identifies HSCs (Figure 6A (a)). Conversely, the genes down-regulated in EVI-1 high leukemias were strongly correlated with a gene set that is highly expressed in progenitors (Figure 6A (b)). We also found another gene set containing long term HSC-enriched genes was significantly correlated to genes up-regulated in EVI-1 high leukemias (Figure 6B).

Additionally, we extracted the expression data of murine normal KSL cells and GMPs, and applied GSEA using two gene sets, which represent EVI-1 high and low MLL-rearranged leukemias, respectively (Supplemental Table S2). As expected, GSEA showed a significant correlation of genes enriched in EVI-1 high leukemias with those in KSL cells (Figure 6C), although the reverse correlation between genes representing EVI-1 low leukemias and GMPs was not significant (Supplemental Figure S3). These results are consistent with our findings that Evi-1 is up-regulated by MLL oncproteins in HSCs, and suggest that EVI-1 high MLL-rearranged leukemias are derived from HSCs.

GSEA also demonstrated strong correlation between the upregulated genes in AMLs with aberrant cytoplasmic localization of nucleophosmin (NPMc+ AMLs) and those in EVI-1 high cases of MLL-rearranged AMLs (Figure 6D (a,b), Supplemental Table S1).

Discussion

Despite the established role of Evi-1 in leukemogenesis, the molecular mechanisms for Evi-1 activation in leukemic cells have been poorly understood. Recently, several clinical studies revealed a positive correlation between EVI-1 overexpression and MLL rearrangements in AML patients [14,15]. Furthermore, we have previously shown that Evi-1 deletion in MLL-ENL-immortalized cells caused distinct reduction of their colony-forming capacity, suggesting a functional interaction between Evi-1 and MLL oncoproteins [3]. In this study, we demonstrated that MLL oncoproteins activate transcription of Evi-1 gene in hematopoietic cells. Importantly, this MLL-mediated Evi-1 activation occurs exclusively in HSCs but not in committed myeloid progenitors.

Mds1 is located approximately 140 kb and 500 kb upstream of the first exon of Evi-1 in human and mouse genome respectively; therefore, the expression of Evi-1a and Mds1-Evi-1 are regulated by different promoters. Nevertheless, MLL oncoproteins bind to the promoters of both Evi-1a and Mds1-Evi-1, and activate their transcription (Figure 3A, 3B, 3D). These findings are consistent with the clinical observations which showed that expression of both EVI-1a and Mds1-EVI-1 are frequently enhanced in MLL-rearranged leukemia [15]. Although some evidence suggests that Evi-1a is oncogenic and Mds1-Evi-1 contributes to tumor suppression [12,41], several reports showed that the activating retroviral insertions in Mds1/Evi-1 locus was involved in long term dominance in hematopoiesis, suggesting a similar function of Mds1/Evi-1 and Evi-1 [42,43]. The specific roles of Evi-1a and Mds1-Evi-1 in MLL-rearranged leukemia should be clarified in future studies.

We found a genomic region, 146 bp in length, which is thought to be crucial for Evi-1a activation by MLL-ENL (Figure 3C). The ChIP assay revealed that DNA-binding of MLL-ENL was enriched near this genomic region, also suggesting the importance of this region for MLL-ENL to regulate Evi-1 (Figure 3D). To identify precise genomic DNA sequence to which MLL binds, we performed an electrophoresis mobility shift assay (EMSA) using three probes from the genomic

region with purified His-tagged protein containing MLL-ENL CXXC domain. In this setting, we observed a sequence-specific shifted band using one of three probes. However, it was not supershifted by adding His-antibodies (data not shown). These results may be due to some technical difficulties in protein-antibody binding in native condition. Therefore, it remains to be determined whether this fragment region is sufficient for MLL-ENL to bind to DNA or some other regions are also involved.

Several studies showed that both HSCs and committed myeloid progenitor cells could be transformed by retroviral transduction of MLL oncoproteins, and they could develop immunophenotypically similar AML [40]. Of note, we found that Evi-1 is activated by MLL-ENL or MLL-AF9 exclusively when it is transduced into KSL cells (Figure 5A, 5B, 5C). Previously, *Chen et al.* also reported that HSCs from MLL-AF9 knockin mice express high levels of Evi-1 [44]. Together with our results, cellular milieu provided by HSCs seems necessary for Evi-1 up-regulation by MLL oncoproteins. One possibility that accounts for these phenomena is that MLL-ENL can bind to the promoter region of Evi-1 only in HSCs. However, we found that MLL-ENL bound to the promoter regions of Evi-1a and Mds1-Evi-1 even in the leukemic cells of low Evi-1 expression by ChIP assay (data not shown). Therefore, binding to Evi-1 promoter alone is not sufficient for activating transcription of Evi-1. Otherwise, the methylation status of Evi-1 promoter may affect the expression of Evi-1. To address this issue, we analyzed the methylation status of Evi-1 promoter in MLL-ENL transformed cells using bisulfite DNA sequencing. However, the methylation status at CpGs was largely low in the Evi-1 promoter, regardless of Evi-1 expression levels (Supplemental Figure S4). Based on these findings, it is unlikely that the expression of Evi-1 is shut down in progenitors by DNA methylation in the promoter. Another possibility is that, undifferentiated HSCs lose some key factors that contribute to activation of Evi-1 irreversibly along with hematopoietic cell differentiation. Significant in this regard is that menin is required for some targets to be activated by MLL oncoproteins [39]. In the current case, however, menin itself is not likely to be a key factor in

MLL-mediated activation of Evi-1, because the MBM is not required for the activation of Evi-1 promoter in luciferase assay (Figure 4C).

In our reporter assay, Jurkat cells provided a condition sufficient for MLL-ENL to activate Evi-1 promoter. However, only an exogenous Evi-1 promoter was activated by MLL-ENL in Jurkat cells, considering that the endogenous Evi-1 expression was not concurrently activated (data not shown). Cellular milieu provided by HSCs seems necessary for the activation of endogenous Evi-1 promoter in the end.

It has been shown that both wild-type MLL and Evi-1 are crucial for proliferation and maintenance of HSCs [2,3,45,46]. Because wild-type MLL and MLL oncoproteins share some transcriptional targets such as HoxA9, we assessed the transcriptional activity of MLL on Evi-1 promoter. Our luciferase reporter assays showed no significant transcriptional activities of wild-type MLL on Evi-1 promoter, suggesting that the wild-type MLL by itself is not sufficient for the activation of Evi-1 (Figure 4A). Given that Evi-1 expression decreases along with normal hematopoietic cell differentiation in spite of the preserved expression of MLL, physiological expression level of wild-type MLL may not be able to activate the expression of Evi-1 by itself.

GSEA analysis using gene expression data of AML samples revealed that EVI-1 high MLL-rearranged leukemias exhibit HSC-like signatures, while genes involved in more differentiated hematopoietic progenitors are enriched in EVI-1 low MLL-rearranged leukemias. Considering that HSCs are more efficient targets for leukemogenic transformation by MLL oncoproteins [40,44], up-regulated Evi-1 may contribute to the propagation of leukemia stem cells (LSCs) in MLL-rearranged leukemias. In support of this concept is our finding that Evi-1 deletion reduces the clonogenic activity most severely in KSL-derived cells (Figure 5E).

In addition to the enrichment of HSC genes in Evi-1 high leukemias, GSEA also revealed that the gene-expression signature of NPMc+ AMLs resembles that of Evi-1 high MLL-rearranged AMLs. Because NPMc+ AMLs display a specific gene expression profile dominated by a HSC

molecular signature [47], the results probably indicates that HSC genes are enriched in Evi-1 high leukemias. Alternatively, Evi-1 overexpression and cytoplasmic NPM may cooperatively contribute to leukemia development, and this possibility should be investigated in the future.

Given that Evi-1 plays an essential role in proliferation and maintenance of HSCs in normal hematopoiesis [3], it is tempting to speculate that activated expression of Evi-1 by MLL oncoproteins results in the propagation of LSCs, which is associated with therapeutic resistance and disease progression. In support of this is a recent report that the adverse effect of EVI1 positivity on prognosis was clinically observed in AML patients with MLL rearrangement [48]. We showed that MLL-ENL transformed cells with up-regulated Evi-1 expression are derived from HSCs (Figure 5A, B), and that their clonogenic potential is highly dependent on Evi-1 (Figure 5E). Collectively, our findings suggest that Evi-1 becomes an attractive therapeutic target in the treatment of Evi-1 high MLL-rearranged leukemias. Additionally, putative key factors collaborating with MLL oncoproteins in undifferentiated hematopoietic cells at the Evi-1 promoter remains unknown. Some clues may be found from the clinical experience that high expression of Evi-1 is frequently observed in leukemias with another MLL-rearrangement, MLL-AF6 [14]. MLL-AF6 aberrantly recruits AF4 and ENL family proteins to its transcriptional target promoters to cause sustained target gene expression, as well as MLL-ENL and MLL-AF9 [49]. These functions common in major MLL-oncoproteins may be involved in activation of Evi-1. Further investigation would clarify how Evi-1 is activated not only in MLL-rearraged leukemias, but also in other leukemias or normal hematopoiesis.

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Authorship

Contribution: S.Arai, S.Goyama, and M.Kurokawa designed the experiments and the study; S.Arai, S.Goyama, M.Nakagawa, Y.Imai, and M.Kurokawa wrote the manuscript; S.Arai, A.Yoshimi, and S.Goyama performed experiments and collected and analyzed data; and M.Shimabe and M.Ichikawa provided important reagents and reviewed the manuscript.

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Correspondence: Mineo Kurokawa, Department of Hematology and Oncology, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan; e-mail: kurokawa-tky@umin.ac.jp

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Figure Legends

Figure 1. Evi-1 is up-regulated in myeloid progenitors immortalized by MLL oncoproteins.

(A) Murine c-Kit$^+$ BM progenitors were retrovirally transduced with pMXs-neo-MLL-ENL, pMXs-neo-MLL-AF9, pMXs-neo-E2A-HLF or pMYs-HoxA9-ires-Meis1. The expression level of Evi-1 in immortalized cells from the third to fourth round of serial replating in semisolid medium was quantified relative to BM MNCs using real-time PCR. Data are shown as mean +/- s.d. *P < 0.05.

(B) Murine c-Kit$^+$ BM progenitors were retrovirally transduced with leukemia oncogenes. Four types of myeloid leukemia genes cloned into MIG were retrovirally transduced into c-Kit$^+$ BM progenitors. Forty-eight hours after the initiation of retroviral transduction, GFP positive cells were isolated, and the expression level of Evi-1 was quantified relative to BM MNCs. Data are shown as mean +/- s.d. *P < 0.05.

(C) Immortalization of c-Kit$^+$ BM progenitors by MLL-ENL-ER is dependent on the presence of 4-OHT. Graph indicates the number of colonies with s.d., generated from 10^4 of pMXs-neo-MLL-ENL-ER transduced BM cells in the presence (white) or absence (black) of 1 μM 4-OHT at each round after retroviral transduction. The number of G418 resistant colonies obtained by transduction of MLL-ENL-ER into 10^4 of BM cells in the presence of 4-OHT is shown in the first round (gray).

(D) The expression level of HoxA9 or Evi-1 in MLL-ENL-ER transformed cells cultured with or without 1 μM 4-OHT for seventy-two hours. The averages of relative expression ratio in 4-OHT (-) cells (black) to 4-OHT (+) cells (white) are shown with s.d. *P < 0.05.

Figure 2. Evi-1 is frequently up-regulated in leukemic cells transformed by MLL fusion protein *in vivo*

(A) Survival curves of sublethally irradiated recipients transplanted with BM cells transduced with either MSCV-neo-MLL-ENL (N=11, blue), or MSCV-cMyc-ires-bcl2 (N=12, red) are shown.

(B) The expression levels of Evi-1 and HoxA9 in leukemic cells transformed by MLL fusion genes

(MLL-ENL (N=11, black dots), or cMyc/bcl2 (N=12, white dots) are indicated. The expression levels of Evi-1 and HoxA9 relative to BM MNCs are shown as dots.

(C) (Left) Gene structures of Evi-1a and Mds1-Evi-1 and positions of primer sets for quantitative RT-PCR are shown. The exons, start codons, and primers are depicted in boxes, standup arrows, and white triangles, respectively. Sequences of primers are presented in Supplemental Methods. (Right) The expression levels of Evi-1a (white) and Mds1-Evi-1 (black) in leukemic cells from four mice with high Evi-1 expression relative to BM MNCs are shown with s.d..

Figure 3. MLL-ENL binds to the promoter regions of both Evi-1a and Mds1-Evi-1.

(A) (Left) Five segments of the Evi-1a promoter were inserted upstream of luciferase cassette of the pGL4-Luc to generate luciferase reporter constructs. Arrows, gray boxes, white boxes, solid lines, and dashed lines represent TSS, exons, highly conserved regions between human and mice, DNA sequences cloned into pGL4-Luc, connection of each DNA segment and luciferase gene, respectively. (Right) Graph shows relative luciferase activity of Evi-1a promoter reporter constructs in Jurkat cell lysates with transiently transfected MLL-ENL (gray), compared to that without MLL-ENL (white). The data were shown as mean +/- s.d. from three independent experiments.

(B) (Left) Three segments of Mds1-Evi-1 promoter were inserted upstream of the luciferase cassette of pGL4-Luc. (Right) Graph shows relative luciferase activity of Mds1/Evi-1 promoter reporter constructs in Jurkat cell lysates with transiently transfected MLL-ENL (gray), compared to that without MLL-ENL (white). The data were shown as mean +/- s.d. from three independent experiments.

(C) (Left) Serial deletions of pGL4-E2265 were constructed. The pGL4-E1957 through pGL4-E1404 constructs are named according to the base length between the N-terminal residue of inserted fragments and TSS of Evi1a on murine genome. The DNA fragment inserted in pGL4-E2265 corresponds to the genomic region between 2265 bp and 1296 bp upstream of the TSS of Evi-1a. (Right) The experiments were performed as described in (A). The data were representative of three

independent experiments, and shown as mean +/- s.d. *P < 0.05.

(D) The enrichment of MLL-ENL to the promoter of Evi-1a and Mds1-Evi-1 was detected by ChIP. Genomic DNA fragments were immunoprecipitated with anti-Flag antibody (lane 2) or normal mouse IgG (lane 3) from formaldehyde-fixed leukemic cells transduced with Flag-MLL-ENL. DNA fragments containing the indicated promoter regions of Evi-1a or Mds1-Evi-1 were amplified by PCR. The position of the amplified regions in Evi-1a or Mds1-Evi-1 promoters is shown in Figure 3A, 3B, or 3C. For controls, each genomic region was amplified from 1% of purified DNA after formaldehyde fixation and sonication (Input, lane 1). Shown are representative data of four experiments.

Figure 4. Reporter assays using MLL-ENL deletion mutants and other oncoproteins.

(A) Transactivation of pGL4-E2265 induced by MLL-AF9, AML1-ETO, PML-RARA, E2A-HLF, HoxA9/Meis1 or wild-type MLL (WT MLL) is shown. Data are presented as a relative fold increase in the mean luciferase activity with s.d. after adjustment for β-galactosidase activity.

(B) Schematics represent the composition of MLL-ENL deletion mutants. Numbers indicated denote the amino acid positions in wild-type MLL and ENL. Positions of CXXC DNA binding motif (red), AT-hooks DNA binding motif (blue), and MBM (yellow) are shown in the schematics of intact MLL-ENL.

(C) Transactivation of pGL4-E2265 induced by intact MLL-ENL or its deletion mutants is shown. Data are presented as described in (A). *P < 0.05 versus MLL-ENL.

Figure 5. MLL-ENL up-regulates Evi-1 expression exclusively in HSCs.

(A) Defined hematopoietic populations were transduced with pMXs-neo-MLL-ENL or pMYs-HoxA9-ires-Meis1, and replated in semisolid medium. The expression level of Evi-1 (gray, scale on the left) and HoxA9 (white, scale on the right) in MLL-ENL-transformed cells from each population (KSL MLL-ENL, CMP MLL-ENL, or GMP MLL-ENL lane) and HoxA9/Meis1-transformed KSL cells (KSL HoxA9/Meis1 lane) was quantified relative to BM MNCs. Data are shown as means

+/- s.d. from two independent experiments. *p < 0.05 versus CMP MLL-ENL, GMP MLL-ENL, or KSL HoxA9/Meis1, respectively.

(B) KSL cells and GMPs were transduced with MIG (KSL/GMP GFP lanes), MIG-MLL-ENL (KSL/GMP MLL-ENL lanes), or MIG-MLL-AF9 (KSL MLL-AF9 lane). After forty-eight hours of transduction, the expression level of Evi-1 in GFP positive cells was quantified relative to BM MNCs using real time PCR, and was compared to that of freshly isolated KSL cells (KSL lane), CMPs, and GMPs. Data are shown as means +/- s.d. from three independent experiments. *p < 0.05.

(C) BM progenitors from 5-FU treated mice were transduced with MIG (GFP KSL lane), MIG-MLL-ENL (MLL-ENL KSL lane), or MIG-MLL-AF9 (MLL-AF9 KSL lane). After thirty-six hours of transduction, the expression level of Evi-1 in GFP positive cells isolated from KSL population was quantified relative to BM MNCs, and was compared to that of freshly isolated KSL cells (KSL lane). Data are shown as means +/- s.d. from three independent experiments. *P < 0.05.

(D) BM KSL cells, CMPs, and GMPs were isolated from $Evi1^{+}$ (white) and $Evi1^{+/-}$ (gray) mice, and transformed by MLL-ENL in the same way as myeloid progenitor transformation assay. Bar graph shows mean colony numbers +/- s.d. in the third round of serial replating from two independent experiments. *P < 0.05

(E) BM KSL cells, CMPs, and GMPs were isolated from Evi-$1^{f/-}$ mice. After they were transformed by MLL-ENL as in (D), they were transduced with either pGCDNsam-eGFP or pGCDNsam-eGFP-iCre. GFP positive cells were isolated and colony forming activity after Evi-1 deletion was assessed in the next round of plating. Bar graph shows colony count ratio of iCre-GFP-transduced cells, compared to GFP-transduced cells. Data are shown as means +/- s.d. from two independent experiments. *p < 0.05.

Figure 6. Gene expression profiles of Evi-1 high leukemias with MLL rearrangement revealed a stem cell-like character.

(A) A strong correlation of upregulated genes in EVI-1 high leukemias with a gene set representing

HSCs and that of down-regulated genes in EVI-1 high leukemias with a gene set typical of progenitors are shown. GSEA of gene expression in human EVI-1 high AMLs with MLL rearrangement (n = 5) as compared to EVI-1 low AMLs with MLL rearrangement (n = 8) using functional gene sets (C2). (a, b) GSEA enrichment plots of the selected gene sets. (a) Upregulated genes in human HSCs [50]. (b) Upregulated genes in hematopoietic late progenitors [32].

The corresponding heat maps represent expression of the leading 30 genes of respective gene sets. Up-regulated and down-regulated genes are presented as red and blue, respectively.

(B) GSEA plots show that expression of genes representing long term HSCs is enriched in EVI-1 high AMLs with MLL rearrangement as compared to EVI-1 low AMLs with MLL rearrangement. NES = 1.33. FDR q-val = 0.141.

(C) GSEA of gene expression in five normal KSL samples and four normal GMP samples using a gene set representing EVI-1 high leukemia with MLL rearrangement. GSEA plots show that expression of genes representing EVI-1 high leukemia is enriched in KSL cells as compared to GMPs. NES = 1.46. FDR q-val = 0.025.

(D) A strong correlation of upregulated genes in EVI-1 high leukemias with those in NPMc+ leukemias. GSEA of gene expression in human EVI-1 high AMLs with MLL rearrangement (n = 5) as compared to EVI-1 low AMLs with MLL rearrangement (n = 8) using functional gene sets (C2). (a, b) GSEA enrichment plots of the selected gene sets. (a) Upregulated genes in NPMc+ leukemias [47]. (b) Downregulated genes in NPMc+ leukemias [47].

Figure 1

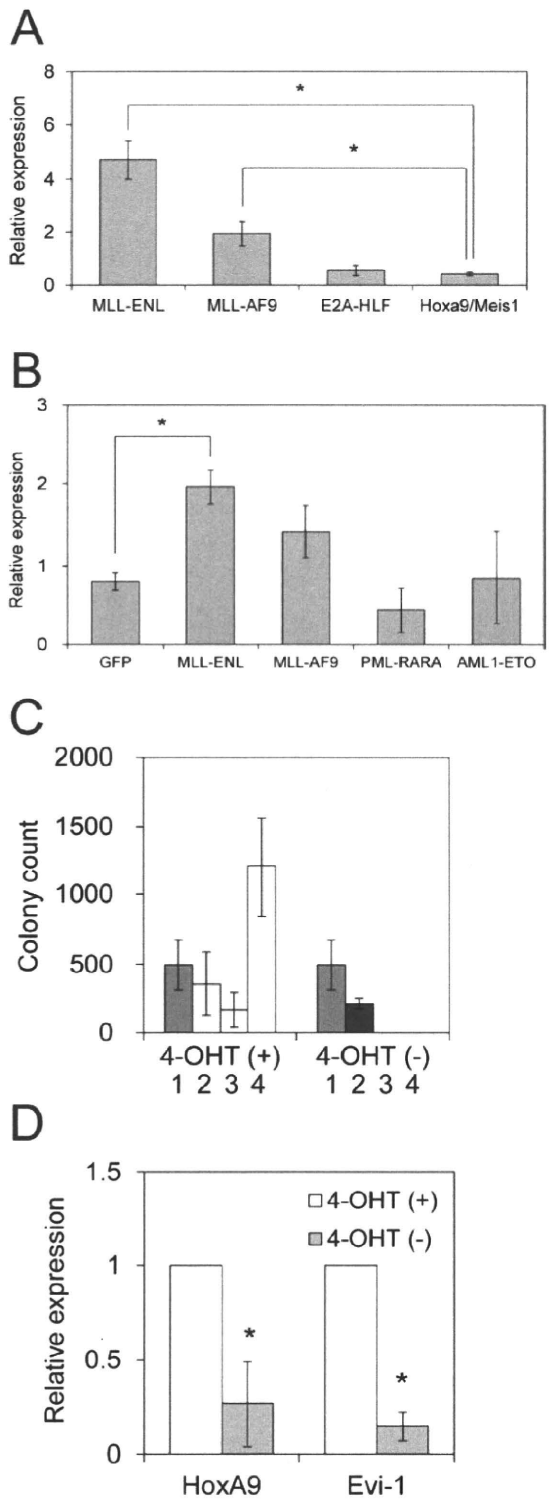

Figure 2

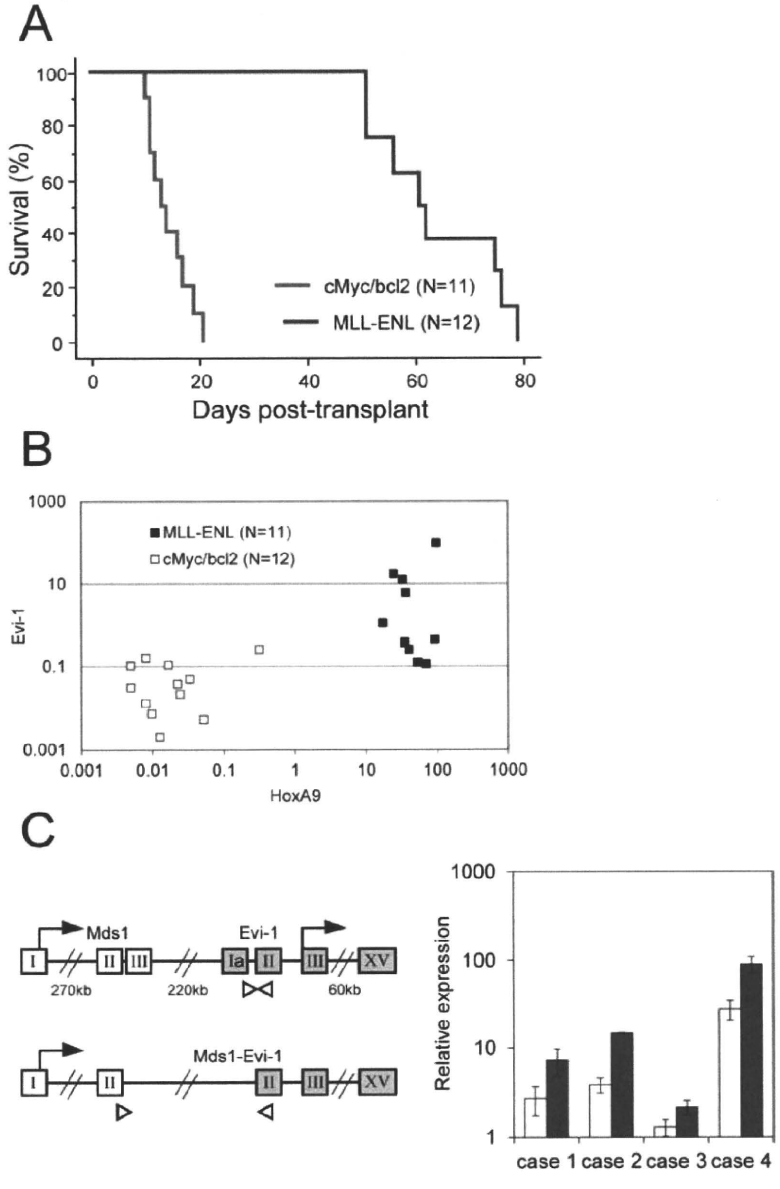

Figure 3