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# Evi-1 Is a Critical Regulator for Hematopoietic Stem Cells and Transformed Leukemic Cells

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## SUMMARY

**Evi-1 has been recognized as one of the dominant oncogenes associated with murine and human myeloid leukemia. Here, we show that hematopoietic stem cells (HSCs) in *Evi-1*-deficient embryos are severely reduced in number with defective proliferative and repopulating capacity. Selective ablation of Evi-1 in Tie2$^+$ cells mimics Evi-1 deficiency, suggesting that Evi-1 function is required in Tie2$^+$ hematopoietic stem/progenitors. Conditional deletion of Evi-1 in the adult hematopoietic system revealed that *Evi-1*-deficient bone marrow HSCs cannot maintain hematopoiesis and lose their repopulating ability. In contrast, Evi-1 is dispensable for blood cell lineage commitment. *Evi-1*$^{+/-}$ mice exhibit the intermediate phenotype for HSC activity, suggesting a gene dosage requirement for Evi-1. We further demonstrate that disruption of Evi-1 in transformed leukemic cells leads to significant loss of their proliferative activity both in vitro and in vivo. Thus, Evi-1 is a common and critical regulator essential for proliferation of embryonic/adult HSCs and transformed leukemic cells.**

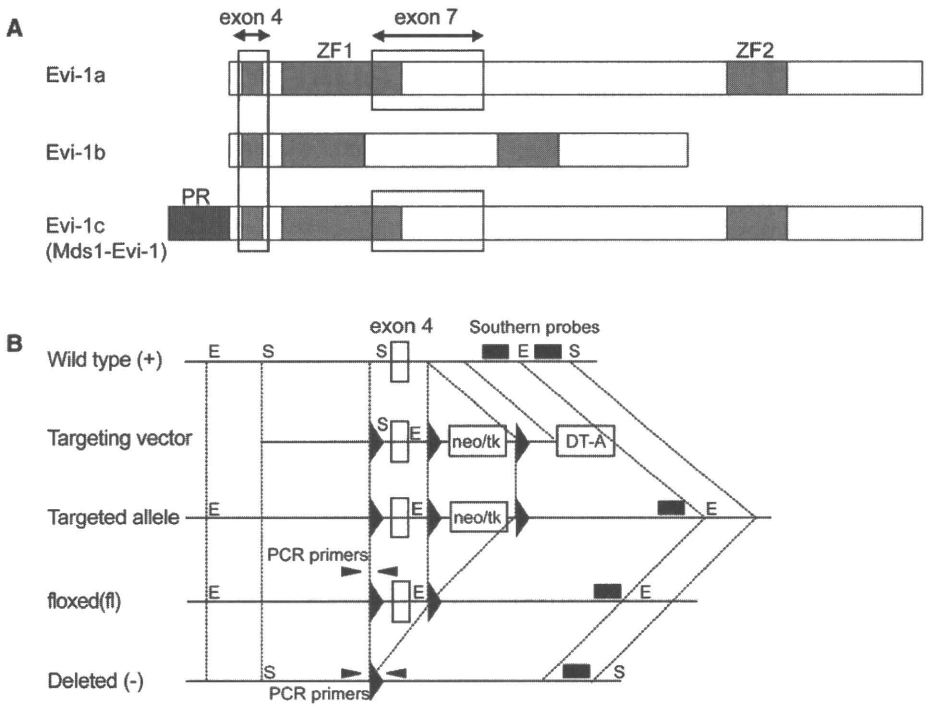
## INTRODUCTION

The ecotropic viral integration site-1 (*Evi-1*) gene was first identified as a common locus of retroviral integration in myeloid tumors in AKXD mice (Mucenski et al., 1988). In humans, *Evi-1* is located on chromosome 3q26, and rearrangements on chromosome 3q26 often activate *Evi-1* expression in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (Ogawa et al., 1996; Suzukawa et al., 1994). Although these rearrangements are infrequent in AML, they are of remarkable prognostic value. Patients with these karyotypes are characterized by the elevated platelet count and lack of response to antileukemic therapy (Pintado et al., 1985). Elevated *Evi-1* expression occurs with high frequency in AML patients without 3q26 abnormalities and is also associated with unfavorable outcomes (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Valk et al., 2004). Thus, Evi-1 is one of the key factors that predict poor survival in leukemia patients.

Evi-1 is a member of the SET/PR domain family of transcription factors, and it contains a total of ten zinc finger motifs organized in two discrete domains, located at the N terminus and toward the C terminus, comprising seven (ZF1) and three (ZF2) repeats, respectively, which have distinct DNA-binding specificities (Delwel et al., 1993; Perkins et al., 1991). The alternative forms generated from the *Evi-1* gene include at least three distinct proteins: Evi-1a, Evi-1b, and Evi-1c (MDS1-Evi-1) (Fears et al., 1996; Hirai, 1999; Figure 1A). Structurally in Evi-1c, a conserved PR domain is located at the N terminus of the Evi-1a. Thus, PR-containing (Evi-1c) and PR-absent (Evi-1a) forms of Evi-1 exist, both of which are expressed in several developing and adult tissues. Although related, functional differences between Evi-1a and Evi-1c have been documented (Nitta et al., 2005; Sood et al., 1999). Another naturally occurring splice variant, designated Evi-1b, has been described, which lacks 324 internal amino acids, including zinc fingers 6 and 7 of ZF1 (Bordereaux et al., 1990). The biological function of Evi-1b is not known thus far.

Previous studies revealed that Evi-1 possesses diverse functions as an oncoprotein. Evi-1 antagonizes growth-inhibitory effects of transforming growth factor-β (TGF-β) by interacting with Smad3 (Kurokawa et al., 1998); protects cells from stress-induced cell death by inhibiting c-Jun N-terminal kinase (JNK) (Kurokawa et al., 2000); increases the expression of endogenous c-Jun and c-*fos*, resulting in activation of AP-1 (Tanaka et al., 1994); and blocks granulocytic differentiation of myeloid cells (Morishita et al., 1992). In addition, Evi-1 interacts with corepressor CtBP, and this interaction contributes to Evi-1-mediated repression of TGF-β signaling (Izutsu et al., 2001). Furthermore, mouse models for Evi-1 overexpression have been established using bone marrow infection and transplantation. These studies showed that activation of Evi-1 in hematopoietic cells leads to myeloid dysplasia while the development of full-blown leukemia requires additional genetic events (Buonamici et al., 2004; Jin et al., 2007).

The mutant mice with disrupted *Evi-1* have been generated (Hoyt et al., 1997). The homozygous embryos tend to exhibit widespread hypocellularity, hemorrhaging, and disruption in the development of paraxial mesenchyme, resulting in the death in utero at approximately embryonic day 10.5 (E10.5). However, these mice carry a targeted deletion of exon 7, resulting in an isoform-specific null for the longer Evi-1 transcripts (Evi-1a and Evi-1c), whereas the truncated form of Evi-1 (Evi-1b) remains unaltered (Figure 1A). Therefore, the remaining expression of




| | wild | Evi-1(+/-) | Evi-1(-/-) | total |
|---|---|---|---|---|
| E9.5 | 2 | 6 | 4 | 12 |
| E10.5 | 8 | 9 | 4 | 21 |
| E11.5 | 4 | 7 | 4 | 15 |
| E12.5 | 5 | 5 | 2 | 12 |
| E13.5 | 8 | 12 | 1 | 21 |
| E14.5 | 11 | 32 | 5 | 48 |
| E15.5 | 5 | 20 | 3 | 28 |
| E16.5 | 6 | 12 | 2 | 20 |
| E18.5 | 4 | 12 | 1* | 17 |
| Pups | 14 | 30 | 0 | 44 |

* Degenerated

**Figure 1. Generation of Evi-1 Mutant Mice**

(A) Schematic representation of Evi-1 isoforms. ZF1, zinc finger domain-1; ZF2, zinc finger domain-2; PR, PR domain.

(B) Schematic representation of gene targeting of the *Evi-1* gene. E, *Eco*RV; S, *Sph*I; neo/tk, PGK-neo/HSV-thymidine kinase positive selection cassette; DT-A, diphtheria toxin A chain negative selection cassette.

(C) Southern blot analysis of *Sph*I- (KO, knockout mice) or *Eco*RV- (cKO, conditional knockout mice) digested genomic tail DNA showing the predicted fragment-length polymorphism.

(D) Expression of Evi-1 and β-actin protein in *Evi-1*$^{+/+}$ and *Evi-1*$^{-/-}$ embryos. Lysates were prepared from mouse embryo fibroblast (MEF) cells from E14.5 embryos and were analyzed by western blotting using anti-Evi-1 antibody (C50E12). *Nonspecific band.

(E) Genotypes of litters obtained by intercrossing *Evi-1*$^{+/-}$.

(F) Gross appearance of embryos at E16.5. The *Evi-1*$^{-/-}$ embryo showed hemorrhaging with the yolk sac exhibiting severe anemia and defective large-vessel development.

Evi-1b might contribute to the complex phenotype observed in these mice.

In contrast to the established role of Evi-1 in leukemia development, the role of Evi-1 in normal hematopoiesis has been poorly understood. Recently, it was reported that *Evi-1* is predominantly expressed in both embryonic and adult hematopoietic stem cells (HSCs), and development of definitive HSCs in the para-aortic splanchnopleural (P-Sp) region was severely impaired in *Evi-1* mutant embryos (Yuasa et al., 2005). In addition, several studies have shown that retroviral vector integration at the *Evi-1* locus can be related to long-term in vivo clonal dominance without necessarily resulting in malignant transformation in mice (Kustikova et al., 2005), nonhuman primates (Calmels et al., 2005), and humans (Ott et al., 2006). These findings suggest that Evi-1 has a role in the regulation of HSCs; however, further functional analysis of Evi-1 has been hampered because of the embryonic lethality of the *Evi-1* mutant mice.

To further investigate the physiological role of Evi-1 in hematopoiesis, we created mutant mice in which exon 4 of the *Evi-1* gene can be deleted by the expression of Cre recombinase, as well as mice in which the same region was completely deleted. Using these mice, we here show that Evi-1 regulates proliferative capacity of HSCs in a dose-dependent manner both during embryogenesis and in adults. We further demonstrate that Evi-1 is also required for proliferation of transformed leukemic cells, and we provide candidate target genes of Evi-1 shared in HSCs and leukemic cells.

## RESULTS

### Generation of Evi-1 Mutant Mice

The mutant mice of Evi-1 were previously generated by disrupting exon 7 of the *Evi-1* gene (Hoyt et al., 1997); however, these mice still retain the Evi-1b, and they lack the longer isoforms of Evi-1 (Evi-1a and Evi-1c). Therefore, we targeted exon 4 of the *Evi-1* gene that is conserved in all of the known isoforms (Figure 1A) and created mutant mice carrying deleted (Evi-1$^{-}$) or loxP-flanked (Evi-1$^{f}$) alleles (Figure 1B). Germline transmission was confirmed by Southern blot analysis of tail DNA (Figure 1C). Disruption of exon 4 of Evi-1 was confirmed by PCR and northern blotting using RNA in embryos and by sequencing the amplified PCR product of *Evi-1*$^{-/-}$ embryo (Figures S1, S2, S3, and S4 available online). Immunoblotting of *Evi-1*$^{-/-}$ embryo using three different antibodies confirmed that all three isoforms of Evi-1 protein are absent in homozygous embryos (Figures 1D and S5).

### C57BL/6 Mice Lacking Three Isoforms of Evi-1 Died between E13.5 and E16.5

First, we analyzed our conventional Evi-1 knockout mice. Newly generated *Evi-1*$^{+/-}$ mice were born and were fertile, exhibiting no morphological abnormalities. In contrast, *Evi-1*$^{-/-}$ pups were not seen. To identify the stage of embryonic development at which the Evi-1 mutation is lethal, E9.5–18.5 embryos were analyzed for their genotype (Figure 1E). Surprisingly, our *Evi-1*$^{-/-}$ mice were alive with normal appearance at E10.5, when the previously developed *Evi-1* mutant mice died. After E13.5, many *Evi-1*$^{-/-}$ embryos exhibited hemorrhaging and subcutaneous edema, with the yolk sacs showing severe anemia and defective large-vessel development (Figure 1F). Some *Evi-1*$^{-/-}$ embryos survived as long as E16.5 with comparable body size to wild-type littermates, but no viable *Evi-1*$^{-/-}$ fetuses were detected after E18.5. Thus, our *Evi-1*$^{-/-}$ mice lacking three isoforms of *Evi-1* survived slightly longer than the prior *Evi-1* mutant mice, but they died between E13.5 and E16.5.

### Decreased Hematopoietic Stem/Progenitor Cells in *Evi-1*-Deficient Embryos

To assess the role of Evi-1 in hematopoiesis, we first examined the capacity of definitive hematopoiesis in *Evi-1*$^{-/-}$ embryos, which occurs in the P-Sp region at E9.5, using an in vitro P-Sp explant/OP9 stromal cell coculture system (Goyama et al., 2004). Consistent with the findings obtained for the prior *Evi-1* mutant mice (Yuasa et al., 2005), the number of HSCs in the *Evi-1*$^{-/-}$ P-Sp cultures was significantly decreased in comparison to the number of HSCs in the wild-type controls (Figure 2A). We next assessed fetal liver (FL) hematopoiesis in *Evi-1*$^{+/-}$ and *Evi-1*$^{-/-}$ mice. The *Evi-1*$^{-/-}$ FL contained nucleated erythrocytes, granulocytes, and B-lymphocytes and was morphologically indistinguishable from the wild-type FL (Figures 2B and S6). However, the population of lineage$^{-}$, c-Kit$^{+}$, Sca-1$^{+}$ cells (LSK cells) or lineage$^{-}$, c-Kit$^{+}$, CD34$^{+}$ cells, which enriches hematopoietic stem/progenitor cells, was severely reduced in *Evi-1*$^{-/-}$ mice. The *Evi-1*$^{+/-}$ FL showed an intermediated phenotype (Figure 2B). The total number of colony-forming cells (CFCs), especially the number of mixed colonies, in *Evi-1*$^{-/-}$ FL cells was also severely decreased (Figure 2C). Together, these results suggest that Evi-1 deletion causes severe reduction in hematopoietic stem/progenitor cells but is compatible with differentiation of progenitors once they are formed.

### Defective HSC Activity in *Evi-1*-Deficient Embryos

To determine whether *Evi-1*$^{-/-}$ embryos possess functional HSCs, we tested the ability of *Evi-1*$^{-/-}$ FL cells to reconstitute the hematopoietic system of recipient mice. FL cells ($2 \times 10^6$ cells) from E14.5 wild-type or *Evi-1*$^{-/-}$ embryos were injected into lethally irradiated (9.5 Gy) recipient mice. All recipients receiving wild-type FL cells survived and remained healthy for at least 1 month; however, no recipients receiving donor cells from *Evi-1*$^{-/-}$ embryos survived beyond 2 weeks (Figure 2D). We further performed the competitive reconstitution assay. FL cells ($1 \times 10^6$ cells) from wild-type or *Evi-1*$^{-/-}$ (Ly5.2) embryos were injected into lethally irradiated recipient mice (Ly5.1) together with $2 \times 10^5$ competitor bone marrow (BM) cells from wild-type mice (Ly5.1). *Evi-1*$^{-/-}$ cells were not detected at all in the recipient mice either 4 or 16 weeks after transplantation. In contrast, wild-type FL cells reconstituted hematopoiesis of the recipients to a larger extent than competitor cells (Figures 2E and S7). Thus, Evi-1 is required for HSC activity to reconstitute the hematopoietic system in recipients.

### Deletion of Evi-1 in Tie2$^{+}$ Cells Mimics Evi-1 Deficiency

Next, we crossed Evi-1$^{f/f}$ mice with Tie2-Cre mice (Li et al., 2006) to selectively disrupt Evi-1 function in Tie2$^{+}$ endothelial and hematopoietic stem/progenitor cells. Most of Tie2-Cre$^{+}$;Evi-1$^{f/f}$ embryos died around E13.5 to E16.5 with hemorrhage and/or subcutaneous edema, as *Evi-1*-deficient embryos do (Figures 3A and 3B). A few Tie2-Cre$^{+}$;Evi-1$^{f/f}$ mice were born alive and grew to adults (Figure 3A), but BM of these mice primarily

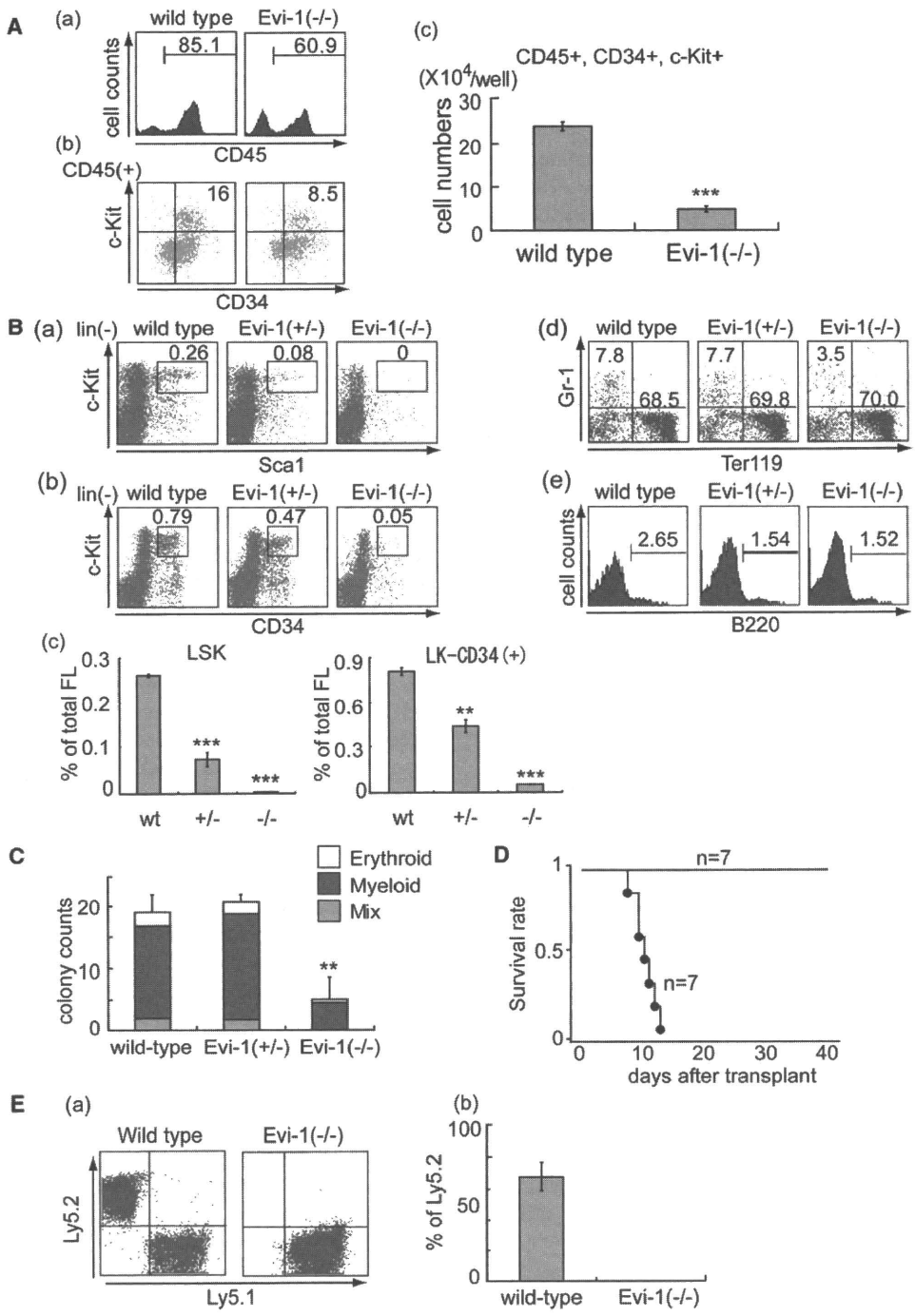



**Figure 2. Decreased Hematopoietic Stem/Progenitor Cells in *Evi-1*$^{-/-}$ Embryos**

(A) Flow cytometric profiles of the cells from the P-Sp cultures harvested after 5 days in culture. (Aa) $CD45^+$ cells in P-Sp culture were gated. (Ab) $CD45^+$ cells were examined for the expression of CD34 and c-Kit. (Ac) The numbers of $CD45^+$, $CD34^+$, c-$Kit^+$ hematopoietic stem/progenitor cells in P-Sp culture derived from wild-type or *Evi-1*$^{-/-}$ mice. n = 3. ***p = 0.0001.

(B) Flow cytometric profiles of wild-type, *Evi-1*$^{+/-}$, and *Evi-1*$^{-/-}$ littermate FL at E14.5. (Ba and Bb) Population of hematopoietic stem/progenitor cells. (Bc) Quantification of flow cytometric analysis. Data are mean ± SD from four mice. P values were calculated as compared with wild-type embryos. **p = 0.0043. ***p < 0.0001. (Bd) Population of myeloid and erythroid cells. (Be) Population of B-lymphocytes. Numbers of each panel represent percentages of the gated population in whole FL cells.

(C) CFCs in wild-type, *Evi-1*$^{+/-}$, and *Evi-1*$^{-/-}$ littermate FL cells. p values were calculated as compared with wild-type embryos. n = 4. **p = 0.0009.

(D) Survival curve of lethally irradiated recipients receiving either wild-type or *Evi-1*$^{-/-}$ FL cells.

(E) Competitive repopulation assays. (Ea) Flow cytometric analysis of peripheral blood cells at 4 weeks after transplantation show extensive contribution of wild-type FL cells (Ly5.2), but no detectable contribution of *Evi-1*$^{-/-}$ cells. (Eb) Cumulative data of donor contribution in peripheral blood of recipients 4 weeks after transplantation. n = 6. Data are mean ± SD.

A

| | | | | Tie2-Cre | | | |
|---|---|---|---|---|---|---|---|
| | Evi-1$^{+/+}$ | Evi-1$^{f/+}$ | Evi-1$^{f/f}$ | Evi-1$^{+/+}$ | Evi-1$^{f/+}$ | Evi-1$^{f/f}$ | total |
| E11.5 | 0 | 2 | 0 | 2 | 4 | 1 | 9 |
| E12.5 | 2 | 3 | 1 | 5 | 3 | 3 | 17 |
| E13.5 | 6 | 2 | 3 | 3 | 5 | 2 | 21 |
| E14.5 | 1 | 3 | 0 | 0 | 3 | 2 | 9 |
| E15.5 | 2 | 6 | 2 | 2 | 1 | 1 | 14 |
| E16.5 | 1 | 3 | 2 | 3 | 2 | 1 | 12 |
| E17.5 | 1 | 5 | 3 | 4 | 3 | 1 | 17 |
| E18.5 | 1 | 2 | 0 | 2 | 2 | 0 | 7 |
| Pups | 22 | 25 | 15 | 19 | 15 | 3 | 96 |

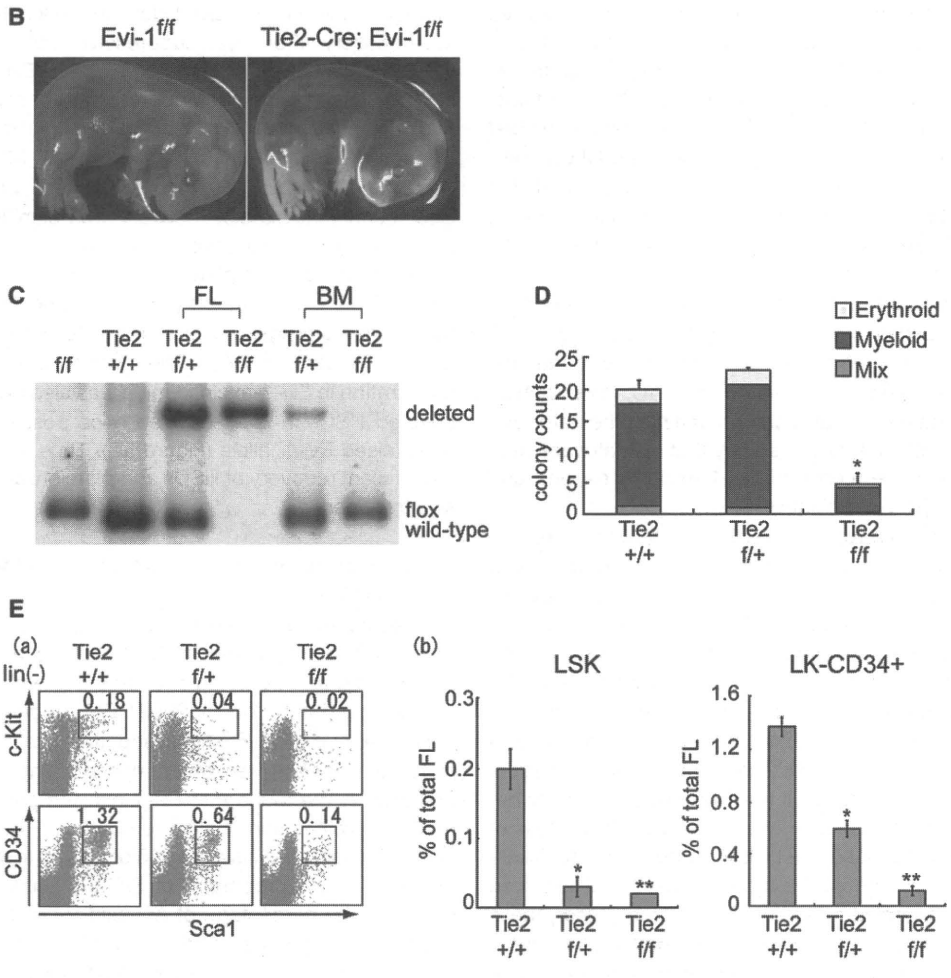



**Figure 3. Selective Ablation of Evi-1 in Tie2$^+$ Cells**

(A) Genotypes of litters obtained by intercrossing Tie2-Cre;Evi-1$^{f/+}$ and Evi-1$^{f/+}$ mice.

(B) Gross appearance of E16.5 fetuses. Tie2-Cre;Evi-1$^{f/f}$ embryo are edematic and show hemorrhaging.

(C) Southern blot genotyping of FL cells (E14.5) and BM cells (1 month after birth). Evi-1 was excised efficiently in E14.5 fetal liver cells of Tie2-Cre;Evi-1$^{f/f}$ embryos, but BM cells of the surviving Tie2-Cre;Evi-1$^{f/f}$ mice exclusively harbored nonexcised Evi-1 allele.

(D) CFCs in Tie2-Cre;*Evi-1*$^{+/+}$, Tie2-Cre;Evi-1$^{f/+}$, Tie2-Cre;Evi-1$^{f/f}$ littermate FL cells. p values were calculated as compared with Tie2-Cre;*Evi-1*$^{+/+}$ embryos. n = 2. *p = 0.0108.

(E) (Ea) Population of hematopoietic stem/progenitors in Tie2-Cre;*Evi-1*$^{+/+}$, Tie2-Cre;Evi-1$^{f/+}$, Tie2-Cre;Evi-1$^{f/f}$ littermate FL cells (E14.5). Numbers of each panel represent percentages of the gated population in whole FL cells. (Eb) Quantification of flow cytometric analysis. Data are mean ± SD from two mice. p values were calculated as compared with Tie2-Cre;*Evi-1*$^{+/+}$ embryos. (LSK) *p = 0.0077. **p = 0.0041. (CD34$^+$-LK) *p = 0.0113. **p = 0.002.

consisted of cells that retain the nonexcised Evi-1$^f$ allele (Figure 3C), indicating that hematopoiesis originated from cells that had escaped Cre-mediated excision. HSCs and CFCs were severely decreased in the FL of Tie2-Cre$^+$;Evi-1$^{f/f}$ embryos (Figures 3D, 3E, and S8). Thus, selective ablation of Evi-1 in Tie2$^+$ cells virtually reproduced all of the phenotypes of *Evi-1*$^{-/-}$ mice,

suggesting that Evi-1 function is required in Tie2$^+$ cells for early development and proliferation of HSCs.

### Evi-1 Is Essential for Proliferation/Maintenance of Adult BM HSCs

To assess a role of Evi-1 in adult hematopoiesis, we then crossed Evi-1$^{f/f}$ mice with Mx-Cre transgenic mice, in which a high level of Cre recombinase is produced by treatment with the interferon inducer pI-pC, leading to recombination in hematopoietic cells of all lineages. Injection of pI-pC did not cause significant differences in white blood cell (WBC) counts or hemoglobin levels among the Mx-Cre;Evi-1$^{f/f}$ (*Evi-1*-excised), Mx-Cre;*Evi-1*$^{+/+}$, and Evi-1$^{f/f}$ mice, and the platelet counts in the *Evi-1*-excised mice modestly declined compared with those in control mice 4 weeks after pI-pC injection (Figure 4A). We next examined the effect of Evi-1 deletion in various hematopoietic cell populations. By 4 weeks after pI-pC injection, *Evi-1*-excised mice exhibited significant decrease in the frequency of HSCs and CFCs compared with control mice (Figures 4B and S9). In contrast, the frequencies of mature myeloid cells (Gr-1$^+$ or Mac-1$^+$ cells), B-lymphocytes (B220$^+$ cells), and T-lymphocytes (CD3$^+$, CD4$^+$, or CD8$^+$ cells) did not appear to be affected (Figure S10) in spite of the efficient excision of *Evi-1* alleles in a majority of these cells (Figure 4C, upper panel). Interestingly, by 12 weeks after pI-pC injection, cells in all hematopoietic populations contained primarily the nonexcised Evi-1$^f$ allele (Figure 4C, lower panel). These results indicate that HSCs can not maintain hematopoiesis in the absence of Evi-1, and it is likely that a small fraction of HSCs that escaped Cre-mediated Evi-1 excision expand and predominate over *Evi-1*-deficient HSCs.

We further assessed a role of Evi-1 for the hematopoietic reconstitution in BM progenitors using Cre-encoding retroviruses. BM progenitors were harvested from wild-type (Ly5.2) or Evi-1$^{f/f}$ (Ly5.2) mice and then infected with GFP (control) or Cre-GFP expressing retrovirus. Evi-1 is completely deleted only in the GFP$^+$, Cre-transduced fraction of Evi-1$^{f/f}$ BM progenitors (Figure 4D). We then injected these progenitors into sublethally irradiated (6.5 Gy) recipient mice (Ly5.1) and assessed the frequency of GFP$^+$ fraction in donor (Ly5.2) cells 2 weeks after transplantation. The infection efficiency among all three groups was similar before transplantation. Remarkably, we found a profound loss of GFP$^+$ cells in Cre-transduced Evi-1$^{f/f}$ BM progenitors after transplantation, although a modest Cre-induced stasis was observed in wild-type BM progenitors (Figure 4D). Taken together, these results suggest that Evi-1 is indispensable for proliferation/maintenance of adult BM HSCs, yet it is dispensable for differentiation into myeloid, erythroid, and lymphoid lines.

### Haploinsufficiency of Evi-1 Perturbs Adult HSC Homeostasis

We then analyzed hematopoiesis of *Evi-1*$^{+/-}$ mice that survive through adulthood without overt abnormalities. Mature blood cell counts in peripheral blood of *Evi-1*$^{+/-}$ mice were comparable to those of wild-type mice (Figure S11). However, *Evi-1*$^{+/-}$ mice had a lower abundance of LSK and CD34$^-$LSK cells, which include short-term and long-term HSCs (Figure 5A). Therefore, we evaluated HSC function by testing the ability of *Evi-1*$^{+/-}$ BM cells to competitively repopulate the adult hematopoietic compartment in lethally irradiated recipient mice. BM cells from *Evi-1*$^{+/-}$ (Ly5.2) mice or wild-type littermates (Ly5.2) were transplanted into lethally irradiated Ly5.1 mice together with wild-type competitor Ly5.1 BM cells at a ratio of 1:1 or 1:10 of test cells to competitor cells. The average contribution of *Evi-1*$^{+/-}$ donor BM cells to total peripheral blood cells was lower than that of wild-type controls at 4 and 16 weeks after transplantation (Figure 5B). These results, together with the observation that *Evi-1*$^{+/-}$ FL had a reduced number of HSCs, suggest a gene dosage requirement for Evi-1 in the regulation of HSCs.

### Delayed Hematopoietic Recovery after 5FU Treatment in *Evi-1*$^{+/-}$ and *Evi-1*-Excised Mice

To assess the effect of Evi-1 dose in the kinetics of hematopoietic recovery after myelosuppressive treatment, we administered a single dose of 5FU to wild-type and *Evi-1*$^{+/-}$ mice and serially followed peripheral blood counts. As shown in Figure 5C, *Evi-1*$^{+/-}$ mice showed a significant delay in the platelet recovery. We performed the same experiment using Mx-Cre;Evi-1$^{f/f}$, Mx-Cre;*Evi-1*$^{+/+}$, and Evi-1$^{f/f}$ mice 7 days after pI-pC injection and also found that platelet recovery was significantly delayed in *Evi-1*-excised mice (Figure 5C). These results indicate that Evi-1 has a specific role in platelet formation. Alternatively, perturbation of HSC compartment in *Evi-1*$^{+/-}$ and *Evi-1*-excised mice may have a stronger influence on platelet recovery than on the recovery of other lineages. The recovery of HSCs after 5FU administration in *Evi-1*-excised mice was also delayed, and the recovered HSCs in *Evi-1*-excised mice possessed primarily the nonexcised Evi-1$^f$ allele (Figure 5D). Thus, Evi-1 is required for the efficient recovery of HSCs and platelets after myelosuppressive treatment.

### Consequences of Evi-1 Deletion on Cell Viability and Proliferation

To characterize the defective proliferation capacity of hematopoietic stem/progenitors in *Evi-1*-excised mice, we assessed proliferation in vitro using a culture system that expands BM HSCs (Zhang and Lodish, 2005). BM progenitors derived from control (Evi-1$^{f/f}$) or *Evi-1*-excised (Mx-Cre;Evi-1$^{f/f}$) mice were cultured in serum-free medium containing SCF, TPO, IGF2, and FGF-1. We found that *Evi-1*-excised progenitors exhibited reduced proliferation relative to controls (Figure 6A). Cell-cycle and apoptosis analysis at day 15 of culture revealed a significant decrease in the proportion of S/G2/M phase cells and an increase in the percentage of Annexin-V-positive cells for *Evi-1*-excised cells (Figures S12 and S13). Accelerated apoptosis and impaired cell cycling will explain the attenuated proliferation of *Evi-1*-excised cells in this culture condition. We then excised Evi-1 in vitro using Cre-encoding retroviruses. BM progenitors were harvested from wild-type or Evi-1$^{f/f}$ mice and infected with GFP (control) or Cre-GFP-expressing retroviruses. These progenitors were cultured in the condition as described above and assessed the frequency of GFP$^+$ fraction every 5 days. This approach also revealed reduced proliferation of GFP$^+$ cells in Cre-transduced Evi-1$^{f/f}$ (*Evi-1*-excised) progenitors (Figures 6B and S14). Taken together, we concluded that Evi-1 is required for sustaining cytokine-dependent proliferation of hematopoietic stem/progenitors.

We next assessed in vivo cell-cycle distribution of FL or BM cells derived from control (Evi-1$^{f/f}$) or *Evi-1*-excised (Mx-Cre;

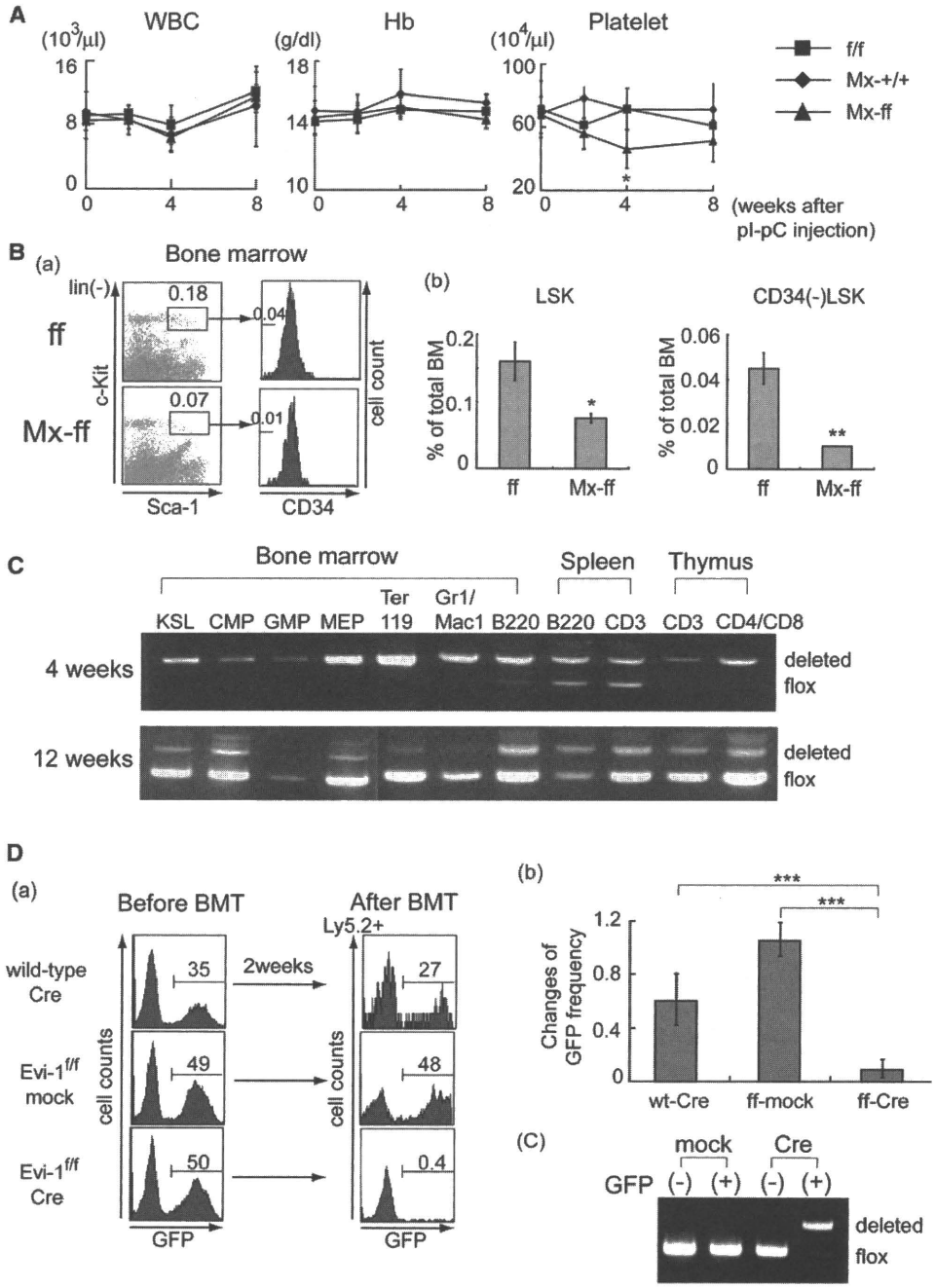



**Figure 4. Disruption of Evi-1 in Adult Hematopoiesis**

(A) Peripheral blood cell counts of Mx-Cre;*Evi-1*$^{+/+}$, Evi-1$^{f/f}$, and Mx-Cre;Evi-1$^{f/f}$ mice injected with pI-pC on weeks 0, 4, and 8. Results are shown as mean ± SD from three (controls) to eight (Mx-Cre;Evi-1$^{f/f}$) mice. p values were calculated as compared with controls. *p = 0.0004.

(B) Flow cytometric analysis of Evi-1$^{f/f}$ or Mx-Cre;Evi-1$^{f/f}$ mice 4 weeks after pI-pC injection. (Ba) Population of HSCs in the BM. Numbers of each panel represent percentages of the gated population in whole BM. (Bb) Quantification of flow cytometric analysis. n = 3. Data are mean ± SD. *p = 0.015. **p = 0.0031.

(C) PCR genotyping of cells from various hematopoietic populations of Mx-Cre;Evi-1$^{ff}$ mice 4 weeks (upper panel) and 12 weeks (lower panel) after pI-pC injection. Efficient excision of *Evi-1* was observed at 4 weeks, but cells in all populations at 12 weeks primarily harbored nonexcised *Evi-1* allele.

(D) (Da) Infection efficiencies before transplantation were compatible among three groups, but GFP$^+$ fraction in Cre-transduced Evi-1$^{ff}$ BM cells (*Evi-1*-excised cells) disappeared after transplantation. (Db) Relative ratios of GFP$^+$ fraction in Ly5.2 donor cells after transplantation compared with those before transplantation. Data are shown as mean ± SD from three independent experiments. Each group contains six mice. ***p < 0.0001. (Dc) PCR analysis of flanked *Evi-1* alleles in control and Cre-transduced BM cells. The flanked *Evi-1* alleles became undetectable in GFP$^+$, Cre-transduced Evi-1$^{f/f}$ BM cells.

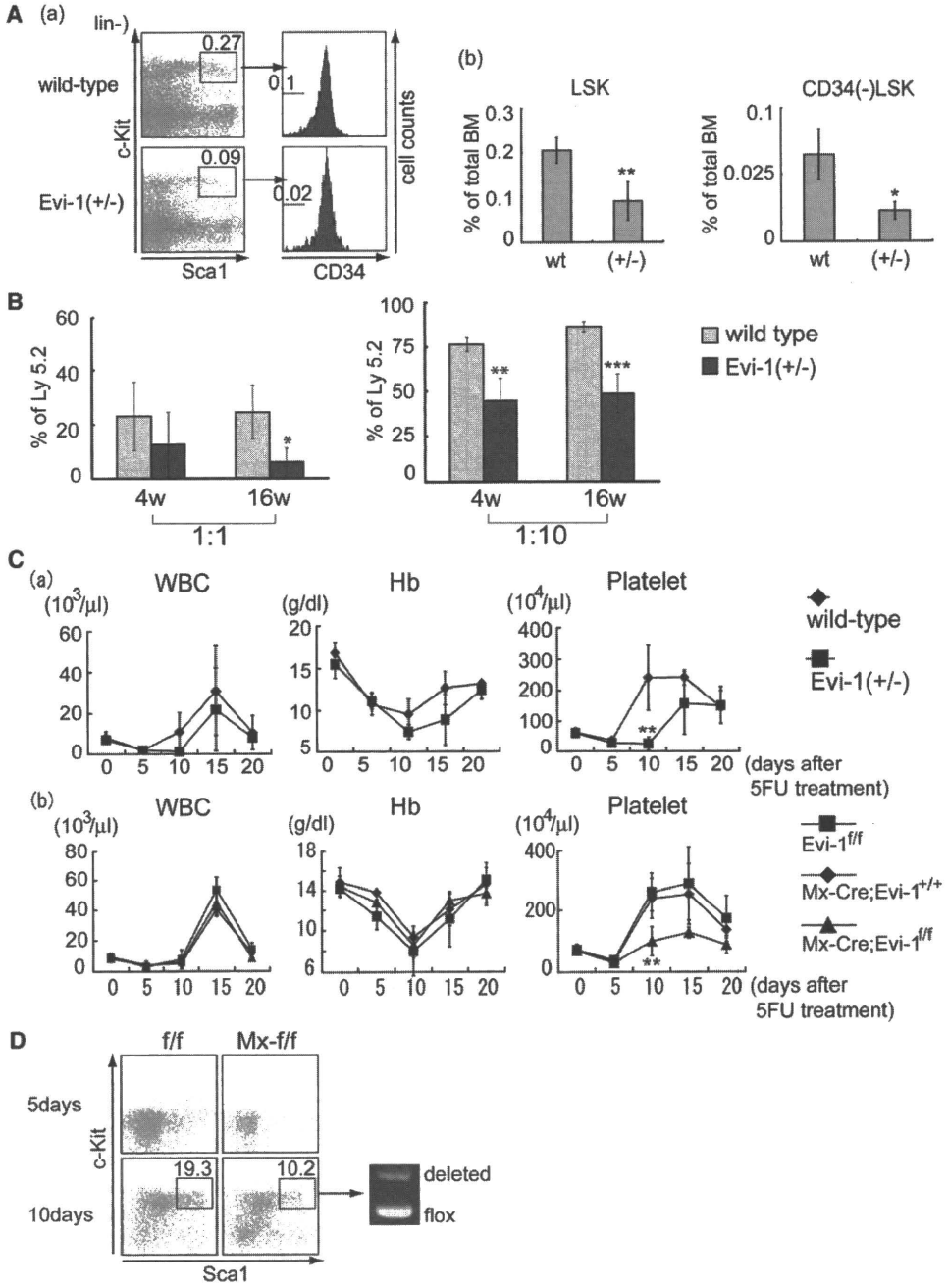



**Figure 5. Defective HSC Activity and Delayed Hematopoietic Recovery in *Evi-1*$^{+/-}$ and *Evi-1*-Excised Mice**

(A) (Aa) *Evi-1*$^{+/-}$ BM exhibited significant decrease in the size of the short-term (ST)-HSC (lin$^-$, Sca-1$^+$, c-Kit$^+$) and long-term (LT)-HSC (CD34$^-$, lin$^-$, Sca-1$^+$, c-Kit$^+$) compartments. (Ab) Quantification of flow cytometric analysis on the size of ST- and LT-HSCs of wild-type and *Evi-1*$^{+/-}$ mice. n = 4. Data are mean ± SD *p = 0.0056. **p = 0.0041.

(B) Competitive transplantation reveals diminished repopulating ability of *Evi-1*$^{+/-}$ BM. n = 4. Data are shown as mean ± SD. 1:1, *p = 0.03242. 1:10, **p = 0.0028, ***p = 0.0005.

(C) Peripheral blood cell recovery after 5FU treatment was followed by serial peripheral blood count monitoring of mice. (Ca) Wild-type and *Evi-1*$^{+/-}$ mice. n = 4. **p = 0.0091. (Cb) Mx-Cre;*Evi-1*$^{+/+}$, Evi-1$^{f/f}$, and Mx-Cre;Evi-1$^{f/f}$ mice 7 days after induced deletion of the floxed genes. p values were calculated as compared with controls. n = 4. **p = 0.0012.

(D) HSC recovery after 5FU treatment of Evi-1$^{f/f}$ and Mx-Cre;Evi-1$^{f/f}$ mice. HSC recovery was modestly delayed in *Evi-1*-excised mice, and re-emerging HSCs had primarily harbored nonexcised *Evi-1* allele. Similar results were obtained in three independent experiments.

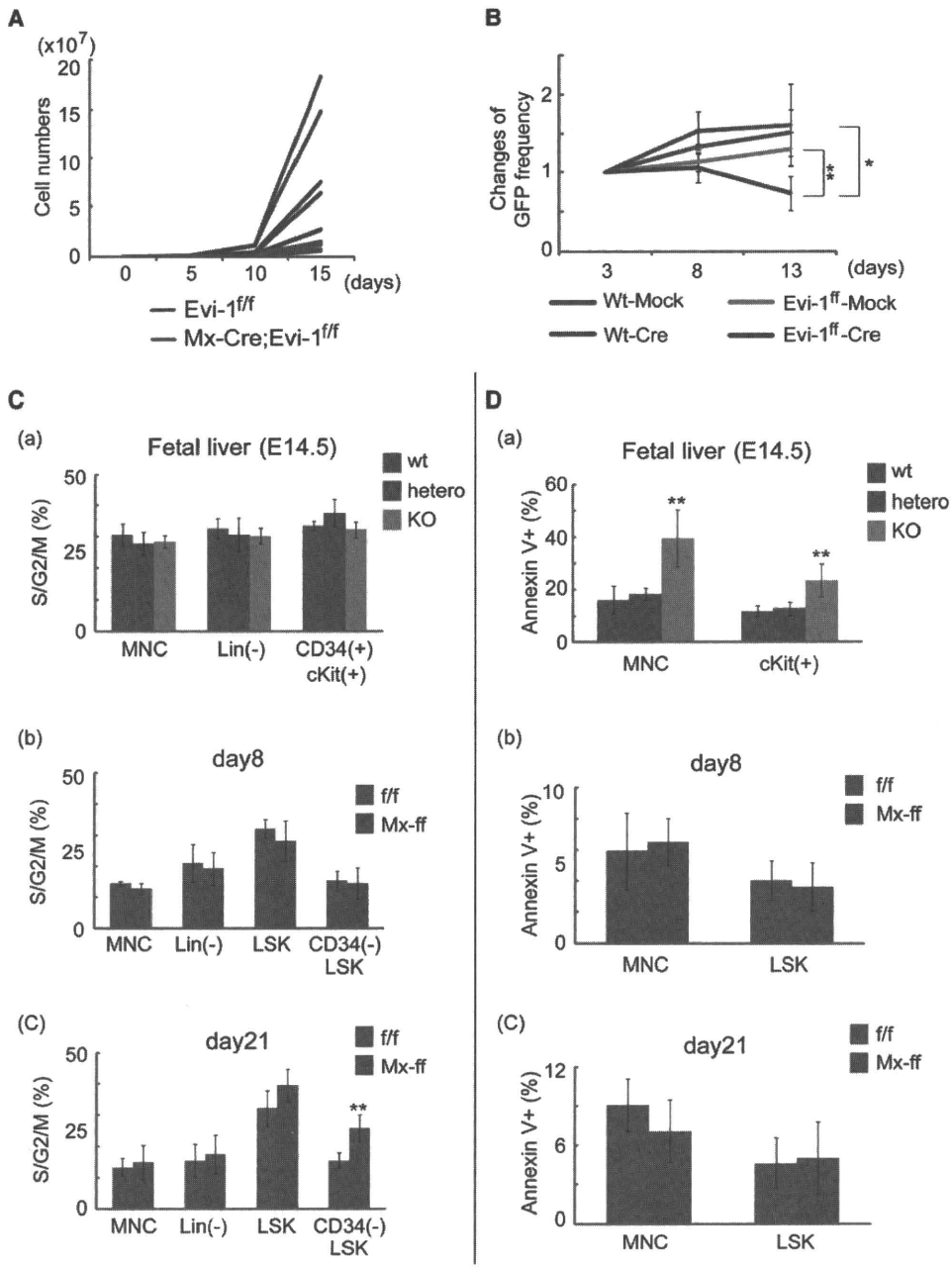



**Figure 6. Effects of Evi-1 Deletion on Cell Death and Proliferation**

(A) Lineage$^-$, c-Kit$^+$ cells were purified from Evi-1$^{f/f}$ or Mx-Cre; Evi-1$^{f/f}$ mice 3–4 weeks after Cre induction. Cells were cultured in liquid medium containing SCF, TPO, IGF-2, and FGF-1. Total cell numbers were monitored every 5 days. Five independent experiments were performed in each group.

(B) BM progenitors derived from wild-type or Evi-1$^{f/f}$ mice were transduced with GFP or Cre-GFP retrovirus and were cultured as in (A). The frequency of GFP$^+$ fraction was assessed every 5 days. Four or five independent experiments were performed in each group. *p = 0.0108. **p = 0.0061.

(C) Cell-cycle status was assessed in various hematopoietic populations of E14.5 FL cells (Ca), BM cells at day 8 post Cre induction (Cb), and BM cells at day 21 post Cre induction (Cc). *Evi-1*-excised FL cells and BM cells at day 8 post Cre induction exhibited normal cell-cycle distribution relative to littermate controls, but *Evi-1*-excised CD34$^-$LSK cells at day 21 post Cre induction showed an increased frequency of S/G2/M phase cells. **p = 0.0015.

(D) Apoptosis was assessed using Annexin-V staining. (Da) *Evi-1*-excised FL cells exhibited an increased frequency of Annexin-V$^+$ cells. (Db and Dc) *Evi-1*-excised BM cells exhibited normal frequency of apoptotic cells both at days 8 and 21 after Cre induction. **p = 0.004 (MNC). p = 0.0021 (c-Kit[+]). MNC, mononuclear cells; Lin, lineage.

Evi-1$^{f/f}$) mice. We found a significant increase in S/G2/M phase CD34$^-$LSK cells in *Evi-1*-excised BM 21 days after Cre induction. In contrast, no difference in cell-cycle distribution was detected in *Evi-1*-excised BM cells immediately after Cre induction (at day 8). Furthermore, Evi-1 deletion had no effect on the cell-cycle distribution of FL cells (Figure 6C). Therefore, the increased

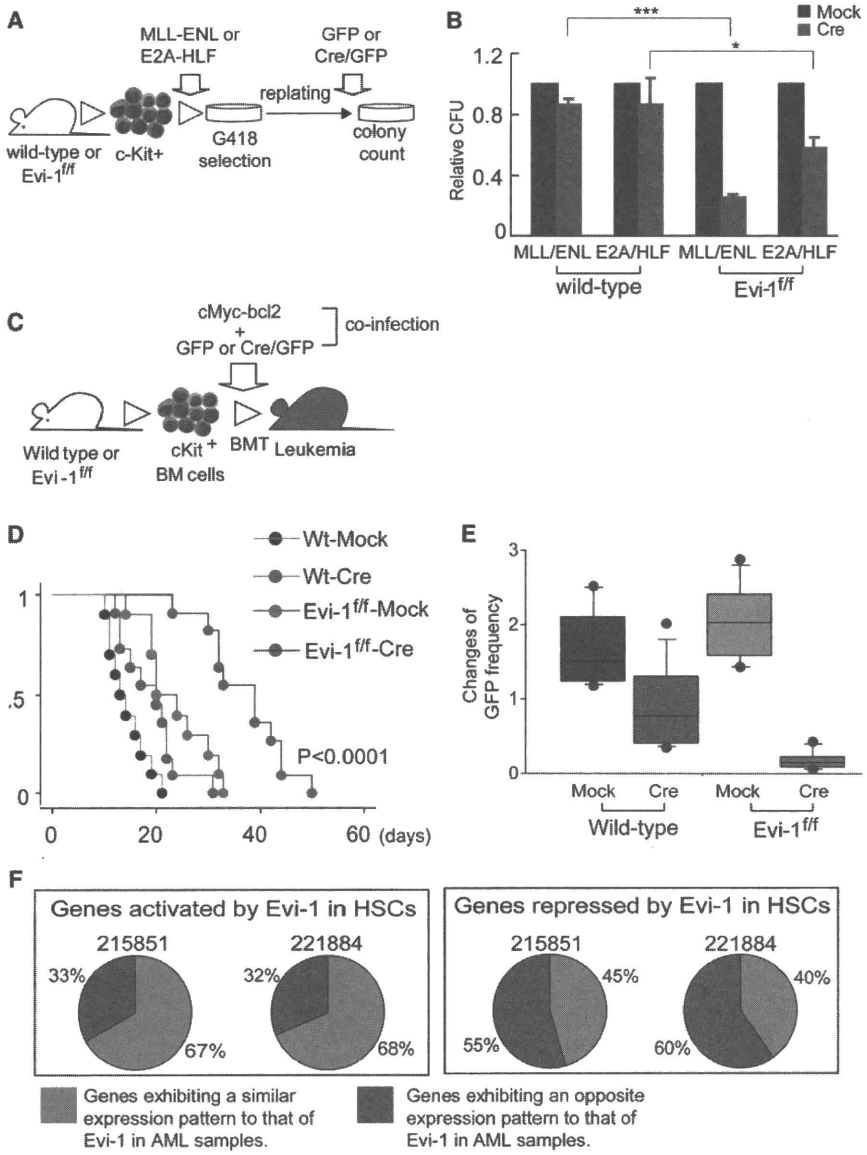



**Figure 7. Evi-1 Determines Proliferative Capacity of Transformed Leukemic Cells**

(A) Schematic representation of the following experiments. BM progenitors from wild-type or Evi-$1^{f/f}$ mice were transduced with MLL/ENL or E2A/HLF oncogenes. Immortalized cells from the third to fifth round of in vitro plating were subsequently transduced with either GFP- or Cre-GFP-expressing retrovirus. $GFP^+$ cells were sorted, and then relative clonogenic activity was assessed in the additional round of plating.

(B) Relative numbers of colonies generated by Cre-transduced cells compared with GFP-transduced cells. Deletion of Evi-1 significantly attenuated clonogenic activities of MLL/ENL and E2A/HLF-transformed cells to 28% and 58% of the controls. Data are shown as mean ± SD from three (MLL/ENL) and four (E2A/HLF) independent experiments. *p = 0.0322. ***p = 0.0001.

(C) Schematic representation of the following experiments. BM progenitors from wild-type or Evi-$1^{f/f}$ mice were transduced with cMyc-bcl2 in combination with GFP or Cre-GFP. Infected progenitors were transplanted into recipients.

(D) Survival curves of mice transplanted with wild-type or Evi-$1^{f/f}$ progenitors transduced by cMyc-bcl2 + GFP or Cre-GFP. Each group contains 10 or 11 mice.

(E) Relative ratios of $GFP^+$ fraction in leukemic cells after transplantation compared with those before transplantation. Peripheral blood cells were isolated from moribund mice, and the frequencies of the $GFP^+$ cells were assessed. Data are shown as a boxplot.

(F) Expression patterns of putative target genes of Evi-1 in cases with AML. Areas of each circular graph represent the percentages of genes exhibiting a similar or opposite expression pattern to that of Evi-1 in AML samples. Results are depicted as for two independent probes (215851 and 221884) used for evaluation of Evi-1 expression.

number of cycling *Evi-1*-excised HSCs at day 21 is probably due to the depletion of quiescent BM HSCs rather than the direct effect of Evi-1 deletion. We then assessed apoptosis in FL or BM cells by Annexin-V-binding to cell surface. Evi-1 deletion significantly increased the rate of cell death in FL cells. However, no significant difference was noted in the percentage of Annexin-V-positive cells in control or *Evi-1*-excised BM cells (Figure 6D). Therefore, the increased cell death would contribute to the defective proliferation of *Evi-1*-excised FL cells but did not account for the homeostasis defects of *Evi-1*-excised BM HSCs. Thus, the effects of Evi-1 deletion on cell cycling and viability in vivo vary in a context-dependent manner.

### Evi-1 Is Required for Efficient Propagation of Transformed Leukemic Cells

Next, we evaluated a role for Evi-1 in myeloid transformation. MLL/ENL (ME) and E2A/HLF (EH) are chimeric genes generated in t(11;19) and t(17;19) leukemias, respectively, both of which are known to transform murine BM cells by distinct molecular mechanisms, resulting in sustained colony formation in the serial replating assay (Ayton and Cleary, 2003; Smith et al., 2002). BM progenitors were harvested from wild-type or Evi-$1^{f/f}$ mice and then transduced with ME or EH. After establishment of sustained clonogenic activity following more than three rounds of replating in methylcellulose medium, the cells were transduced with GFP or Cre-GFP-expressing retroviruses. Then, $GFP^+$ cells were sorted and subjected to another round of replating to evaluate relative clonogenic activity (Figure 7A). Disruption of Evi-1 in ME-transformed progenitors caused significant reduction in colony numbers to about 30% of the control, whereas Cre expression in ME-transformed wild-type progenitors resulted in marginal decrease of clonogenic activity. Evi-1 deletion also modestly reduced clonogenic activity in EH-transformed cells to about 60% of the control (Figure 7B).

We next examined a role for Evi-1 in in vivo leukemia development using transplantation of the cells transduced by cMyc-bcl2

into sublethally irradiated syngeneic mice. This murine leukemia model is useful for evaluating the in vivo effects of Evi-1 deletion on the leukemogenetic activity because these mice developed leukemia with very short latencies. BM progenitors purified from wild-type or Evi-$1^{f/f}$ mice were cotransduced with cMyc-bcl2 together with GFP or Cre-GFP-expressing retroviruses. We then injected these progenitors ($1 \times 10^6$) into recipient mice and assessed their survival (Figure 7C). All the recipient mice developed leukemia as described previously (Luo et al., 2005; Figure S15); however, the mice transplanted with *Evi-1*-excised cells died with significantly longer latencies (Figure 7D). Furthermore, we found significant loss of $GFP^+$ cells in the peripheral blood of the leukemic mice transplanted with cMyc-bcl2- and Cre-transduced Evi-$1^{f/f}$ (*Evi-1*-excised) progenitors, suggesting impaired contribution of *Evi-1*-excised cells to leukemia, although a modest Cre-induced stasis was observed as for wild-type progenitors (Figure 7E). We also used the two-step transplantation model to assess the effect of Evi-1 deletion on the maintenance of leukemia. We first transplanted wild-type or Evi-$1^{f/f}$ progenitors transduced with cMyc-bcl2 into recipients. Leukemic cells were then isolated from the spleens of moribund primary cMyc-Bcl2 mice, and the cells were transduced with GFP or Cre-GFP-expressing retroviruses. Then, the $GFP^+$ cells were sorted and transplanted into secondary recipients to evaluate the leukemogenic activity (Figure S16). This approach again demonstrated that the mice transplanted with *Evi-1*-excised leukemic cells died with significantly longer latencies (Figure S17). Thus, Evi-1 is required for efficient propagation of transformed leukemic cells both in vitro and in vivo.

### Putative Target Genes of Evi-1 in HSCs and AML Samples

To identify the common target genes of Evi-1 shared in HSCs and leukemic cells, we first carried out genome-wide transcriptional analysis using GFP or Cre-GFP-infected Evi-$1^{f/f}$ HSCs. LSK cells derived from Evi-$1^{f/f}$ mice were transduced with GFP or Cre-GFP-expressing retroviruses, and $GFP^+$ cells were sorted. Evi-1 deletion in Cre-GFP-transduced cells was confirmed by PCR (Figure S18). Linear amplification of RNA obtained from $GFP^+$ cells and subsequent gene-expression profiling identified 378 downregulated and 446 upregulated genes in *Evi-1*-deleted HSCs. Transcripts were considered upregulated or downregulated if the change in transcript level as a multiple of the control was >1.2. We then assessed the correlation between expression of Evi-1 and these potential target genes in AML samples using gene-expression data of 285 individuals with AML (Valk et al., 2004; Figure S19). We found that expression of the genes activated by Evi-1 in HSCs tends to coincide with that of Evi-1 in AML samples, whereas many of the genes repressed by Evi-1 in HSCs showed an expression pattern opposite to that of Evi-1 in AML (Figure 7F). Thus, our approach revealed the existence of the common regulatory pathway underlying Evi-1-mediated hematopoiesis and leukemogenesis and provides putative target genes of Evi-1 in both HSCs and leukemic cells. The potential Evi-1 target genes include genes whose expression is decreased in the *Evi-1*$^{-/-}$ P-Sp region (Gata1, Gata2, and Angpt1) (Yuasa et al., 2005), genes involved in the regulation of HSCs (Gata2, Angpt1, Mpl, Jag2, Pbx1, and Setbp1), and genes related to platelet formation (Gata1, Mpl, Itga2b, and Itgb3) (Tables S1 and S2). We evaluated the expression levels of some of the putative target genes of Evi-1 in LSK cells transduced with GFP or Cre-GFP using quantitative PCR method, and we found that they showed a similar tendency to the results obtained by the microarray analysis (Figure S20).

## DISCUSSION

Although Evi-1 has been recognized as a crucial gene that promotes leukemogenesis, a physiological role of Evi-1 in vivo, particularly in terms of adult hematopoiesis, is poorly understood. In this study, we generated Evi-1 mutant mice and showed that Evi-1 is a common regulator essential for proliferation of hematopoietic stem/progenitor cells both during fetal development and in adults. Evi-1 function is required for establishment of definitive HSCs in $Tie2^+$ cells of developing embryos. Furthermore, *Evi-1*$^{+/-}$ mice exhibited the intermediate phenotype as for the number of HSCs, as well as hematopoietic reconstitution activity of the BM cells, suggesting a gene dosage requirement for Evi-1 in the regulation of HSCs. We also found that the platelet counts modestly declined in *Evi-1*-excised mice, and platelet recovery after the myelosuppressive treatment was significantly delayed in *Evi-1*$^{+/-}$ and *Evi-1*-excised mice. Although the underlying mechanism responsible for this defective platelet formation remains to be identified, this phenotype may reflect the clinical feature of elevated platelet counts in AML/MDS cases carrying 3q26 rearrangements.

The *Evi-1*$^{-/-}$ mice we have created survived longer than the previously developed *Evi-1* mutant mice. In the prior *Evi-1* mutant mice, the expression of Evi-1b (shorter isoform) is unaffected. In addition, the previous Evi-1 knockout was established with ES-D3 cells with chimeras subsequently mated to CF-1 mice. Therefore, both the presence of Evi-1b in the prior *Evi-1* mutant mice and dissimilar genetic backgrounds may contribute to the different stages of death.

An emerging concept in the field of cancer biology is that a rare population of "cancer stem cells" exists among the heterogeneous group of cancer cells (Bonnet and Dick, 1997). Cancer stem cells have the abilities to self-renew and differentiate into multiple cell types, and these cells persist in tumors as a distinct population that likely causes disease relapse. Therefore, elimination of the cancer stem cell compartment is necessary and potentially sufficient for cure of the cancer. Several factors that govern the fate of adult stem cells also play a significant role in the regulation of cancer stem cells, such as Wnt, Notch, hedgehog, and Bmi-1 (Huntly and Gilliland, 2005; Lessard and Sauvageau, 2003). Of note, retrovirus-induced Evi-1 activation in hematopoietic cells leads to long-term in vivo clonal dominance of the infected cells (Calmels et al., 2005; Kustikova et al., 2005; Ott et al., 2006), and elevated expression of Evi-1 is associated with poor treatment outcomes in leukemia patients (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Valk et al., 2004). Therefore, together with the findings in this report, we hypothesized that activation of Evi-1 enhances proliferation/survival of leukemia stem cells and, thus, confers drug resistance on various types of leukemia. In support of this is our observation that Evi-1 deletion in transformed leukemic cells leads to significant loss of their proliferative activity both in vitro and in vivo. Therefore, it is tempting to speculate that Evi-1 induces leukemia

development with cooperative oncogenes by enhancing self-renewing capacity in leukemic cells. Consequently, therapies designed to target Evi-1 will be an attractive option in the treatment of leukemia patients, especially in the treatment of those with a poor prognosis.

The presence of zinc fingers that are able to bind to specific sequences of DNA suggests that Evi-1 is a transcriptional regulator; however, target genes of Evi-1 have been poorly identified thus far. Here, we identified candidate Evi-1 target genes using gene-expression-profiling analysis in HSCs combined with the gene-expression data of AML samples. Our analysis revealed the existence of common target genes of Evi-1 in HSCs and leukemic cells. Notably, many genes involved in the HSC regulation, including *Gata2*, *Angpt1*, *Mpl*, *Jag2*, *Pbx1*, and *Setbp1*, are activated by Evi-1, implying that Evi-1 acts as a key regulator in HSCs and leukemic cells. Although it has yet to be determined whether these are primary targets of Evi-1, these data provide a starting point for further studies to uncover the underlying mechanisms for Evi-1-mediated hematopoiesis and leukemogenesis.

Finally, the functions of Evi-1 that we identified in the current study provide an insight into homeostasis and malignant transformation of various types of organs other than the hematopoietic system. Evi-1 is expressed in several other organs, including kidney and ovary (Morishita et al., 1990). Furthermore, aberrant expression of Evi-1 has been frequently found in a variety of solid tumors (Sugita et al., 2000). Thus, Evi-1 may play an important role in stem cell regulation and cancer development in a broad spectrum of tissues.

## EXPERIMENTAL PROCEDURES

### Mice

Evi-1 mutant mice were generated as described in the Supplemental Experimental Procedures. For conditional deletion of Evi-1 in vivo, mice possessing the Evi-1$^{f}$ allele were mated to Tie2-cre or Mx-cre transgenic mice. Mx-cre expression was induced by intraperitoneally injecting 500 μg of pIpC, on 3 alternate days, into 6- to 8-week-old mice. Times after pI-pC injection are counted from the first day of injection. Competitive repopulation assay was performed using the Ly5 congenic mouse system. Mice were kept at the Animal Center for Biomedical Research, University of Tokyo, according to institutional guidelines.

### Flow Cytometric Analysis

A list of antibodies is provided in the Supplemental Experimental Procedures. Cells were sorted with a FACSAria, and analysis was performed on FACSCaliber or LSRII (BD Biosciences). To analyze the cell-cycle status, cells were first stained with lineage and stem/progenitor markers, followed by staining with 10 ng/ml Hoechst 33342 at 37°C for 75 min. Apoptosis was assayed by staining cells with lineage and stem/progenitor markers, followed by Annexin-V and propidium iodide (PI) staining.

### P-Sp Culture

P-Sp culture was performed as described previously (Goyama et al., 2004).

### Colony-Forming Assay

Colony-forming assay was performed in MethoCult GF M3434 (Stem Cell Technologies) with 2 × $10^4$ FL or BM cells and scored for mixed, myeloid, erythroid colony formation at 7 days.

### Retrovirus Production

To produce oncoprotein-expressing retrovirus, Plat-E packaging cells (Kitamura et al., 2003) were transiently transfected with retroviral constructs (see the Supplemental Experimental Procedures), as described previously (Goyama et al., 2004). To produce GFP- or Cre-GFP-expressing retrovirus, we used ψMP34 packaging cells (Takara) transduced with pGCDNsam-eGFP or pGCDNsam-eGFP-iCre.

### Transplantation Assays

For FL transplantation, FL cells from E14.5 wild-type or *Evi-1*$^{-/-}$ embryos (both Ly5.2) alone (noncompetitive transplants) or mixed with BM cells from wild-type mice (Ly5.1) (competitive transplants) were injected into lethally irradiated (9.5 Gy) recipient mice (Ly5.1). For BM transplantation using Cre-expressing retrovirus, c-Kit$^+$ BM progenitors from wild-type or Evi-1$^{f/f}$ mice (Ly5.2) were transduced with GFP- or Cre-GFP-expressing retroviruses and injected into sublethally irradiated (6.5 Gy) recipients (Ly5.1). For competitive transplantation using wild-type or *Evi-1*$^{+/-}$ BM cells, nucleated BM cells (Ly5.2) from each genotype were admixed in 1:1 or 1:10 ratio with competitor BM cells (Ly5.1) and injected into lethally irradiated (9.5 Gy) recipients (Ly5.1).

### 5FU Treatment

We used 5FU (200 mg/kg, i.p.) for myelosuppression. Peripheral blood cell counts were monitored every 5 days in each mouse.

### BM Culture Assays

Lineage$^-$, c-Kit$^+$ BM progenitors were plated at a density of $10^5$/ml in StemSpan serum-free medium (StemCell Technologies) supplemented with 10 μg/ml heparin, 10 ng/ml mouse SCF, 20 ng/ml mouse TPO, 20 ng/ml mouse IGF-2, and 10 ng/ml human FGF-1. Cell counts were monitored every 5 days. For the culture using Cre-expressing retroviruses, c-Kit$^+$ BM progenitors transduced with GFP or Cre-GFP were cultured in the identical condition.

### Myeloid Progenitor Transformation Assays

BM progenitors (c-Kit$^+$ cells) from wild-type or Evi-1$^{f/f}$ mice were transduced with MLL/ENL or E2A/HLF oncogenes. Immortalized cells (5 × $10^4$) from the third to fifth round of in vitro plating were subsequently transduced with either GFP- or Cre-GFP-expressing retrovirus. GFP$^+$ cells were sorted, and, then, relative clonogenic activity was assessed in the additional round of plating.

### Leukemogenesis Assays In Vivo

BM progenitors (c-Kit$^+$ cells) were transduced with cMyc-bcl2 together with GFP or Cre-GFP under identical conditions to those for myeloid progenitor transformation assays. Infected progenitors (1 × $10^6$) were injected into sublethally irradiated (6.5 Gy) recipients. For the two-step transplantation assay, BM progenitors transduced with cMyc-bcl2 were injected into recipients as described above. When transplanted mice exhibited signs of ill health, they were euthanized and their mononuclear spleen cells were isolated. Leukemic cells were then transduced with GFP- or Cre-GFP-expressing retroviruses. Secondary transplantation was performed by injecting 1 × $10^5$ sorted GFP$^+$ cells into sublethally irradiated (6.5 Gy) recipients.

### Microarray Analysis and Gene-Expression Analysis of Individuals with AML

For gene-expression profiling, total RNA was extracted from sorted cells and amplified (NuGEN Technologies, Inc.). Labeled cDNA was hybridized to the Mouse Genome 430 2.0 Array (Affymetrix). Normalization and analysis of chip data were performed using DNA-Chip Analyzer (www.dchip.org) (Li and Wong, 2001). The expression pattern of the genes in leukemic cells was assessed using gene-expression data of 285 individuals with AML (http://www.ncbi.nlm.nih.gov/geo, accession number GSE1159 [NCBI GEO]) (Valk et al., 2004). See the Supplemental Experimental Procedures for detailed analysis.

### Statistical Analysis

Statistical significance of differences between parameters was assessed using a two-tailed unpaired t test. The survival distributions were compared using a log-rank test.

**ACCESSION NUMBERS**

All microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE11557.

**SUPPLEMENTAL DATA**

Supplemental Data for this article include 20 figures, 2 tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/3/2/207/DC1/.

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ORIGINAL ARTICLE

# Lenalidomide is active in Japanese patients with symptomatic anemia in low- or intermediate-1 risk myelodysplastic syndromes with a deletion 5q abnormality

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**Abstract** Lenalidomide is an immunomodulatory agent recently reported to be effective in the treatment of transfusion-dependent anemia due to low- or intermediate-1 risk myelodysplastic syndromes (MDS) associated with a deletion 5q (del 5q) cytogenetic abnormality. We conducted a multicenter, single-arm clinical trial to evaluate the safety and efficacy of lenalidomide in Japanese patients with anemia in low- or intermediate-1 risk MDS associated with the del 5q cytogenetic abnormality. Eleven patients (5 with transfusion-dependent anemia; 6 with transfusion-independent symptomatic anemia) received once daily oral administrations of 10 mg of lenalidomide for 21 consecutive days in a 28-day treatment cycle. The efficacy was assessed by the IWG criteria. At an interim analysis after ≥24 weeks of therapy, hemoglobin increase was noted in all 11 patients, with a median increase of 6.0 g/dL (range, 0.9–10.9) from the baseline. All transfusion-dependent patients achieved

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transfusion independence. Histopathologic and cytogenetic improvement was also noted. Neutropenia and thrombocytopenia were the most common adverse events related to lenalidomide. The adverse events were manageable, and no patients experienced serious adverse events or adverse events requiring treatment discontinuation. The results indicate that lenalidomide can be a useful agent for treating Japanese patients with anemia associated with low- or intermediate-1 risk MDS with the del 5q cytogenetic abnormality.

**Keywords** Lenalidomide · Myelodysplastic syndromes · Chromosome 5q deletion

## 1 Introduction

Myelodysplastic syndromes (MDS) are a group of acquired hematologic malignancies characterized by peripheral cytopenias and dysplastic morphology together with normocellular or hyperplasic bone marrow [1]. Anemia is the most common clinical manifestation of MDS, which may influence the quality of life of patients. MDS affects approximately 5 of 100,000 people and is more common in middle-aged and elderly populations [2]. Furthermore, MDS progresses to acute leukemia over a period of several months to several years in 20–40% of the entire MDS population.

The administration of erythropoiesis-stimulating agent (ESA) alone or in combination with granulocyte colony-stimulating factors can alleviate MDS-associated anemia, although the benefits of the treatments are generally limited to patients with low serum erythropoietin concentration with lesser need for red blood cell (RBC) transfusions [3, 4]. Therefore, supportive RBC transfusion is the mainstay of treatment for symptomatic anemia in MDS [5]. However, long-term transfusion therapy may cause iron-overload disorder, which may lead to organ damage most commonly involving the liver, heart and pancreas [6]. Thus, concomitant administration of iron-chelators is necessary, which is an additional burden for the patients.

Lenalidomide is an immunomodulatory compound originally developed by Celgene Corporation. Lenalidomide has a variety of pharmacologic actions, including stimulation of T lymphocytes and natural-killer cells, inhibition of angiogenesis, suppression of tumor-cell growth, regulation of stem cell differentiation, and suppression of inflammation and hyperalgesia [7]. A US-clinical study evaluated the effect of lenalidomide in patients with transfusion-dependent anemia of low- or intermediate-1-risk MDS, having a cytogenetic abnormality in the long arm of chromosome 5 (del 5q), by the International Prognostic Scoring System (IPSS) [8]. In the US clinical study MDS-003, ninety-nine (67%) of 148 patients achieved transfusion independence. The median increase in the blood hemoglobin concentration from the baseline was 5.4 g/dL. Cytogenetic response was observed in 62 (73%) of 85 patients in whom chromosomal examinations were performed; the abnormal bone marrow cells detected at the baseline were absent in 38 patients [9]. Lenalidomide at a 10 mg per day starting daily dosage was well tolerated with predictable and manageable adverse events. The most common adverse events were granulocytopenia and thrombocytopenia requiring temporarily withholding lenalidomide therapy with or without resumption at a reduced daily dosing.

Lenalidomide has been approved by the US Food and Drug Administration (FDA) as a treatment for transfusion-dependent anemia in low- or intermediate-1-risk MDS with del 5q. The National Comprehensive Cancer Network (NCCN) clinical practice guidelines for MDS (Version 1, 2009) [10] recommend the use of lenalidomide for the treatment of anemia associated with MDS. The incidence of MDS with del 5q is quite rare in Japan [11] and less frequent than in the West, as has also been suggested in other Asian countries [12]. Of 425 Japanese MDS patients registered in the Japan National Research Group on Idiopathic Bone Marrow Failure Syndromes, 50 cases (11.8%) had chromosome 5 abnormalities and the estimated rate of MDS with del 5q and 5q- syndrome among MDS patients was 8.4 and 1.3%, respectively. Lenalidomide is not yet in clinical use in Japan.

We conducted a multicenter, single-arm, open-label clinical trial to assess the efficacy and safety of lenalidomide treatment in Japanese patients with anemia in low- or intermediate-1-risk MDS with del 5q.

## 2 Design and materials

### 2.1 Study design and treatment

The study design is illustrated in Fig. 1. The study was a multicenter, single-arm, open-label study. The treatment cycle consisted of 28 days: the patients received 10 mg of lenalidomide once daily for 21 consecutive days followed by a drug holiday for 7 days. On occurrence of dose-limiting adverse events the dose was reduced or the treatment interrupted according to the protocol. Treatment with lenalidomide was to be continued for a maximum of 156 weeks until disease progression or disease recurrence after a response to the treatment. The treatment was terminated if there was no response at 32 weeks after the initiation of treatment. The study protocol was approved by IRB.

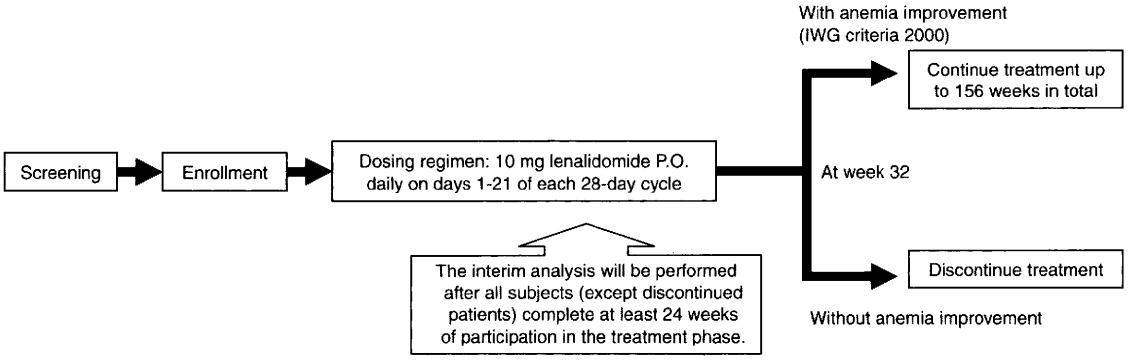


**Fig. 1** Study design. This trial was designed as a multicenter, single-arm, open-label study. A treatment cycle consisted of 28 days: the patients received 10 mg lenalidomide once daily for 21 consecutive days and had drug holiday for 7 days. Treatment with lenalidomide was to be continued for a maximum of 156 weeks until disease progression or recurrence after a response to the treatment. The treatment was terminated if there was no response in terms of the efficacy end points at 32 weeks after the initiation of treatment

## 2.2 Patients

The study subjects were MDS patients aged 20 years or more who met all of the following criteria: del 5q with or without additional chromosomal abnormalities; low- or intermediate-1 risk MDS by the IPSS score; transfusion-dependent anemia or symptomatic anemia with a blood hemoglobin concentration of less than 10.0 g/dL [12]. Written informed consent was obtained from all the patients before enrollment in the study.

The patients were excluded from the study if they met any of the following criteria: pregnancy or lactation; neutrophil count of less than 750/μL, platelet count of less than 50,000/μL, serum creatinine level of greater than 2.5 mg/dL, or serum AST or ALT levels greater than 3 times the upper normal limit; a history of deep venous thrombosis or pulmonary embolism; or treatments of MDS immediately before the initiation of the study, including chemotherapeutics, immunomodulators, antithymocyte globulin, erythropoiesis-stimulating factors and stem cell transplantation.

## 2.3 Clinical and laboratory evaluations

The efficacy end points of the study were improvement in anemia, duration of the improvement, change in blood hemoglobin concentration, cytogenetic response and bone marrow response. The criteria of improvement in anemia in this study followed that of the International Working Group (IWG) 2000 criteria [13] and were defined as transfusion independence lasting for 8 consecutive weeks after the treatment in a transfusion-dependent anemic patient requiring at least 4.5 units (equivalent to 2 units in Western countries) of RBC transfusion in the 8 weeks preceding lenalidomide treatment, or a greater than 2.0 g/dL increase from the baseline in the blood hemoglobin concentration that lasted for 8 consecutive weeks after the treatment in a transfusion-independent symptomatic anemic patient requiring less than 4.5 units of RBC transfusion preceding initiation of lenalidomide treatment. Morphologic and cytogenetic examinations of the bone marrow were performed using aspiration/biopsy specimen. Cytogenetic analysis was performed with both conventional G-banding and fluorescence in situ hybridization (FISH) procedure. For G-banding analysis, chromosomes were pretreated by trypsin, stained with Giemsa and karyotyped according to the recommendation of the ISCN (2005) [14]. For intrerphase analysis, the BAC clone containing *EGR1* gene (Vysis LSI EGR1 (5q31) SpectrumOrange probe; Abott Molecular) was used as a FISH probe. FISH signals were evaluated in 100 nuclei per slides. Separate panels of independent reviewers assessed the cytogenetic response, the bone marrow response and reanalyzed the IPSS classification.

The safety evaluation consisted of assessments of the adverse events in terms of terminology, frequency, severity as graded by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) Version 3.0 and relationship with lenalidomide treatment.

The pharmacokinetics of lenalidomide in Japanese patients was also evaluated. The concentrations of unchanged lenalidomide in plasma on the first 5 days of treatment were used to calculate the pharmacokinetic parameters, including the maximum plasma concentration ($C_{max}$), time to maximum plasma concentration ($t_{max}$), area under the plasma concentration–time curve (AUC), elimination half-life ($t_{1/2}$) and total clearance (CL/*F*).

## 2.4 Statistical analysis

Since the present study was a single-arm, open-label study, only descriptive analyses were performed.

## 3 Results

### 3.1 Patients

Fourteen patients were screened; 11 patients received the lenalidomide treatment, and 3 patients were found ineligible for the study. This report presents the interim analysis results from the data obtained until the data cutoff date (15 October 2008), when all 11 patients had received the study treatment for at least 24 weeks. The median duration of the study at the data cutoff date was 48.1 weeks (range, 28.1–56.1 weeks). None of the patients were withdrawn from the study.

The demographics of the patients (4 males and 7 females) treated with lenalidomide are listed in Table 1. The average age of the patients was 71.8 years. The median duration of the disease was 1.9 years; 2 and 9 patients had low- and intermediate-1-risk MDS by the IPSS score, respectively. 5 patients had transfusion-dependent anemia requiring at least 4.5 units (equivalent to 2 units in Western countries) of RBC transfusion in the 8 weeks before treatment, and 6 patients had transfusion-independent symptomatic anemia requiring less than 4.5 units of RBC transfusion in the same period. The FAB subtype classification was refractory anemia (RA) in 9 patients and RA with excess blasts (RAEB) in 2 patients. According to the WHO classification, 8 patients had 5q-syndrome, 2 patients had RAEB-1 and 1 patient had refractory cytopenia with multi-lineage dysplasia (RCMD). A total of 7 patients had received treatment for MDS, 4 patients each receiving alfacalcidol and menatetrenone.

The median amount of blood transfusion at the baseline was 8.0 units for patients with transfusion-dependent anemia and 1.0 unit for those with transfusion-independent symptomatic anemia. The median blood hemoglobin concentration at the baseline was 7.1 g/dL for patients with transfusion-dependent anemia and 6.4 g/dL for those with transfusion-independent symptomatic anemia.

### 3.2 Anemia improvement

Anemia improvement was observed in all the 11 patients who were treated with lenalidomide (Table 2). The 5 patients with transfusion-dependent anemia at the baseline had increased blood hemoglobin concentration after the treatment, and achieved transfusion independence; the median blood hemoglobin concentration increased from 7.1 g/dL at the baseline to 12.7 g/dL with treatment, with a median increase of hemoglobin of 6.0 g/dL from the baseline. In the 6 patients with transfusion-independent symptomatic anemia, the median blood hemoglobin concentration increased from 6.4 g/dL at the baseline to 12.7 g/dL, with a median increase of hemoglobin of 5.3 g/dL from the baseline. The median time to the anemia improvement was 6.3 weeks (range, 3.1–31.1 weeks). The improvement persisted in all the patients on the data cutoff date. The median duration until the interim analysis was 41.0 weeks (range, 17.1–46.1 weeks).

The changes in blood hemoglobin concentrations of the patients who received lenalidomide are shown in Fig. 2. The median blood hemoglobin concentration was 7.0 g/dL at the baseline, and 12.7 g/dL at the maximum during the

**Table 1** Patient characteristics

| Subject no. | Age/sex | Height (cm) | Weight (kg) | Duration of MDS (years) | IPSS[a] Group (independent review) | FAB classification of MDS | WHO classification of MDS | Baseline hemoglobin (g/dL) | Baseline transfusion dependency | Baseline transfusion units[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| 0020001 | 73/male | 162.0 | 58.4 | 2.7 | Intermediate-1 | RAEB | RAEB-1 | 5.50 | Yes | 8 |
| 0040001 | 64/female | 151.0 | 61.2 | 0.2 | Intermediate-1 | RA | 5q-syndrome | 6.05 | No | 4 |
| 0040002 | 83/female | 144.5 | 44.5 | 4.2 | Intermediate-1 | RA | 5q-syndrome | 6.60 | No | 0 |
| 0040003 | 76/female | 147.5 | 50.6 | 0.9 | Intermediate-1 | RA | 5q-syndrome | 6.25 | No | 2 |
| 0050001 | 68/male | 170.5 | 74.9 | 2.1 | Low | RA | 5q-syndrome | 4.70 | No | 2 |
| 0060001 | 72/female | 146.0 | 42.6 | 1.0 | Intermediate-1 | RA | 5q-syndrome | 8.00 | Yes | 8 |
| 0080001 | 74/female | 151.0 | 52.8 | 4.8 | Intermediate-1 | RAEB | RAEB-1 | 7.10 | Yes | 12 |
| 0080002 | 69/female | 160.3 | 63.0 | 1.8 | Intermediate-1 | RA | 5q-syndrome | 9.10 | No | 0 |
| 0080003 | 65/female | 155.7 | 56.7 | 0.6 | Intermediate-1 | RA | 5q-syndrome | 6.95 | Yes | 6 |
| 0090001 | 79/male | 164.4 | 51.2 | 2.1 | Intermediate-1 | RA | RCMD | 7.55 | No | 0 |
| 0090002 | 67/male | 159.2 | 64.9 | 1.9 | Low | RA | 5q-syndrome | 7.80 | Yes | 6 |

*MDS* myelodysplastic syndromes, *IPSS* International Prognostic Scoring System, *FAB* French–American–British, *WHO* World Health Organization classification, *RAEB* refractory anemia with excess blasts, *RA* refractory anemia, *RCMD* refractory cytopenia with multi-lineage dysplasia

[a] IPSS Group = sum of marrow blast + karyotype + cytopenia score

[b] RBC transfusion units within 56 days before the start of the study drug. In Japan, 1 unit of RBC contains 200 mL of whole blood

anemia improvement period; the median change from the baseline was 6.0 g/dL.

### 3.3 Cytogenetic response

The cytogenetic responses are shown in Table 3. All 11 patients treated with lenalidomide had abnormal metaphases with del(5q) at baseline. Abnormal metaphases were eliminated at the time of the completion of cycle 6 (on day 169) in three of 10 evaluable patients. Furthermore, no 5q abnormality was detectable by interphase FISH in two patients (0050001 and 0090001) on day 169. Additional cytogenetic abnormality, which had not been observed at baseline was detected in two patients (0040002 and 0080002) during the treatment.

**Table 2** Anemia improvement with lenalidomide

| Response | No. of patients | Responder *n* (%) |
|---|---|---|
| Overall improvement in anemia | 11 | 11 (100.0) |
| RBC transfusion independence and increase in hemoglobin level of ≥1 g/dL[a] | 5 | 5 (100.0) |
| Increase in hemoglobin level of >2 g/dL[b] | 6 | 6 (100.0) |
| Hemoglobin (g/dL) | | |
| Baseline[c] median (range) | 11 | 7.0 (4.7–9.1) |
| Response[d] median (range) | 11 | 12.7 (8.9–15.6) |
| Change median (range) | 11 | 6.0 (0.9–10.9) |

[a] Transfusion independence and an increase in hemoglobin of at least 1 g/dL from the baseline for 8 consecutive weeks for patients who were RBC transfusion dependent at baseline

[b] >2 g/dL increase in hemoglobin without transfusion from the baseline for 8 consecutive weeks for patients who were RBC transfusion independent with hemoglobin <10 g/dL at baseline

[c] The baseline hemoglobin concentration was the mean of the two most recent hemoglobin measurements prior to the start of lenalidomide treatment

[d] The response hemoglobin concentration was the maximum value during improvement in anemia

### 3.4 Bone marrow response

The bone marrow cellularity normalized at cycle 6 in 5 patients: 2 patients with a hypercellular bone marrow and 3 patients who had a hypocellular bone marrow before treatment. On the other hand, 2 patients with normocellular bone marrow before the treatment had hypocellular bone marrows at cycle 6.

No patients had a change in terms of worsening in the FAB or WHO subtype. The number of BM erythroblasts increased with treatment. Therefore, the proportion of BM myeloblasts declined to less than 5% in 2 patients. In 2 patients, the FAB subtype changed from RAEB at the baseline to RA. By the WHO subtype, 2 patients with RAEB-1 at the baseline changed to 5q deletion syndrome in one patient and RCMD in the other.

### 3.5 Adverse events and dose adjustment

Adverse events were reported in all 11 patients treated with lenalidomide. The grade 3 or higher lenalidomide-related adverse events were neutropenia ($n = 10$), leukopenia ($n = 6$), lymphopenia ($n = 3$), thrombocytopenia ($n = 1$), and hypertension ($n = 1$) (Table 4). None of the patients had grade 3 or higher hemorrhagic adverse events or infections, or discontinued the treatment because of adverse events. A serious compression fracture was reported in 1 patient, which was considered to be unrelated to lenalidomide treatment. Clinically significant changes were not observed in any other safety parameters, including laboratory tests, vital signs, and ECG. A total of 8 patients had

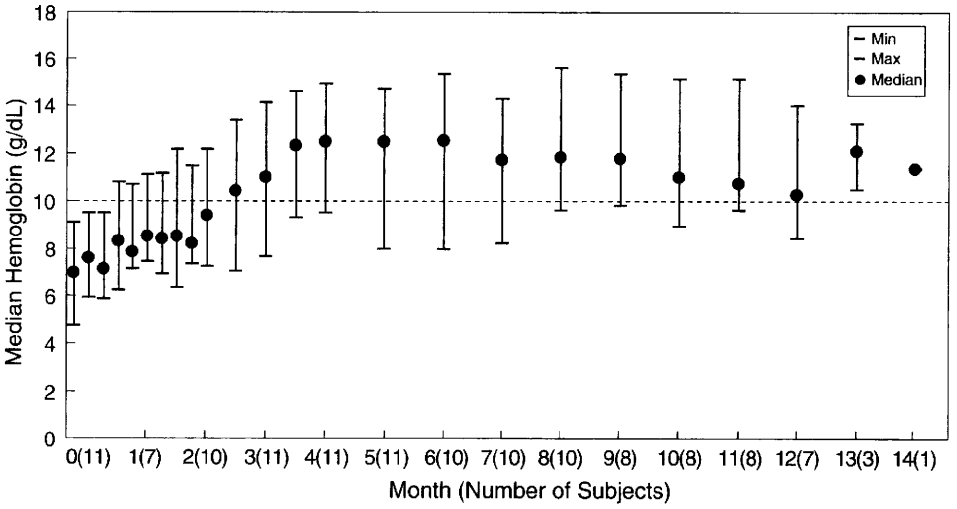


**Fig. 2** Hemoglobin levels over treatment time. The median hemoglobin concentration was 7.0 g/dL at the baseline, and 12.7 g/dL at the maximum during the anemia improvement period; the median change from the baseline was 6.0 g/dL

**Table 3** Cytogenetic response, as assessed by interphase FISH and G-banding procedure

| Subject no. | No. of nuclei with one EGR1 signal[a] | | | No. of abnormal chromosomes with del (5q)[b] | | | |
|---|---|---|---|---|---|---|---|
| | Screening | Day85 | Day169 | Karyotype | Screening | Day 85 | Day 169 |
| 0020001 | 74 | 25 | ND | 46,XY,del(5)(q13q33) | 19 | 11 | ND |
| 0040001 | 51 | 10 | 4 | 46,XX,del(5)(q13q33) | 8 | 2 | 2 |
| 0040002 | 72 | 0 | 7 | 46,XX,del(5)(q13q33) | 17 | 1 | 3 |
| | | | | 46,idem,add(11)(q13) | 0 | 1 | 0 |
| 0040003 | 83 | 11 | 4 | 46,XX,del(5)(q13q33) | 20 | 5 | 0 |
| 0050001 | 67 | 3 | 0 | 46,XY,del(5)(q22q31) | 8 | 1 | 0 |
| 0060001 | 37 | 33 | 65 | 46,XX,del(5)(q31q35) | 19 | 5 | 11 |
| 0080001 | 77 | 57 | 23 | 46,XX,del(5)(q13q33), inv(9)(p12q13) | 20 | 13 | 5 |
| 0080002 | 41 | 6 | 5 | 46,XX,del(5)(q13q33) | 14 | 0 | 1 |
| | | | | 46,XX,del(5)(q13q33), del(20)(q11.2q13.3) | 0 | 1 | 4 |
| 0080003 | 22 | 5 | 53 | 46,XX,del(5)(q13q33) | 9 | 1 | 14 |
| 0090001 | 23 | 10 | 0 | 46,XY,del(5)(q13q33) | 7 | 0 | 0 |
| | | | | 46,XY,der(5)del(5)(q13q33)t(5;?)(q35;?) | 9 | 6 | 0 |
| 0090002 | 60 | 13 | 49 | 46,XY,del(5)(q15q33) | 19 | 3 | 13 |

*ND* no data

[a] With interphase FISH in each patient, 100 nuclei were analyzed

[b] With G-banding analysis in each patient, 20 metaphase cells were analyzed

**Table 4** Treatment-related adverse events with NCI CTCAE grades 3 or 4

| System organ class/ preferred term[a] | Initial dose (10 mg) ($N = 11$) $n$ (%) |
|---|---|
| Subjects with at least one NCI CTCAE grade 3 or 4-related adverse event | 11 (100.0) |
| Blood and lymphatic system disorders | 11 (100.0) |
| Neutropenia | 10 (90.9) |
| Leukopenia | 6 (54.6) |
| Lymphopenia | 3 (27.2) |
| Thrombocytopenia | 1 (9.0) |
| Vascular disorders | 1 (9.0) |
| Hypertension | 1 (9.0) |

*NCI CTCAE* National Cancer Institute Common Terminology Criteria for Adverse Events

[a] Preferred terms and system organ classes are coded using the MedDRA dictionary (version 10.0). A subject with multiple occurrences of an adverse event is counted only once in the preferred term category

reduced lenalidomide dosages from 10 mg daily to 5 mg daily (21 consecutive days). On the data cutoff, lenalidomide dosage for these 8 patients was 5 mg daily. The reason for the dose reduction was neutropenia (≥grade 3; $n = 8$). Furthermore, administration was temporarily interrupted in 8 patients due to AEs. The reasons for the dose interruption were neutropenia (≥grade 3, $n = 8$) and abnormal laboratory value of TSH (grade 2, $n = 1$).

### 3.6 Pharmacokinetic evaluation

Blood lenalidomide pharmacokinetics was evaluated during the first five days of therapy. Serum specimens were successfully collected in 6 patients.

The mean $C_{max}$ values were 136 ng/mL at day 1 and 149 ng/mL at day 4 of lenalidomide treatment. The mean $AUC_{\tau}$ values were 866.5 ng h/mL at day 1 and 877.9 ng h/mL at day 4. The ratios of the $C_{max}$ and $AUC_{\tau}$ values at day 4 to those at day 1 were 1.16 and 1.04, respectively. The results did not suggest drug accumulation after repeated administrations. The $t_{max}$ values were 2.52 h at day1 and 2.93 h at day 4 of the treatment, with no significant change. Other pharmacokinetic parameters at day 4 were also similar to those at day 1; the $t_{1/2}$ values were 3.26 and 3.57 h, and the CL/*F* values were 189.8 and 189.9 mL/min at day 1 and day 4, respectively.

## 4 Discussion

Chronic anemia compromises the quality of life in MDS patients. In addition, chronic blood transfusion therapy may lead to complications such as iron-overload disorder and cardiac failure. The need for blood transfusions also correlates with the risk of progression to acute myeloid leukemia, resulting in a poor prognosis in patients with lower-risk MDS [5].

In this study, lenalidomide treatment resulted in rapid increase in the hemoglobin (median time to anemia