACUUAUCACdTdT-3′ (sense) and 5′-GUGAUAAGUUUCGCAGCUCdTdT-3′ (antisense), and nonspecific control IX (Dharmacon) as control-siRNA, 5′-AUUGUAUGCGAUCGC AGACdTdT-3′ (sense) and 5′-GUCUGCGAUCGCAUACAAUdTdT-3′ (antisense). These siRNA duplexes were manufactured and supplied by Dharmacon. Transfection was done with Lipofectamine 2000 (Invitrogen) as described previously (55). At 72 h after transfection, the cells were counted and subjected to Western blot analysis.

Retroviral constructs and retrovirus production. A series of pMXs-neo constructs (40) harboring the *MLL-SEPT6* and *MLL-ENL* short form (MEs), which had a FLAG epitope tag at the C terminus, were modified by the replacement of their neo cassettes with the puro cassette of pMXs-puro (25) to generate a series of pMXs-puro constructs.

Packaging cells, PlatE, were transfected with retroviral constructs using FuGENE 6 (Roche Diagnostics) according to the manufacturer's recommendations, and appropriate expression of the transgenes was confirmed by Western blot analysis as described previously (40). Retroviruses were harvested 48 h after transfection as described previously (36).

Myeloid immortalization assays of hematopoietic progenitors in vitro and leukemogenesis assays in vivo. The oncogenic potential of MLL-SEPT6 and MLL-ENLs fusion proteins in vitro in the presence or absence of *Sept6* was analyzed by myeloid immortalization assays of hematopoietic progenitors harvested from *Sept6*-deficient (KO) mice or their wild-type (WT) male mice
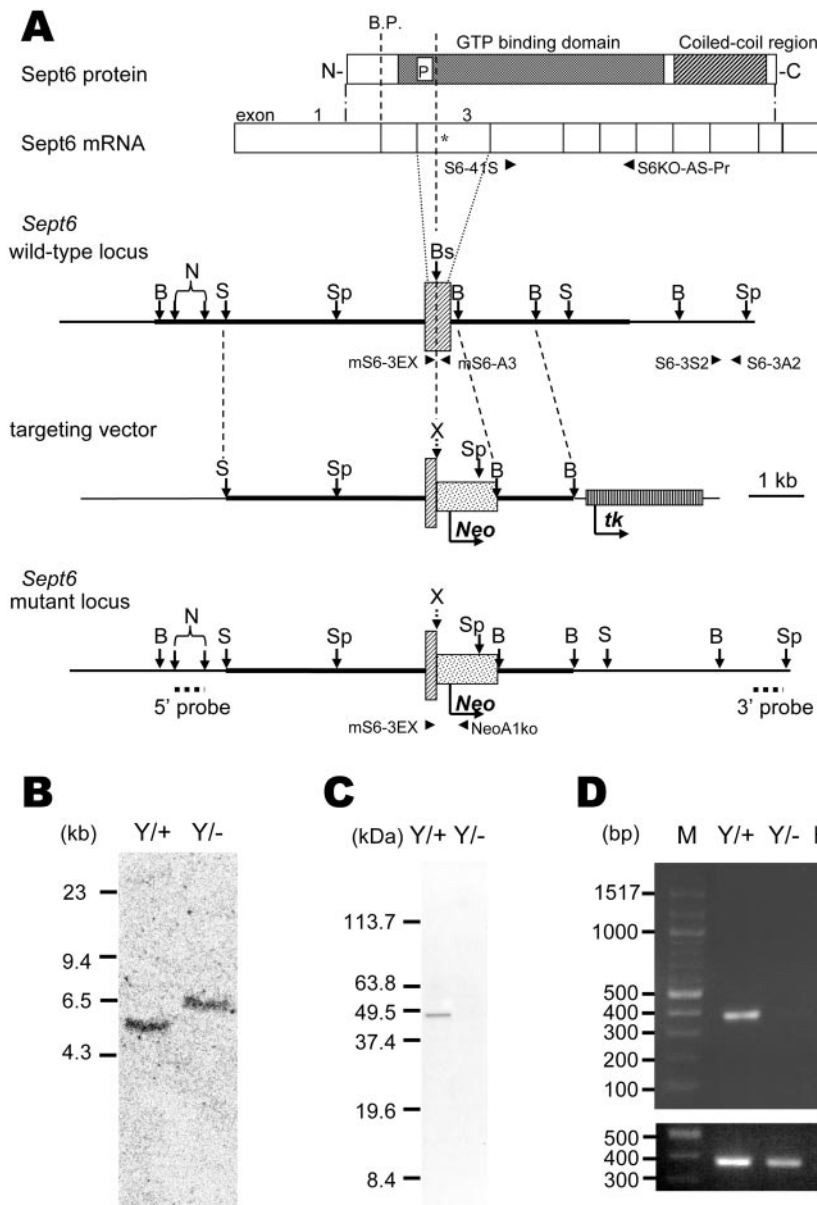
FIG. 1. Targeted disruption of *Sept6*. (A) A schematic representation of the targeting strategy. The structure of Sept6 products (protein and mRNA) (top), the wild-type locus, the targeting construct, and the targeted locus are shown. Genomic fragments, including exon 3 (a box with diagonal lines) of *Sept6*, that were obtained by the screening of the genomic library and the probes used for a Southern blot analysis are indicated by bold horizontal and dotted lines, respectively. The restriction sites and a converted restriction site are indicated by vertical solid and dotted arrows, respectively. The orientations of the neomycin resistance gene (*Neo*) and the herpes simplex virus thymidine kinase gene (*tk*) are indicated by horizontal arrows. The primers used are indicated by horizontal arrowheads. The location of P-loop motif (P), the second ATG (∗), and a breakpoint in fusion with MLL (B.P.) are shown in Sept6 protein and mRNA. B, BamHI; Bs, BstXI; N, NheI; S, SacI; Sp, SphI; X, XhoI. (B) Homologous recombination in the male ES cells by Southern blot analysis. BamHI- or SphI-digested genomic DNA extracted from the ES cells was hybridized with the 5′ probe or the 3′ probe, resulting in the wild-type 5.5- and targeted 6.3-kb bands or the wild-type (Y/+) 7.4- and targeted (Y/−) 5.3-kb bands (data not shown), respectively. (C and D) The expression of Sept6 protein in the brain (C) and mRNA in MEFs (D, upper panel) derived from the wild-type (Y/+) and disrupted (Y/−) mice by Western blot analysis using the anti-Sept6-1 antibody and RT-PCR analysis using primers S6-41S and S6KO-AS-Pr, respectively. The expression levels of β_2 microglobulin (D, lower panel) were used as internal standards of the RT-PCR analysis. M, 100-bp DNA ladder (New England Biolabs).

(Ly-5.2) littermates as described previously (40), except for using pMXs-puro retroviral constructs and 1 µg/ml of puromycin instead of the pMXs-neo constructs and G418, respectively.

The leukemogenic potential of MLL-SEPT6 and MLL-ENLs fusion proteins in vivo in the presence or absence of *Sept6* was also analyzed by modified leukemogenesis assays as described previously (40). In brief, bone marrow he-

matopoietic progenitors from *Sept6*-deficient mice or their littermate wild-type mice (Ly-5.2) were mock transduced or retrovirally transduced with *MLL-SEPT6* or *MLL-ENLs* in vitro, and 10^5 progenitor cells from each transduction were intravenously injected into F$_1$ offspring of Ly-5.1 C57BL/6 and Ly-5.2 129/SvJ mice together with a radioprotective dose of 2×10^5 bone marrow cells harvested from the same kind of mice (Ly-5.1/5.2) as the recipients. Before injection, the
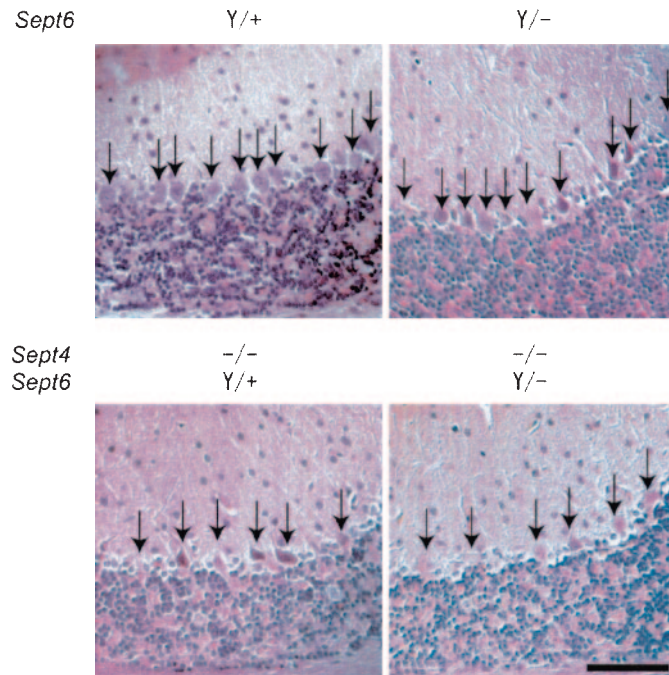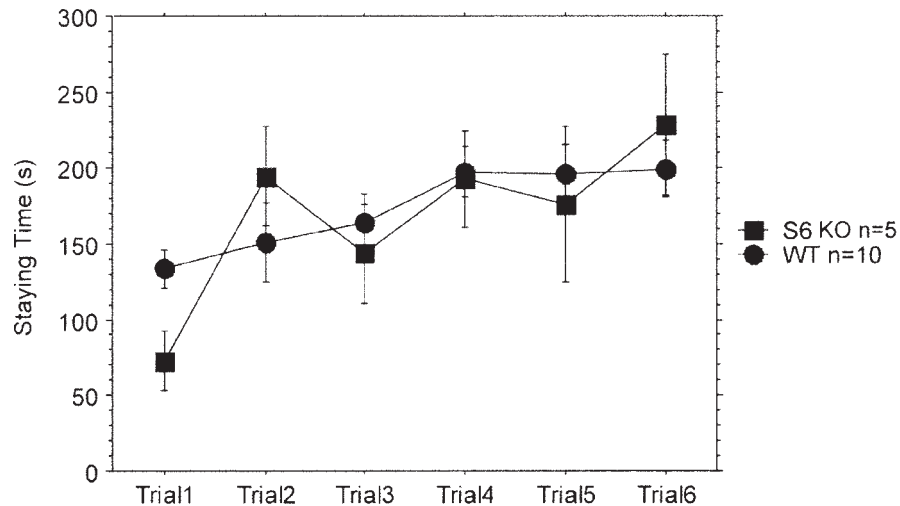
FIG. 2. A neurological analysis of *Sept6*- and *Sept4/Sept6*-deficient mice. (A) Sagittally sectioned, hematoxylin-eosin-stained cerebella in the adult mice. *Sept6* deficiency did not lead to any anatomical abnormalities of the cerebellum (compare upper two panels). Despite a paucity of Purkinje cells and granule cells in *Sept4*$^{-/-}$ cerebella (lower left panel), the loss of Sept6 did not cause additional changes in the cerebellum (lower right panel). Arrows, Purkinje cells. Bar, 40 μm. (B) Results of rotarod task. Wild-type or *Sept6*-deficient mice were placed on the rotarod drum (3-cm diameter) starting at 4 rpm and then slowly accelerating to 40 rpm over 300s. We recorded the latency until mice fell out of the drum, and the results and data were analyzed by an analysis of variance.

recipient mice had been administered a lethal dose of 9.5 Gy total-body γ irradiation. Probabilities of murine overall survival were estimated using the Kaplan-Meier method and then were compared using the log rank test.

Morphological and immunophenotyping analysis. Mice were euthanized, and their tissue samples were analyzed. Circulating blood cells were counted, and tissue specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin as described previously (37). Cytospin preparations of bone marrow cells and blood smears were stained with Wright-Giemsa or peroxidase stain to assess cell morphology. Immunopheno-

typing by fluorescence-activated cell sorting was done using a FACSCalibur (BD Biosciences) as described previously (40). All animal studies were approved by the Animal Care Committee of the Institute of Medical Science of the University of Tokyo.

Reverse transcription (RT)-PCR and genomic PCR. Total RNA was extracted from tissues, MEFs, the immortalized cells, or murine pro-B cell line Ba/F3 and reverse transcribed to cDNA with random hexamers as described previously (51). The conditions, reagents for RT-PCR, and the primers specific for β$_2$ micro-globulin (β$_2$MG), *Hoxa7*, *Hoxa9*, and *Meis1* were all as previously described (37,

TABLE 1. Peripheral blood cell counts in *Sept6*-deficient and wild-type mice

Genotype	Cell counts for[a]:				
	WBC/µl	RBC (10^4/µl)	Hb (g/dl)	Ht (%)	Plt (10^4/µl)
Sept6 deficient ($n = 5$)	$11,500 \pm 4,000$	963 ± 30	16.4 ± 0.5	56.6 ± 2.6	54.2 ± 22.5
Wild type ($n = 5$)	$11,000 \pm 3,600$	953 ± 83	16.8 ± 1.2	56.8 ± 4.8	56.8 ± 20.0

[a] The averages with standard deviations are shown. WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit; Plt, platelets.

40). To detect the transcripts of *Sept1*, *Sept6*, *Sept8* (total and variant 2), and *Sept11*, PCR amplification was run for 24 cycles using primers as follows: mS1-S, 5′-GTTGGTGCAGACCCACCTTCA-3′, and mS1-AS, 5′-GCACATCTGATT GCTCGCCCT-3′; S6-41S, 5′-GAGGAGCTGAAGATCCGA AGAGTA-3′, and S6KO-AS-Pr, 5′-CCTCGTTTTCAACCTGCACAGTC-3′; Sept8-S1, 5′-GATCCGCCGTTCCCTCTTTG-3′, and Sept8-AS1, 5′-CCGTTC CTTCTCCTTCAGCTCAA-3′ (for total transcript); mS8-S, 5′-GGAGTTCCT AAGCGAGCTGCA-3′, and mS8v2-AS, 5′-CTCAGAGGAATCCTTCCCTCC A-3′ (for transcript variant 2); and Sept11-S1, 5′-CCGACACCATCGCCAAAA ACGAAT-3′, and Sept11-AS1, 5′-GCCTCTTTCTCCTTCACCCTCAT-3′. In addition, to detect transcripts of *Sept5* (variants 1 and 2), *Sept8* (variant 1), *Sept9* (types b, c, and d), and *Sept10*, PCR amplification was run for 28 cycles using the following primers: mS5v1-S, 5′-GCGACCCCAGAGGACAAACA-3′, and mS5-ASc, 5′-GCGTGTCCACAATGGTGAGCTTT-3′ (for transcript variant 1); mS5v2-S, 5′-AGGCCCAGAGGCGACTGAA-3′, and mS5-ASc (for transcript variant 2); mS8-S and mS8v1-AS, 5′-TCCCGGGCTGAGGGTCAAAA-3′ (for transcript variant 1); mS9b-S, 5′-GAACACAACCGGAGTCC-CTGA-3′, and mS9-AS, 5′-CTCGAAGCCCTGTTTCATAGCCT-3′ (for type b); mS9c-S, 5′-CCCGACTTTCAGCTGCTGG-3′, and mS9-AS (for type c); mS9d-S, 5′-AGATCCAGGTGCCCAAGCCA-3′, and mS9-AS (for type d); and mS10-S, 5′-GGGGGAGACTGGAATTGGAAAATC-3′, and mS10-AS, 5′-GGAGCA ATGAAGTAGAGGCACAC-3′.

To carry out the genotyping of offspring and MEFs, 40 ng of genomic DNA was amplified under the same conditions as RT-PCR except for using three primers, 80 pmol of mS6-S3Ex and 40 pmol of NeoA1ko and mS6-A3. To generate the 3′ probe used in screening, genomic DNA from nonmanipulated ES cells was also amplified in the same way as RT-PCR by using primers S6-3S2 and S6-3A2. The primers used were as follows: mS6-S3Ex, 5′-GGGAGACAGGTT TGGGCAAGT-3′; NeoA1ko, 5′-AGGCCACACGCGTCACCTTA-3′; mS6-A3, 5′-CCTCCTTGTTGATCTGATCCCCAA-3′; S6-3S2, 5′-CTTGGGTTA TTGATGTGTGCTACTTTC-3′; and S6-3A2, 5′-AGAGATATGCTGTAT GGGTGGTCAAT-3′.

RESULTS

Targeted disruption of *Sept6*. To delineate the physiological roles of *Sept6* in vivo, we generated *Sept6*-deficient mice by gene targeting. A targeting vector was constructed to replace most of the third exon, including the second ATG which starts from 6 bp downstream of the P-loop motif within the GTP-binding domain, with the *Neo* cassette (Fig. 1A). An ES clone was demonstrated to have the predicted homologous recombination (Fig. 1B), and it was used to obtain chimeric founder male mice. The chimeric mice were backcrossed to C57BL/6 (Ly-5.2) female mice to generate heterozygous (*Sept6*$^{+/-}$) female mice and wild-type male mice in the F$_1$ generation. After these F$_1$ mice were interbred to generate knockout (*Sept6*$^{Y/-}$) male mice in the F$_2$ generation, *Sept6*$^{Y/-}$ male and *Sept6*$^{+/-}$ female mice were interbred to generate homozygous (*Sept6*$^{-/-}$) female mice in the F$_3$ generation. Genotyping was carried out by PCR of genomic DNA (data not shown). A *Sept6* deficiency in vivo was confirmed by Western blot analysis of the brain using the anti-Sept6-1 antibody that recognizes epitopes within the carboxyl-terminal region and RT-PCR of total RNA extracted from MEFs using primers S6-41S and S6KO-AS-Pr, covering exons 4 to 6 (Fig. 1A, C, and D).

Disruption of *Sept6* causes no gross abnormality in growth and development. *Sept6*-deficient mice were born with predicted Mendelian frequencies in both sexes, developed without growth retardation, were fertile, and had as long a life span as their littermates within the observation period of 18 months. Although Sept6 was ubiquitously expressed, no macroscopic or microscopic differences in several organs, including the brain, thymus, heart, lung, stomach, intestine, colon, liver, pancreas, kidney, spleen, genitalia, and bone, between deficient mice and littermates were observed (Fig. 2A and data not shown). In addition, to detect subtle neurological disorders, the acquisition of skilled behavior in the rotarod task was examined, but there were no statistical differences between *Sept6*-deficient and wild-type mice (Fig. 2B). Since the fusion of SEPT6 with MLL develops infant acute myeloid leukemia in humans (39), both the bone marrow and peripheral blood were also analyzed. Neither any significant differences in the morphology of cytospin preparations of the bone marrow cells and blood smears nor those in the blood cell counts were observed (Table 1 and data not shown). These results indicate that *Sept6* is therefore not essential for normal development and normal hematopoiesis at steady state.

No quantitative changes of other septins are found in *Sept6* deficiency. A previous report disclosed that the depletion of *Sept2* or *Sept7* using RNAi in vitro led to a perturbation of F-actin, resulting in the changes of localization of actin bundles (23). To investigate the molecular alterations followed by *Sept6* deficiency in vitro, actin bundles which interact with septin complexes containing Sept2, Sept6, and Sept7 and microtubules which interact with other septins were imaged by immunofluorescent confocal microscopy in a primary culture of the fetal brain. In the *Sept6*-deficient cells, the organization of F-actin and α-tubulin was not perturbed, and their localization and morphology also did not differ from the control cells (Fig. 3). Although Sept6 staining was not successful due to background signals (data not shown), the expression levels of Sept6 were confirmed by Western blot analysis of the lysates extracted from these cells using the anti-Sept6-2 (Fig. 4A). These results, together with no gross abnormalities in *Sept6*-deficient mice, suggested the functional redundancy of septin family molecules as shown in *Sept5*-deficient mice (43).

To examine whether *Sept6* deficiency leads to compensatory changes in the expression of septins Sept2 and Sept7, which coassemble into a physiological complex with Sept6 (23), the levels of these septins were first analyzed. Each lysate was extracted from brains, cells in primary brain culture, and MEFs derived from *Sept6*-deficient mice or wild-type littermates. A Western blot analysis of equal amounts of these lysates using the anti-Sept2 or anti-Sept7 antibody revealed no significant differences in the expression levels of these two septins
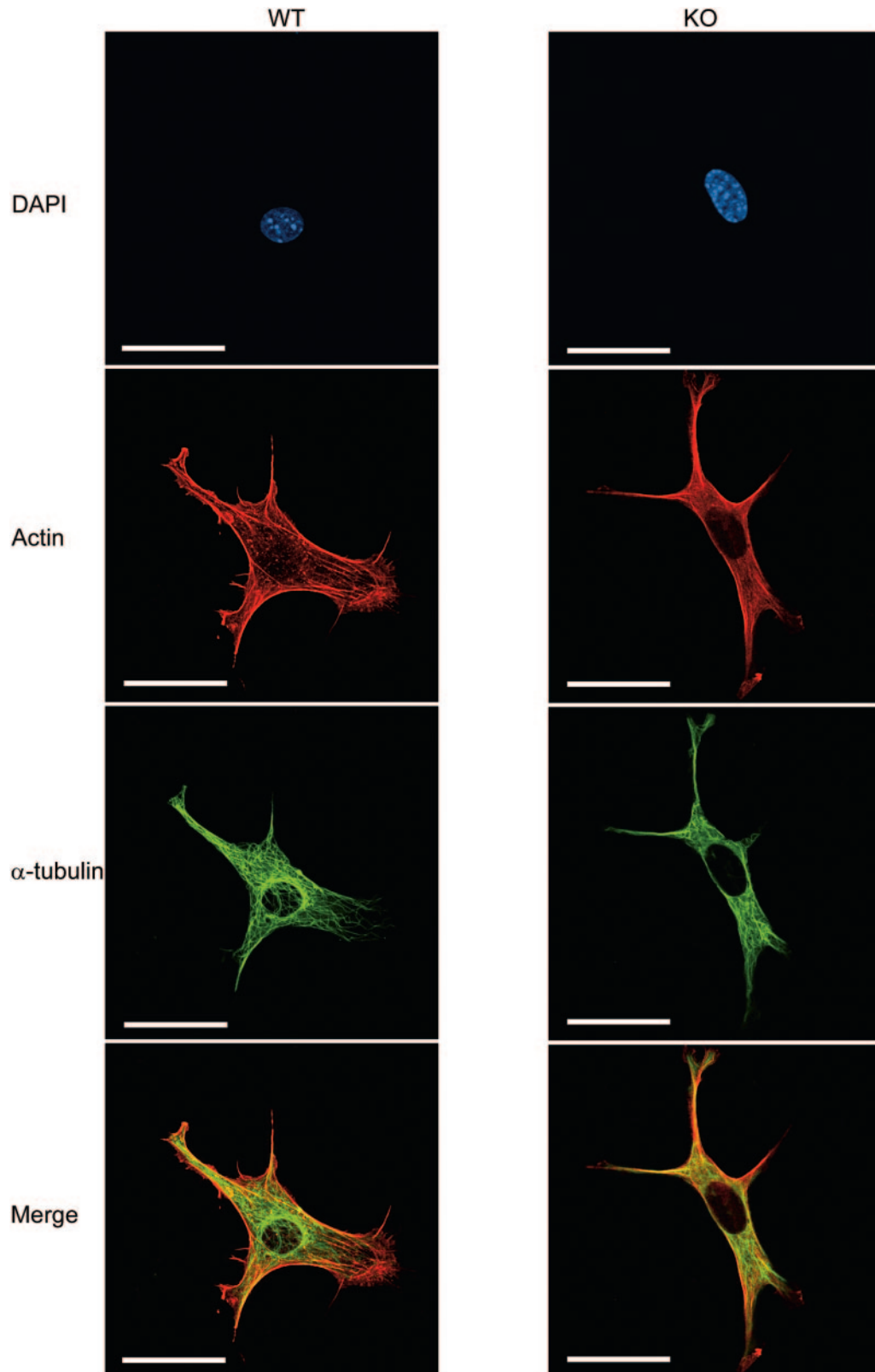
FIG. 3. Cytoskeleton of actin bundles and microtubules in primary cultures of fetal brains derived from the KO and WT embryos. The localization and morphology of F-actin (red) and α-tubulin (green) in the cells were analyzed by immunofluorescent confocal microscopy. The nuclei were visualized with 4′,6′-diamidino-2-phenylindole (DAPI) dihydrochloride (top panels). Bar, 30 μm.
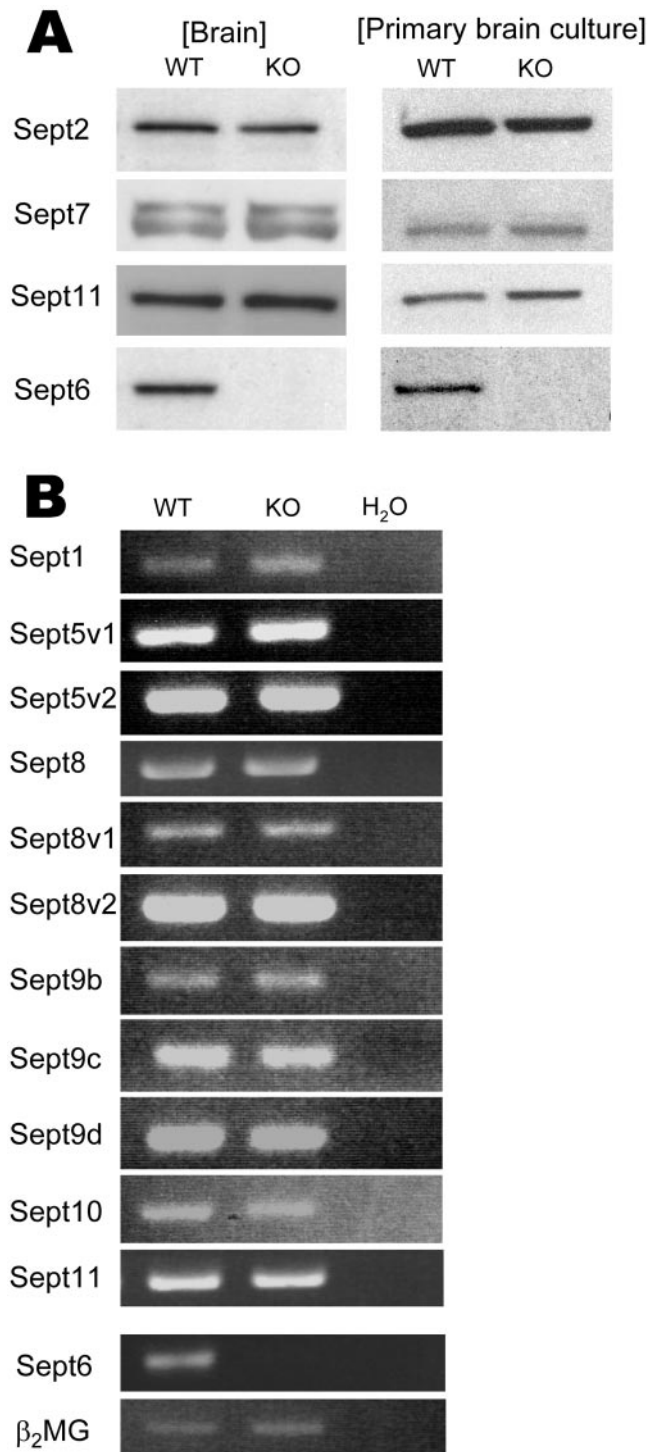
FIG. 4. No detectable compensation by other septin genes in the *Sept6*-deficient brain tissue specimens. (A) The expression levels of Sept2, Sept7, and Sept11 in the brain or the primary brain culture cells by a Western blot analysis. The expression of Sept6 protein in the brain was analyzed with the anti-Sept6-1 antibody, while that in the primary brain culture cells was analyzed with the anti-Sept6-2 antibody. (B) The expression profile of septins in the brain by an RT-PCR analysis. Isoforms of *Sept5* (variants 1 and 2), *Sept8* (variants 1 and 2), and *Sept9* (type b, c, and d) were examined with isoform-specific primers. The expression levels of β_2MG were used as internal standard bases for RT-PCR analysis.
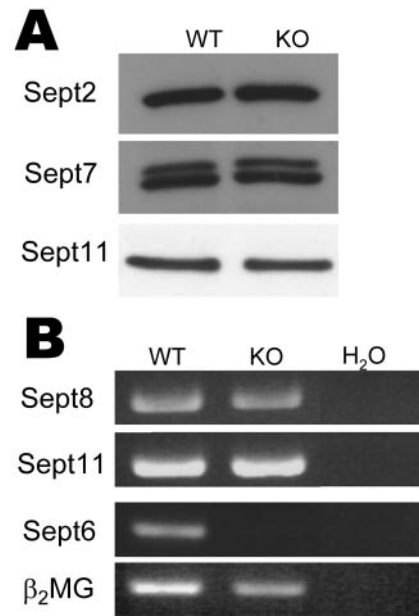

FIG. 5. No detectable compensation by other septin genes in the *Sept6*-deficient MEFs. (A and B) The expression levels of Sept2, Sept7, Sept8, and Sept11 in the MEFs by Western blot (A) and RT-PCR analyses (B). Sept6 in the MEFs could not be successfully detected by a Western blot analysis due to nonspecific bands (data not shown), although it could be detected by RT-PCR analysis as shown in panel B.

(Fig. 4A and 5A), although detection of Sept6 was hampered by the presence of other nonspecific bands in the lysate of MEFs (data not shown). The expression levels of *Sept6* transcript could be confirmed by RT-PCR of total RNAs extracted from these MEFs (Fig. 5B). Since other septins homologous to Sept6 might compensate for its deficiency, Sept11, Sept8, and Sept10, which share the sequences most homologous to Sept6 among septins, were next examined by Western blot analysis of the same lysates using the anti-Sept11 antibody and by RT-PCR of total RNAs extracted from brains and MEFs using primers Sept11-S1 and Sept11-AS1, Sept8-S1 and Sept8-AS1, or mS10-S and mS10-AS. But no significant differences were detected (Fig. 4A and B and 5B). Furthermore, the relative ratios of the individual septin splice variants were investigated in search of a possibility that changes in the ratios could lead to functional compensation in loss of *Sept6*, as it was reported that *Sept9* gene isoforms were associated with neoplasia. Because septin often possesses not a few transcript variants by the extensive alternative splicing, the septin isoforms carrying at least partially different amino acids, like *Sept8* gene variants 1 and 2, which can be retrieved through public database, were targeted. In addition to *Sept8*, other septin genes, *Sept1*, *-4*, *-5*, and *-9*, which are expressed in the brain, were also tested. A Western blot analysis of the lysates extracted from the cerebellum using an antibody against major isoforms of Sept4 (18) revealed neither significant differences in expression levels of the total Sept4 between Sept6-deficient mice and wild-type littermates nor significant changes in the relative ratio of Sept4 isoforms (Fig. 6A). Similarly, RT-PCR of the same RNAs extracted from the brain using several pairs of primers, some of which are isoform specific, detected neither significant differ-
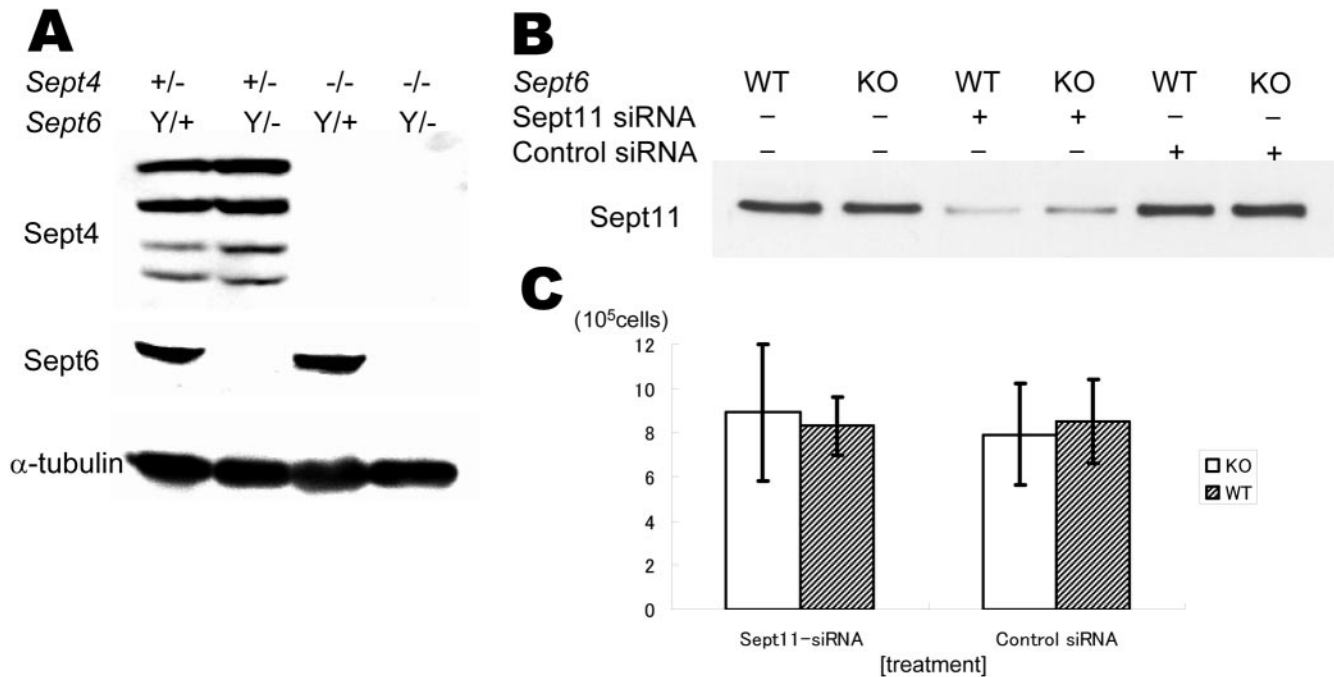
FIG. 6. *Sept4* knockout or *Sept11* knockdown on *Sept6* deficiency. (A) The expression of Sept4 and Sept6 in the cerebellum derived from *Sept4*/*Sept6*-deficient mice and control littermates by a Western blot analysis using the anti-Sept6-1 and anti-Sept4 antibody. The expression levels of α-tubulin were used as internal standards. Genotypes of *Sept4* and *Sept6* are shown above the top panel. (B and C) Growth of the KO and WT MEFs with depletion of Sept11 using siRNA. A Western blot analysis of the lysates extracted from the cells using the anti-Sept11 antibody in the presence (+) or absence (−) of each siRNA transfected is shown in panel B. The bar graph shows the number of MEFs 72 h after each siRNA transfection (average plus or minus standard deviation) (C).

ences in the expression levels of the total *Sept1*, *-5*, and *-9* nor significant changes in the relative ratio of *Sept5*, *-8*, and *-9* gene isoforms (Fig. 4B). These results suggested that the *Sept6* deficiency was not compensated for by the changes in the total expression levels of other septin genes or relative ratios of the individual septin isoforms, at least in the brain.

It is possible that loss of a single septin gene, *Sept6*, might not lead to evident dysfunction in vivo. Therefore, we designed functional loss or reduction of another septin in addition to Sept6. Since Sept6 and Sept4 are contained in the septin complex which was purified from the mouse brain (23) and reduction of the expression level of Sept6 has been found in *Sept4*-deficient mouse testes as we have recently reported (18), we generated *Sept6*/*Sept4*-deficient mice by crossing *Sept6*-deficient and *Sept4*-deficient mice. *Sept6*/*Sept4* deficiency in vivo was confirmed by a Western blot analysis of the brain tissue using the anti-Sept6-1 and anti-Sept4 antibody (Fig. 6A). *Sept6*/*Sept4*-deficient mice exhibited the same phenotypes, including male infertility (18) and anatomical abnormalities in the cerebellum (Fig. 2A), as those of *Sept4*-deficient mice (M. Ihara, A. Hagiwara, J. Monypenny, A. Kinoshita, A. Kitano, A. Tanigaki, S. Itohara, M. Noda, and M. Kinoshita, unpublished data), thus indicating that *Sept6* is dispensable in vivo irrespective of the presence or absence of *Sept4*. Furthermore, a perturbation of the most homologous septin, Sept11, was also designed using RNAi in *Sept6*-deficient or wild-type MEFs. In this experimental system, a Western blot analysis of the lysates extracted from transfected MEFs using the anti-Sept11 antibody demonstrated that Sept11 decreased to approximately

20% of the control level 72 h after transfection with siRNA (Fig. 6B). In this condition, neither morphological nor proliferative differences were observed in the presence or absence of Sept6 (Fig. 6C and data not shown). Although careful consideration is needed for the possibility that the remnant functional activity of Sept11 with siRNA treatment may be enough for the cellular function even in the absence of Sept6, these results raised another possibility, that Sept6 and Sept11 are dispensable for the cell.

Disruption of *Sept6* affects neither immortalization of murine hematopoietic progenitors in vitro nor leukemogenesis in vivo by the MLL-SEPT6 fusion protein. Recently, we have demonstrated that the MLL-SEPT6 fusion protein can induce immortalization of murine hematopoietic progenitors in vitro and development of no acute leukemia but MPD in vivo and that secondary genotoxic stress, such as *FLT3*-internal tandem duplication, and MLL fusion protein MLL-ENL as well as MLL-SEPT6 synergize to transform those progenitors in vitro and develop acute leukemia in vivo (40). Since *Sept6*, which is one of the translocation partners of *MLL*, was not found to be involved in normal hematopoiesis, we applied *Sept6*-deficient hematopoietic progenitors to an in vitro myeloid immortalization assay and an in vivo leukemogenesis assay in order to determine whether a *Sept6* deficiency played an important role, such as the secondary essential genotoxic stress, in leukemogenesis by *MLL-SEPT6* through the loss of the translocation partner expression that occurs in human male acute myeloid leukemia with *MLL-SEPT6*. *Sept6*-deficient or wild-type murine hematopoietic progenitors enriched with 5-fluorouracil treatment
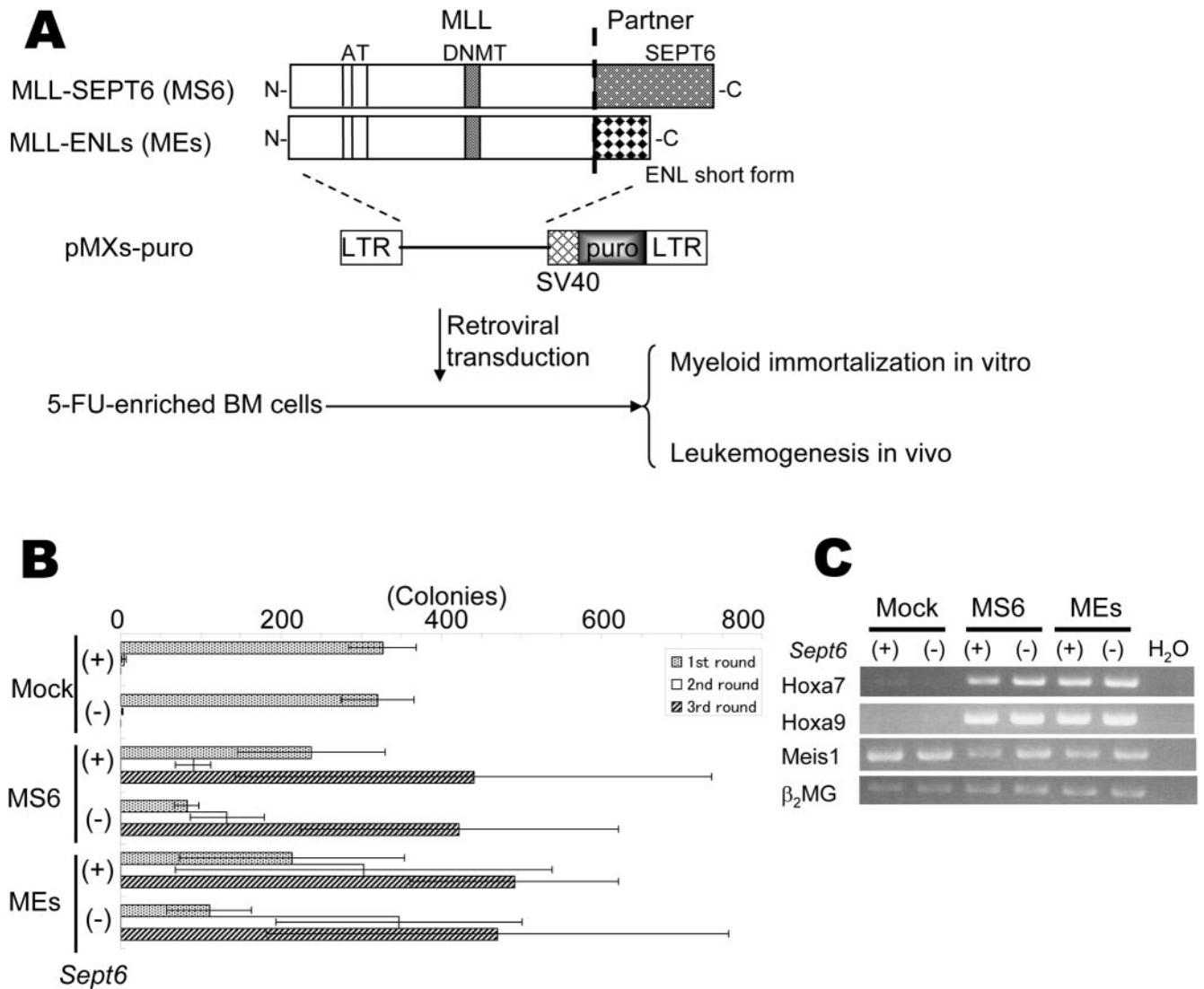
FIG. 7. Myeloid immortalization of murine hematopoietic progenitors by MLL-SEPT6 (MS6) or MEs fusion proteins via the aberrant expression of *Hox* genes. (A) A schematic representation of the retroviral constructs employed and experimental strategy. AT, AT hooks; DNMT, DNA methyltransferase domain; LTR, long terminal repeat; SV40, SV40 early promoter; puro, puromycin resistance gene; 5-FU, 5-fluorouracil. (B) Myeloid immortalization assay using the pMXs-puro constructs shown in panel A. Bar graph shows the number of colonies obtained after each round of replating in methylcellulose (average plus or minus standard deviation). (C) The expression levels of *Hoxa7*, *Hoxa9*, and *Meis1* by RT-PCR in the cells from third-round cultures. The expression levels of β2MG were used as an internal standard. The genotypes of *Sept6* are indicated by wild type (+) or *Sept6* deficiency (−) in panels B and C.

were analyzed by a myeloid immortalization assay using retroviruses harboring *MLL-SEPT6* or *MLL-ENL*s as one of the representative *MLL* fusion genes or vector alone (named MS6, MEs, or mock) after confirming their expression in packaging cells by Western blot analysis (Fig. 7A and data not shown). Initial plating demonstrated that the *Sept6*-deficient cells transduced with *MLL* fusion genes generated half of the colonies with a morphology similar to the wild-type cells, while both *Sept6*-deficient and wild-type cells that were mock transduced formed very similar colonies in both number and morphology. However, in serial replating, the *Sept6*-deficient cells transduced with *MLL* fusion genes generated and maintained an increased number of compact colonies similar to that of the wild-type cells, while both cells that were mock transduced

rapidly failed to form any colonies (Fig. 7B). Wright-Giemsa-stained cytospin preparations of the cells comprising these compact colonies in the presence or absence of *Sept6* showed almost identical features consistent with myelomonocytic blasts (40) (data not shown). RT-PCR of total RNAs extracted from these colonies at the third round also confirmed that the expression of target genes of MLL fusion protein, such as *Hoxa7* and *Hoxa9*, was upregulated in the deficient cells transduced with *MLL* fusion genes as in the wild-type cells, while the expression of *Meis1* was not upregulated in the presence or absence of *Sept6*, as described previously (40) (Fig. 7C).

Furthermore, the hematopoietic progenitors that were mock transduced or transduced with *MLL* fusion genes were also directly transplanted into lethally irradiated F₁ offspring be-
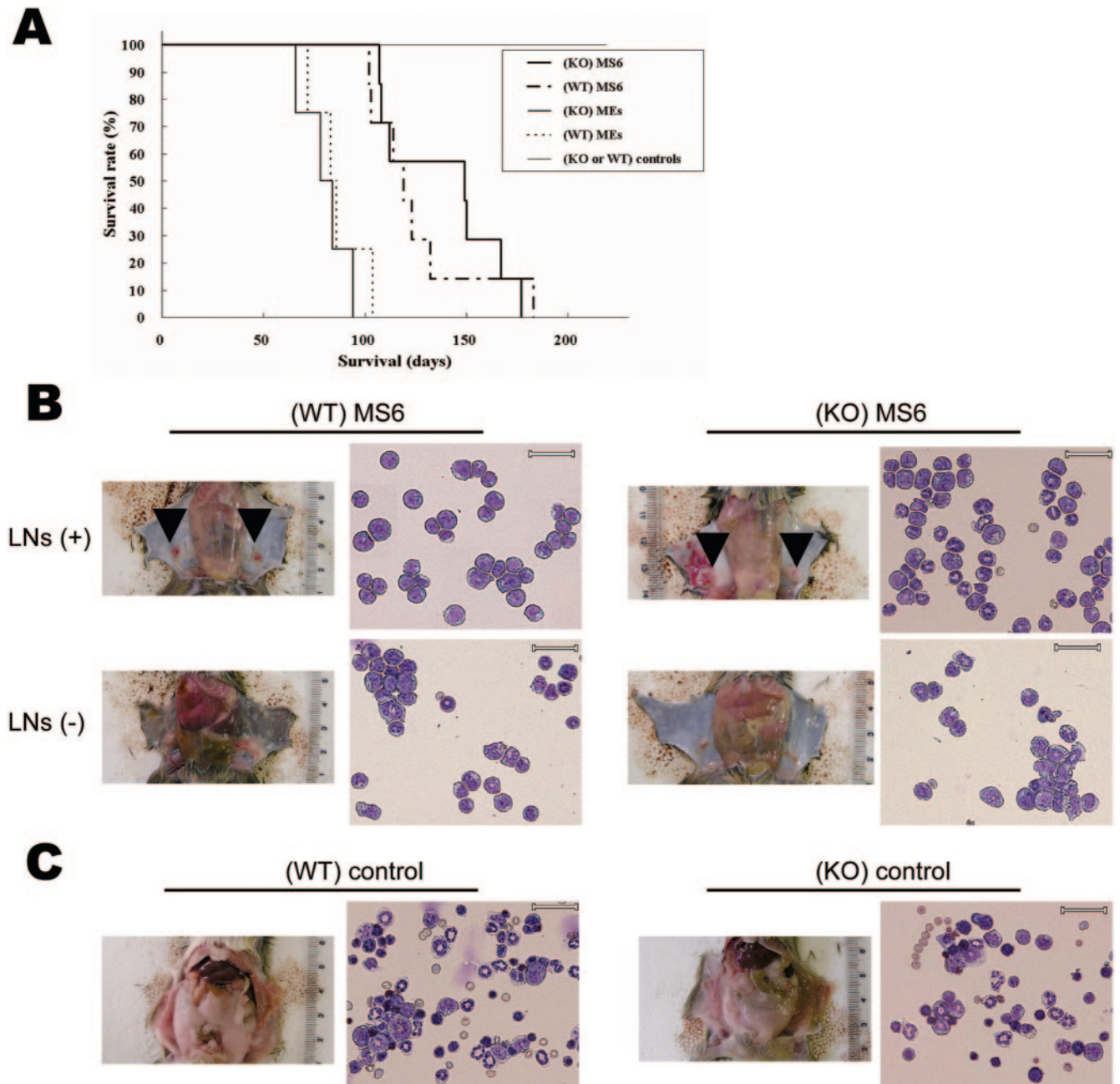
FIG. 8. Leukemogenesis induced by *MLL-SEPT6* (MS6) or MEs using KO or WT hematopoietic progenitors. (A) The survival curves of mice transplanted with (KO or WT) controls (*n* was three pairs), (KO)MS6 or (WT)MS6 (*n* was seven pairs), and (KO)MEs or (WT)MEs (*n* was four pairs). (B and C) Representative macroscopic images and cytospin preparations of bone marrow of the morbid mice transplanted with MS6 (B), having lymphadenopathy [LNs (+)] or not [LNs (−)], and of the control mice (C). The arrowheads show lymphadenopathy. Cytospin preparations were stained with Wright-Giemsa. Bar, 30 μm.

tween Ly-5.1 C57BL/6 and Ly-5.2 129/SvJ mice. No harmful graft-versus-host disease occurred with this strategy. Notably, mice receiving the *Sept6*-deficient cells transduced with *MLL-SEPT6* [(KO)MS6] as well as *MLL-ENL*s [(KO)MEs] died with latencies as long as those receiving the wild-type cells [(WT)MS6 and (WT)MEs], although a slight (but statistically not significant) delay in lethality was seen for (KO)MS6 mice in comparison to (WT)MS6 mice (Fig. 8A and Table 2). Both genotypes of the morbid MS6 and MEs mice exhibited mod-

erate splenomegaly with various degrees of leukocytosis, anemia, and thrombocytopenia, and the MEs mice also had mild hepatomegaly, but no significant differences between KO and WT groups were found (Fig. 8A and B and Table 2). Some of the morbid (WT) and (KO)MS6 mice also exhibited remarkable lymphadenopathy (Fig. 8B). It seemed that the lymphadenopathy was sometimes more severe in the (KO)MS6 mice than in the (WT)MS6 mice, but the tendency could not be statistically analyzed due to an insufficient number of morbid mice with

TABLE 2. Characteristics of the morbid mice transplanted with *Sept6*-deficient or wild-type hematopoietic progenitors after transduction with *MLL-SEPT6* or *MLL-ENL*s

Mouse	Value for characteristic[a]					
	Latency (days)	Liver (g)	Spleen (g)	WBC/μl	Hb (g/dl)	Plt (10⁴/μl)
(WT)MS6 (*n* = 7)	125 ± 28	1.32 ± 0.15	0.37 ± 0.15	101,000 ± 47,000	5.5 ± 3.0	8.4 ± 5.3
(KO)MS6 (*n* = 7)	139 ± 29	1.73 ± 0.66	0.37 ± 0.16	129,000 ± 106,000	3.8 ± 0.9	9.8 ± 4.7
(WT)MEs (*n* = 4)	83 ± 16	2.5 ± 0.39	0.66 ± 0.13	71,000 ± 22,000	5.9 ± 0.7	7.1 ± 2.5
(KO)MEs (*n* = 4)	81 ± 12	2.34 ± 0.90	0.69 ± 0.19	196,000 ± 170,000	6.5 ± 0.7	2.7[b]
(WT) control (*n* = 3)	NA	1.87 ± 0.23	0.08 ± 0.01	13,000 ± 3,000	16.6 ± 0.3	90 ± 6.8
(KO) control (*n* = 3)	NA	1.63 ± 0.22	0.07 ± 0.01	13,000 ± 4,000	18.6 ± 1.0	84 ± 15

[a] Averages with standard deviations are shown. WBC, white blood cells; Hb, hemoglobin; Plt, platelets; NA, not applicable.
[b] Platelet count of only one morbid mouse.

lymphadenopathy. A histopathologic analysis revealed that bone marrow and peripheral blood cells derived from both genotypes of the morbid MS6 and MEs mice had similar morphological features of myeloid hyperplasia consisting predominantly of mature granulocytic elements (Fig. 8B) but that, infrequently, these cells derived from (WT)MS6 mice had morphological features of homogeneous immature lymphoblastic elements (Fig. 8B) which were confirmed by peroxidase staining (data not shown). This difference between myeloid and lymphoid lineages did not correlate with the presence of lymphadenopathy. These elements severely infiltrated the spleen and the liver as observed in MPD mice induced by the MLL-SEPT6 and MLL-ENLs fusion proteins previously reported (40) and the swollen lymph nodes (data not shown).

An immunophenotyping analysis by a fluorescence-activated cell sorter demonstrated that almost all of the bone marrow cells derived from the morbid (WT) and (KO)MS6 mice were negative for Ly-5.1, thus indicating that these cells originated in donor cells transduced with *MLL-SEPT6* (Fig. 9A). This analysis also revealed that those cells derived from MS6 mice without lymphadenopathy were positive for CD11b, frequently positive for Gr-1, and negative for B220, CD3, Sca1, and Ter119 but that those cells from MS6 mice with lymphadenopathy were positive for B220, CD11b, and sometimes Gr-1 but were negative for CD3, Sca1, and Ter119 (Fig. 9A and data not shown). Irrespective of their lineages, these bone marrow cells were positive for c-kit to various extents (data not shown). While the representative (WT)MS6 mouse exhibiting immature lymphoblastic features with mild lymphadenopathy had one major population of Ly-5.1-negative (donor-derived) bone marrow cells expressing both B220 and CD11b, the representative (KO)MS6 mouse exhibiting not immature lymphoblastic features but instead myeloid hyperplasic features with moderate lymphadenopathy had at least two populations of donor-derived bone marrow cells, namely, that expressing CD11b but not B220 and that expressing both B220 and CD11b (Fig. 9B). These results indicated that, irrespective of the presence of lymphadenopathy, the majority of the morbid (WT) and (KO)MS6 mice developed MPD as previously reported (40) or sometimes MPD partly expressing B220 but that few of the (WT)MS6 mice developed acute lymphoblastic leukemia-expressing myelomonocytic surface markers. Therefore, a deficiency in the *MLL*-associated translocation partner *Sept6* thus caused no deteriorative effects of MPD, namely, neither an acceleration of latency nor the development of acute leukemia, in concert with *MLL-SEPT6* itself.

DISCUSSION

The *Sept6*-deficient mice established in the present study provided an intriguing finding that the loss of *Sept6* by a genetically targeted disruption led to no substantial developmental consequences. Indeed, the formation of actin bundles, which interact with septin complexes, including Sept6 (23), and microtubules in primary culture cells of fetal brain did not change. A loss-of-function analysis in vivo highlighted the indispensable roles of tissue-specific septins, including Sept4, reported very recently (18), as well as Sept5 (7). On the other hand, recent studies disclosed that a depletion of ubiquitous septin Sept2 or Sept7 leads to a defect in chromosome congression and segregation (49) or a disorganization of actin bundles (23) in vitro, but still little is understood about the role of ubiquitous septins in vivo. Hence, our first approach to a ubiquitous septin, Sept6, using gene targeting has conferred helpful clues to understanding the whole mammalian septin system in vivo.

We found no changes in expression levels of other septins or relative ratios of the individual septins in *Sept6* deficiency. Previous studies in vitro demonstrated that a 1:1:1 association of SeptX (where X is 1, 2, 4, or 5), Sept6, and Sept7 can occur, but that, in a complex of Sept2/Sept6/Sept7, the Sept6 seemed to be replaceable with Sept11 and probably with Sept8 or Sept10 (24). On the basis of these data in vitro, our results raised the possibility that abundant pools of septins, such as Sept11, Sept8, and/or Sept10, might substitute for Sept6 in septin complexes in vivo. However, most, but not all, of the transcripts of *Sept11* were found to be dispensable in *Sept6*-deficient MEFs in our experiments using RNAi. To address this issue of the replaceable septins, either more-efficient depletion or additional genetic targeting of these septins is further needed. Interestingly, a genetic loss of both *Sept4* and *Sept6*, which are coexpressed in the brain and testis, induced no additional effects compared with the individual loss of each septin gene. These findings might reflect the complex diversity of the septin network in mammals.

The *Sept6* deficiency in mice did not shorten their life spans, nor was there spontaneous malignancy, including leukemia. It is well known that some septin genes, including *SEPT9/Sept9* and *SEPT4/Sept4*, are implicated in oncogenesis, including leukemogenesis (21). However, whether the septins serve as oncogenes or tumor suppressor genes remains controversial (14). Recent studies have demonstrated the profile of SEPT9 isoforms (3, 44, 47, 48) to vary among tumor cell lines, and the
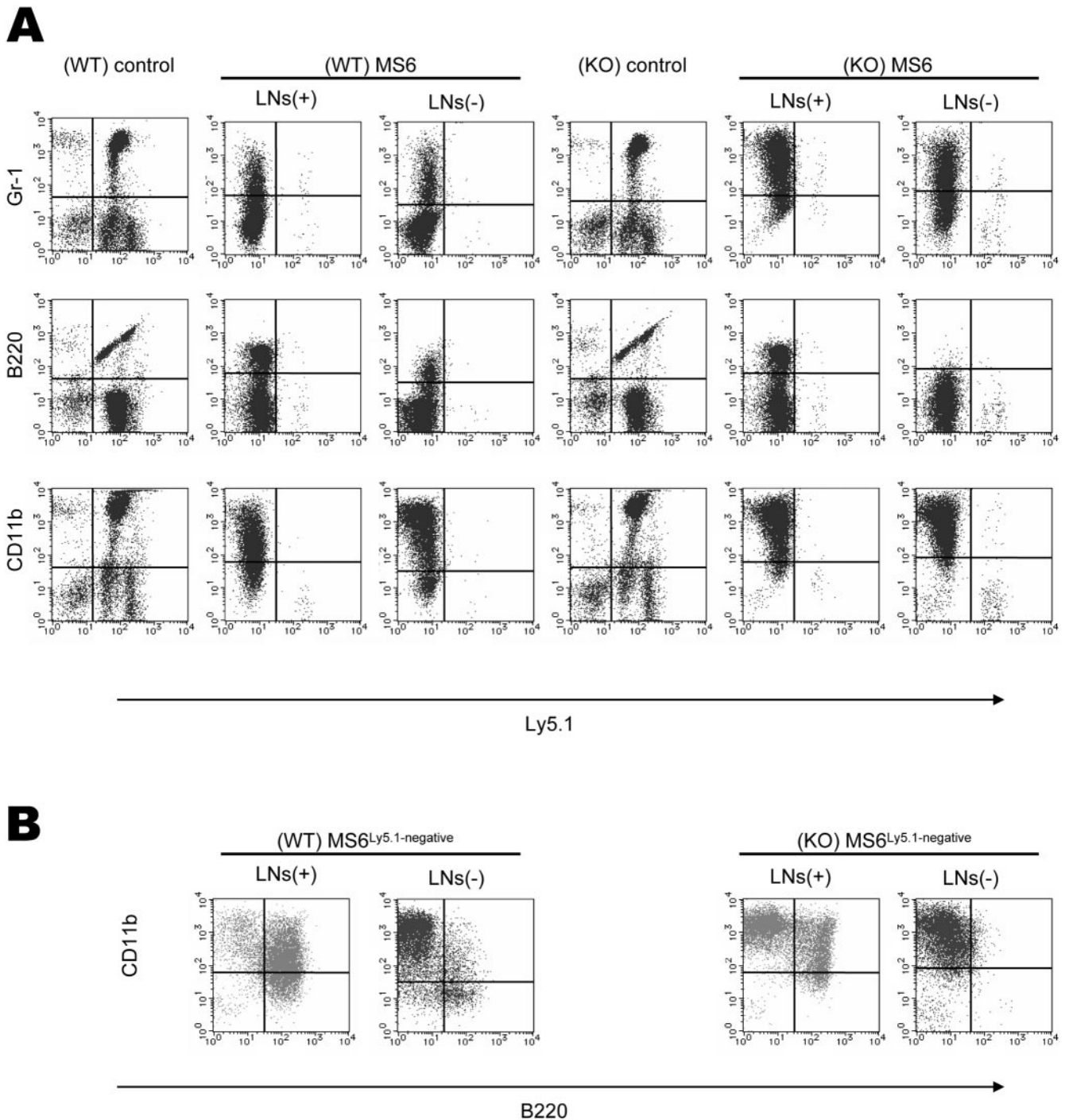
FIG. 9. The immunophenotype of bone marrow cells obtained from representative morbid mice having lymphadenopathy [LNs(+)] or not [LNs(−)], which were transplanted with *MLL-SEPT6* (MS6)-transduced KO or WT progenitors. (A) The expression of lineage surface markers versus Ly-5.1, indicating the origin of cells. The dot plots show each surface antigen labeled with the corresponding phycoerythrin-conjugated monoclonal antibody versus expression of Ly-5.1 labeled with fluorescein isothiocyanate-conjugated monoclonal anti-CD45.1 antibody. (B) Multilineage populations in the donor-derived (Ly-5.1-negative) cells. The dot plots show myeloid antigen (CD11b) labeled with phycoerythrin-conjugated monoclonal antibody versus lymphoid antigen (B220) labeled with allophycocyanin-conjugated monoclonal antibody.

genetic loss of *Sept4* in vivo has been reported to induce no spontaneous malignancy (18). *SEPT6* encodes at least four isoforms which are slightly different from each other in the C terminus (39) by alternative splicing, and the expression pro-

filing of *SEPT6* in several leukemic cell lines demonstrated an expression pattern identical to that in various tissues except for the brain, irrespective of their lineages (39). We recently demonstrated that homo-oligomerization of MLL-SEPT6 through

the SEPT6 moiety in the nucleus is essential for its oncogenic activity in the presence of *Sept6* (40). Meanwhile, whether genetic loss of *SEPT6* resulting from male leukemic cells with *MLL-SEPT6* might contribute to the leukemogenesis in the same way as several septin genes implicated in oncogenesis remains to be elucidated (14). Our observation of *Sept6*-deficient mice indicated that, like *Sept4* deficiency, *Sept6* deficiency alone is not sufficient to induce spontaneous malignancy, including leukemia, but it did not exclude any contribution to the occurrence of malignancy in specific conditions.

Therefore, we have focused on synergistic effects of *Sept6* deficiency in *MLL-SEPT6*- or *MLL-ENL*-mediated leukemogenesis and demonstrated that *Sept6* deficiency did not confer any significant contribution to leukemogenesis. Previous studies have illustrated how a translocation partner as a part of MLL fusion protein contributes to the mechanisms of leukemogenesis by MLL fusion protein (1, 6, 17, 28, 38, 40) rather than the aspect of dysfunction caused by the disruption of the translocation partner. It has been hypothetically proposed that the dysfunction itself might affect the *MLL*-mediated leukemogenesis (1, 17). The establishment of multistep leukemogenesis by MLL fusion protein and secondary genotoxic stress, as we previously reported (40), and which has also been supported by a recent analysis of another MLL fusion gene (57), led us toward another hypothesis regarding the disruption as a candidate for the secondary genotoxic stress. To date, the difficulty in developing an appropriate model system in vivo, mainly due to phenotypes induced by a deficiency of translocation partner genes (4, 7, 9, 19, 31, 53, 56), made it impossible to clarify the roles of this dysfunction in leukemogenesis. We have overcome such a difficulty by using hematopoietic progenitors derived from mice with normal hematopoiesis despite deficiency in *Sept6*. The present strategy is capable of reproducing not only male cases with genomic loss of *SEPT6* but also mimicking some female cases, because it is theoretically considered that *SEPT6* is either expressed or not expressed in an original leukemic clone. Our findings, based on both in vitro myeloid immortalization assay and in vivo leukemogenesis assay, suggest that the dysfunction associated with deficiency of a translocation partner such as *SEPT6* confers little contribution to the phenotype of the disease per se and does not serve as a secondary genotoxic stress. These results are not incompatible with the implications in the inducible *Mll-Enl* mice (11). In addition, the same in vitro and in vivo assays with a transduction of *MLL-ENL* did not reveal any significant differences, thus implying that a *Sept6* deficiency may not tend to induce acute leukemia. It is noteworthy that *MLL-SEPT6* can induce MPD with expression of lymphoid surface antigen and acute lymphoblastic leukemia with expression of myeloid surface antigen in our in vivo leukemogenesis assay using *Sept6*-deficient and wild-type progenitors, respectively. Since some studies of *MLL*-mediated leukemogenesis using the chimeric background of C57BL/6 and 129Sv/J reproduced lymphoid (5, 8) as well as myeloid malignancy, *MLL* fusion genes may develop hematological malignancies of multiple lineages in various strains of mice.

In conclusion, this study has demonstrated for the first time that genetic loss of a fusion partner of *MLL* does not contribute to the leukemogenesis associated with the 11q23 translocation. The normal development and life spans of *Sept6*-deficient mice suggest the robustness of the mammalian septin system. Future studies using mice lacking multiple septin genes should help to clarify the diverse aspects of the septin biology, while also providing novel insights into their implications in human malignancies.

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