Evi-1 promotes para-aortic splanchnopleural hematopoiesis through up-regulation of GATA-2 and repression of TGF-β signaling

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(Received November 28, 2007/Revised March 21, 2008/Accepted March 30, 2008/Online publication April 30, 2008)

Evi-1 is a zinc-finger transcriptional factor whose inappropriate expression leads to leukemic transformation in mice and humans. Recently, it has been shown that Evi-1 regulates proliferation of hematopoietic stem/progenitor cells at embryonic stage via GATA-2 up-regulation; however, detailed mechanisms underlying Evi-1-mediated early hematopoiesis are not fully understood. We therefore evaluated hematopoietic potential of Evi-1 mutants using a cultivation system of murine para-aortic splanchnopleural (P-Sp) regions, and found that both the first zinc finger domain and the acidic domain were required for Evi-1-mediated hematopoiesis. The hematopoietic potential of Evi-1 mutants was likely to be related to its ability to up-regulate GATA-2 expression. We also showed that the decreased colony forming capacity of *Evi-1*-deficient P-Sp cells was successfully recovered by inhibition of TGF-β signaling, using ALK5 inhibitor or retroviral transfer of dominant-negative-type Smad3. Our findings suggest that Evi-1 promotes hematopoietic stem/progenitor expansion at the embryonic stage through up-regulation of GATA-2 and repression of TGF-β signaling. (*Cancer Sci* 2008; 99: 1407–1413)

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.[1] Evi-1 is highly expressed in cases with human acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) as a consequence of chromosomal rearrangements involving 3q26, where Evi-1 is mapped.[2–5] Alterations such as t(2;3)(p15;q26), inv(3)(q21;q26), t(3;3)(q21;q26), t(3;7)(q27;q22), t(3;12)(q26;p13), t(3;13)(q26;p13-14), and t(3;17)(q26;q22) are representative, but there are some cases with Evi-1 deregulation without any 3q26 rearrangements.[6] The most frequent ones are inv(3)(q21;q26) and t(3;3)(q21;q26), and cases with these two anomalies have elevated platelet counts, marked hyperplasia with dysplastic megakaryocytes, and poor prognosis, which are characterized as 3q21q26 syndrome.[7] These findings indicate that Evi-1 plays a critical role in human leukemogenesis.

The alternative forms generated from *Evi-1* gene encode two distinct proteins, Evi-1a and Evi-1c, with the latter also called MDS1-Evi-1.[8] Structurally in Evi-1c, amino(N)-terminally to the Evi-1a moiety lies a 188 amino-acid region of homology called the PR domain. The PR domain (*P*RDI-BF1-*R*IZ1 homologous domain) has been found in at least 17 kinds of diverse proteins to date, and a large body of evidence suggests that the PR-containing forms contribute to tumor suppression, while the PR-absent forms are oncogenic.[9–12] Consistent with this principle, it has been shown clinically that the expression of Evi-1a (PR-absent form) is associated with highly aggressive AML, but that of Evi-1c (PR-containing form) is not.[13,14] As all PR domain proteins, Evi-1 contains several zinc-finger motifs which are grouped in the first zinc-finger domain (seven motifs) and the second zinc-finger domain (three motifs). Between these two zinc-finger domains, there is a proline-rich repression domain and at the C-terminus there is a highly acidic stretch.[15]

We have 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, protects cells from stress-induced cell death by inhibiting c-Jun N-terminal kinase (JNK), and increases the expression of endogenous c-Jun and c-Fos resulting in activation of AP-1.[16–18] We have also shown that Evi-1 interacts with corepressor CtBP and this interaction contributes to Evi-1-mediated repression of TGF-β signaling.[19] Furthermore, we found that Evi-1a, an oncogenic form of the *Evi-1*, forms a homo-oligomeric complex. This oligomerization is inhibited in Evi-1c by the presence of PR domain, which results in attenuated potential to repress TGF-β signaling.[20]

Mice deficient in Evi-1 die during embryogenesis with widespread hypocellularity, hemorrhaging, and disruption in the development of the heart, somite, and neural crest-derived cells.[21] It was recently reported that Evi-1 is expressed at a high level in the para-aortic splanchnopleural (P-Sp) region, from which definitive hematopoiesis originates. Hematopoietic stem cells (HSCs) in *Evi-1*-deficient embryos are decreased in number with defective proliferation capacity, and this defective proliferation of *Evi-1*-deficient P-Sp cells is successfully rescued *in vitro* by retroviral transfer of GATA-2.[22] However, detailed molecular mechanisms underlying Evi-1-mediated hematopoiesis remain to be elucidated.

In the present study, we used a coculture system of cells derived from the P-Sp region with a layer of a stromal cell line OP9, on which hematopoietic cell development is efficiently induced.[23] In this culture system, *Evi-1*-deficient P-Sp-derived cells showed a severely decreased colony forming capacity. This defect was overcome by reactivating Evi-1 retrovirally. Using this assay, we examined the hematopoietic potential of a series of Evi-1 mutants and found that the first zinc-finger domain and the acidic domain are required for the hematopoietic rescue of *Evi-1*-deficient P-Sp cells. These two domains were also related to some extent for GATA-2 up-regulation, suggesting that GATA-2 is one of the key molecules in Evi-1-mediated hematopoiesis. Furthermore, we found that the blocking of TGF-β signaling is also able to recover the hematopoietic defect of *Evi-1*-deficient P-Sp cells. These findings suggest that Evi-1 promotes hematopoietic stem/progenitor expansion during embryogenesis through up-regulation of GATA-2 and inhibition of TGF-β signaling.

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doi: 10.1111/j.1349-7006.2008.00842.x

Materials and Methods

Mice and embryos. We newly generated *Evi-1*-deficient mice by deleting exon 4 of *Evi-1* (S Goyama, manuscript submitted in September 2007) and the mice were crossed onto the C57BL/6 background. To generate embryos, timed mating was set up between *Evi-1* heterozygous mice. The time at midday (12:00) was taken to be 0.5 dpc for the plugged mice. Mice were kept at the Animal Center for Biomedical Research, University of Tokyo, according to institutional guidelines.

***In vitro* P-Sp culture.** P-Sp culture was performed as described previously,[23] with minor modifications. In brief, isolated P-Sp regions of E9.5 embryos were dissociated by incubation with 250 protease units (PU)/mL dispase (Godo Shusei, Tokyo, Japan) for 20 min and cell dissociation buffer (Gibco BRL, Carlsbad, CA, USA) for 20 min at 37°C, and washed once in phosphate-buffered saline, followed by vigorous pipetting. Approximately 5×10^4 P-Sp-derived cells were suspended in 300 μL of serum-free StemPro media (Life Technologies, Gaithersburg, MD, USA) supplemented with 50 ng/mL stem cell factor, 5 ng/mL interleukin 3 (IL-3; gifts from Kirin Brewery, Takasaki, Japan), and 10 ng/mL mouse oncostatin M (R&D Systems, Minneapolis, MN, USA). Single-cell suspensions were seeded on preplated OP9 stromal cells,[24] in the 24-well plate, followed by incubation at 37°C in humidified 5% CO_2 air. Images were taken with a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan).

Plasmid construction. The complementary DNAs (cDNAs) for Flag-tagged human Evi-1a, Evi-1c, Evi-1ΔZF1, Evi-1ΔZF2, Evi-1ΔRep, Evi-1ΔAD, and Evi-1-DL/AS were inserted into the SalI-BamHI sites of the pGCsam, upstream of the internal ribosomal entry site (IRES) and *GFP* reporter gene.[16,22] Runx1/AML1,[23] cDNA and Flag-tagged GATA-2, and PU.1[25] cDNAs were inserted into the pMYs/IRES-EGFP retrovirus vector.[26] Flag-tagged Bmi-1 cDNA was inserted into pGCsam. Dominant-negative human Smad3ΔSSVS in the pCMV5F expression vector was given by Rik Derynck (University of California at San Francisco) and subcloned into the SalI-BamHI sites of the pGCsam.[27]

Retroviral transduction. Plat-E packaging cells[28] (2×10^6) were transiently transfected with 3 μg of retrovirus vectors, mixed with 9 μL of FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA), and incubated at 37°C. Supernatant containing retrovirus was collected 48 h after transfection and used immediately for infection. Retroviral transduction of the cells derived from *Evi-1*-deficient P-Sp regions was performed as described previously.[23] In brief, viral supernatant was added to P-Sp cells on a layer of OP9 stromal cells together with 8 μg/mL polybrene (Sigma, St Louis, MO, USA). After 72-h incubation, virus-containing medium was replaced by the original culture media. The cells were incubated for another three days and processed for analyses. To confirm the expression of proteins, NIH3T3 cells were also infected with the same retroviruses. Infection efficiency was evaluated by the positive rate of green fluorescent protein (GFP).

Colony forming cell (CFC) assay. Wild-type and *Evi-1*-deficient P-Sp-derived cells with OP9 stromal layer were used for CFC assays. Cells (5×10^4) were plated into MethoCult GF M3434 medium (StemCell Technologies, Vancouver, BC, Canada) and cultured at 37°C in a 5% CO_2 air. The number of colonies was counted at day 7. Error bars in the figures indicate standard error of the mean (SEM).

Flow cytometry. Flow cytometric analysis was performed in a FACScalibur with Cellquest program (Becton Dickinson, San Jose, CA, USA) after addition of propidium iodide to exclude dead cells.

Quantitative real-time polymerase chain reaction (PCR) analysis. From each culture dish, P-Sp-derived cultured cells were harvested on day 3. After RNA isolation using RNeasy reagents (Qiagen, Hilden, Germany), transcription into cDNA was performed with SuperScript III (Invitrogen). All PCRs were carried out in the ABI-7000 sequence detection system with SYBR Green PCR Core reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, Country). Primer sequences used for analyses are as follows: *GATA-2* forward, ATCCACCCTTCCTCCAGTCT; reverse, CGGGAGCCAAGAGTATGTTC; *Gapdh* forward, GAATCTACTGGCGTCTTCACC; reverse, GTCATGAGCCCTTCCACGATGC. Each assay was performed in triplicate and the results were normalized to *Gapdh* levels.

Western blot analysis. Retrovirus-infected NIH3T3 cells were lyzed in radioimmunoprecipitation assay buffer.[29] Whole-cell lysates containing 80 μg of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidine difluoride membrane (Immobilon; Millipore, Bedford, MA, USA). Immunoblotting was performed with anti-Flag antibody,[18] or anti-AML1 polyclonal antibody (Calbiochem, San Diego, CA, USA) using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Transcriptional response assay. Analysis of luciferase activities was performed as described previously.[16] To determine the TGF-β-mediated transcription response, HepG2 cells were transfected with the p3TP-Lux reporter plasmid along with the effector plasmid pME18S, pME18S-Evi-1a or pME18S-GATA-2, and cells were treated with 200 pM TGF-β (Roche Diagnostics) for 24 h before harvesting. The cells were harvested 48 h after transfection and assayed for the luciferase activity by means of the luciferase assay system (Promega, Madison, WI Country) and a luminometer (Lumat, Wildebad, Germany). The data were normalized to the β-galactosidase activity.

Statistical analysis. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered significant.

Results

Decreased colony-forming capacity of hematopoietic cells derived from *Evi*-1-deficient P-Sp regions. In this study we used newly generated *Evi-1*-deficient mice (S Goyama, manuscript submitted in September 2007), and we first examined whether our new *Evi-1*-deficient mice showed a similar phenotype to that of the previously reported Evi-1 mutant mice[21] in P-Sp hematopoiesis. *Evi-1*-deficient P-Sp cells gave rise to hematopoietic cells when overlayed on the OP9 stromal cells (Fig. 1a), but showed severely decreased colony-forming capacity (Fig. 1b). To examine whether reactivation of Evi-1 can rescue this hematopoietic defect, we retrovirally infected *Evi-1*-deficient P-Sp cells with empty vector and Evi-1a, seeded in semisolid medium at day 7 and cultured for another 7 days. Transduction of empty vector had no effect on *Evi-1*-deficient P-Sp cells, but Evi-1 transduction resulted in increasing hematopoietic colonies (Fig. 1b). Almost all colonies generated in this culture system were granulocyte/macrophage-type colonies. Thus, we confirmed that Evi-1 plays a significant role in the proliferation of hematopoietic cells derived from the P-Sp region using new *Evi-1*-deficient mice. Furthermore, the defective colony-forming capacity of *Evi-1*-deficient P-Sp cells can be complemented by retrovirus-mediated reactivation of Evi-1 in this experimental system.

Functional implication of Evi-1 in early hematopoietic development. Using this system, we then analyzed the hematopoietic potential of various Evi-1 mutants. We generated retroviruses that express two types of Evi-1 isoforms and a series of mutants that harbor deletion of the functional domains or substitution of specific residues (Fig. 2a). NIH3T3 cells were infected with these retroviruses and the titer of the viruses was evaluated by flow

doi: 10.1111/j.1349-7006.2008.00842.x

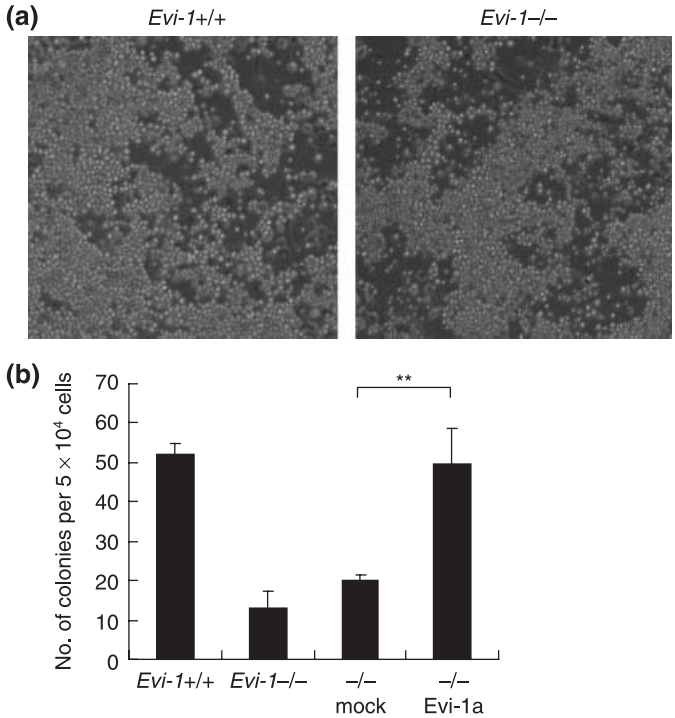


Fig. 1. Decreased colony forming capacity of ecotropic viral integration site-1 (*Evi-1*)–deficient P-Sp cells. P-Sp cells from wild-type (*Evi-1*+/+) and *Evi-1*-deficient (*Evi-1–/–*) embryos at E9.5 were cultured for 7 days on OP9 cells. *Evi-1–/–* P-Sp cells (right panel) could generate hematopoietic cells. Shown at 100× original magnification (a). *Evi-1–/–* P-Sp cells showed decreased colony forming capacity compared with *Evi-1*+/+P-Sp cells. Retroviral transfer of Evi-1a could rescue this hematopoietic defect of *Evi-1–/–* P-Sp culture, while control vector could not. Error bars indicate SEM of two independent experiments, each in duplicate. *P*-values were calculated as compared with *Evi-1*+/+ P-Sp-derived colonies (b). $^{**}P < 0.005$.

cytometric measurement of GFP-positive cells. Figure 2b shows that the titers of Evi-1 mutants are comparable although they are lower than that of the mock virus. Coincidentally, retrovirus-mediated expression of each mutant in infected cells was confirmed by Western blotting (Fig. 2c).

We first examined the hematopoietic potential of Evi-1c, a PR-domain-containing form of Evi-1. Evi-1c has been associated with slower cellular growth or with terminal differentiation, and cannot efficiently repress TGF-β signaling.[20] Surprisingly, Evi-1c could restore the colony forming capacity of *Evi-1*-deficient P-Sp cells as efficiently as Evi-1a, suggesting that Evi-1c possesses normal hematopoietic potential in spite of its reduced oncogenic activity (Fig. 3).

Evi-1 has several distinct domains with defined biochemical functions. The first zinc-finger domain recognizes a consensus sequence of 15 nucleotides consisting of GA(C/T)AAGA(T/C)AAGATAA *in vitro*, and is essential for repressing TGF-β signaling by binding to Smad3.[16] The first zinc-finger domain is also required to protect cells from stress-induced cell death by inhibiting JNK activity.[17] The second zinc-finger domain recognizes a consensus sequence of GAAGATGAG, and is essential for AP-1 activation.[18] The region between amino acid 608 and 732 of Evi-1, which we call the repression domain, is required for the efficient repression of TGF-β signaling, although it does not contribute to binding to Smad3. The region containing CtBP-binding-motif-like sequences, PLDLS at amino acid 584, is responsible for the interaction with CtBP1.[19] In addition, Evi-1 contains a highly acidic domain at the C-terminus, but its function is not clear thus far. In order to identify the functional domains required for Evi-1-mediated hematopoiesis, we then examined a series of Evi-1 mutants for hematopoietic proliferation.

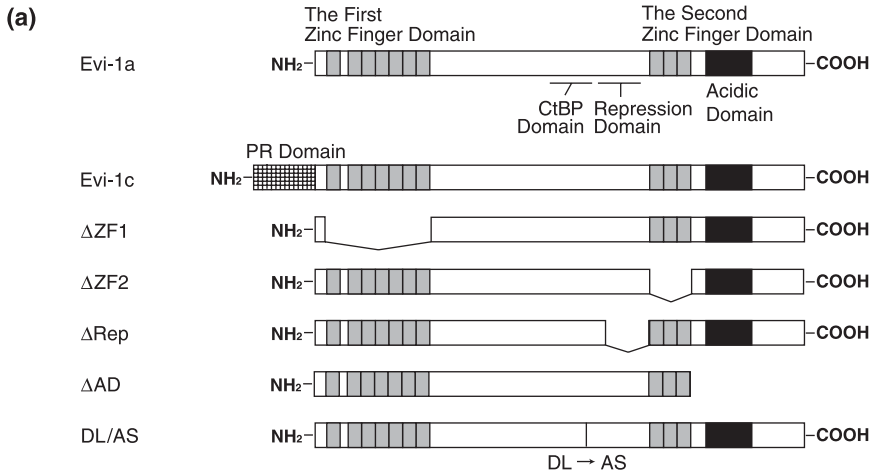
As shown in Figure 3, infection of retroviruses containing ΔZF2, ΔRep, and DL/AS of Evi-1 efficiently overcome the defect of *Evi-1*-deficient phenotype, giving a similar colony-forming capacity with that of Evi-1a-transduced *Evi-1*-deficieint P-Sp cells. In contrast, other mutants, such as ΔZF1 and ΔAD could not recover the defect. We also employed DL/AS, a mutant that harbors a point mutation causing substitution of DL with AS at amino acid 584. Although the mutant (DL/AS) loses its ability to interact with CtBP, it rescued the hematopoietic defect of *Evi-1*-deficient P-Sp cells. These results suggest that both the first zinc-finger domain and the acidic domain of Evi-1 are required for Evi-1-mediated hematopoiesis, while the second zinc-finger domain, the repression domain, or the CtBP-binding domain is dispensable.

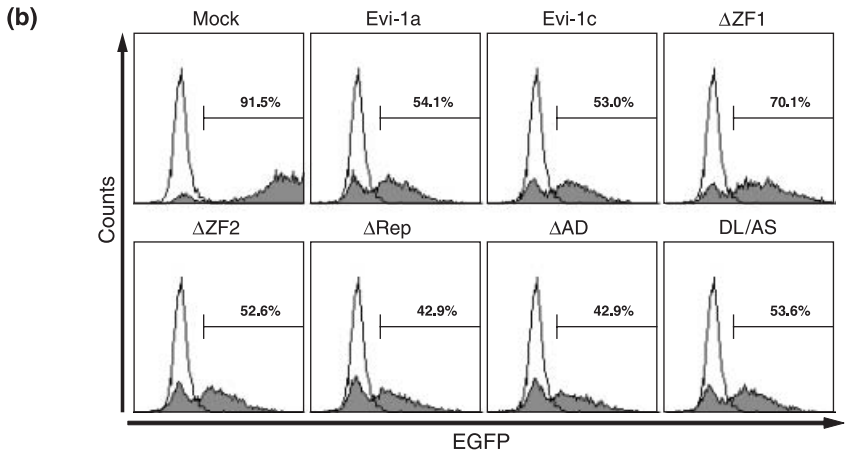
Enforced expression of GATA-2 overcomes hematopoietic defects of *Evi*-1-deficient P-Sp regions. Next, we investigated downstream target genes of Evi-1 in P-Sp hematopoiesis using several genes involved in early hematopoietic development and HSC regulation (*GATA-2*, *Runx1/AML1*, *PU.1*, and *Bmi-1*). We retrovirally infected *Evi-1*-deficient P-Sp cells with these genes and assessed their colony forming capacity. Titers of the retrovirus containing these genes were similar to each other as evaluated by infecting NIH3T3 cells with these viruses (Fig. 4a). Expression of individual proteins was confirmed by Western blot analysis (Fig. 4b). Consistent with the observation in the previous report, GATA-2-transduced *Evi-1*-deficieint P-Sp cells showed an increase of colony forming capacity, while transduction of mock or Runx1/AML1 did not affect the capacity. In addition, PU.1 and Bmi-1 enhanced the colony-formation capacity of *Evi-1*-deficieint P-Sp cells to some extent (Fig. 4c).

To evaluate the ability of Evi-1 mutants to up-regulate GATA-2 expression in P-Sp cells, we performed quantitative real-time PCR (RT-PCR) analysis. Wild-type P-Sp cells were infected with Evi-1 and its mutants, and the mRNA levels of GATA-2 in these cells were assessed after an additional two-day culture. As shown in Figure 4d, GATA-2 expression tended to be higher in P-Sp cells transduced with Evi-1c, ΔZF2, ΔRep, or DL/AS. In contrast, Mock, ΔZF1, or ΔAD transduction did not increase GATA-2 expression. These results indicate that the hematopoietic potential of Evi-1 mutants was partly related to its ability to up-regulate GATA-2 expression.

Repression of TGF-β signaling is involved in *Evi*-1-mediated hematopoiesis. It has been shown that TGF-β is one of the most potent endogenous negative regulators of hematopoiesis.[30] In addition, we have revealed that Evi-1 represses TGF-β signaling by interacting with Smad3.[16] Therefore, we assessed whether the hematopoietic phenotype of *Evi-1*-deficient P-Sp cells is relevant to excessive TGF-β signaling.

We first used an ALK5 inhibitor, LY364947,[31] (LY) to block TGF-β signaling. We added LY at 0.01, 0.1, 1 μmol/L concentration to the P-Sp culture medium and evaluated their colony-forming capacity. As shown in Figure 5a, a 1-μmol/L LY application restored the colony-forming capacity of *Evi-1*-deficient P-Sp cells. We then employed Smad3ΔSSVS, dominant-negative-type Smad3 (DNSmad3), to mimic the inhibition of Smad3 by Evi-1.[27] Retroviral transduction of DNSmad3 could also recover the hematopoietic defect of *Evi-1*-deficient P-Sp cells (Fig. 5b). The titer of DNSmad3 retrovirus was evaluated by infecting NIH3T3 cells with these viruses (Fig. 5c), and the expression of DNSmad3 protein was confirmed by Western blotting (Fig. 5d). Thus excessive TGF-β signaling is responsible, at least in part, for the hematopoietic defect of *Evi-1*-deficient P-Sp cells.





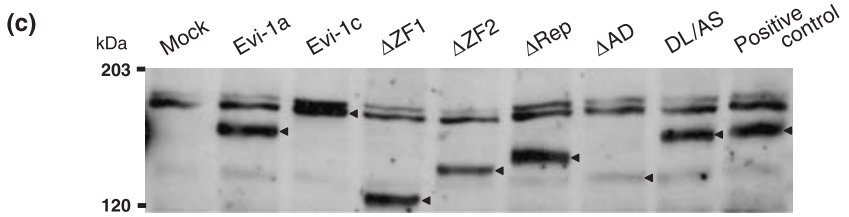


Fig. 2. Generation of retroviruses that express ecotropic viral integration site-1 (Evi-1) or its mutants. Structures of Evi-1a, its isoform Evi-1c, and mutants are depicted. ZF1 indicates the first zinc-finger domain; ZF2, the second zinc-finger domain; Rep, the repression domain; AD, the acidic domain. DL/AS mutant has a point mutation that abolishes CtBP binding. All constructs were tagged with a FLAG epitope at the N-terminus of Evi-1 (a). Flow cytometric analysis of EGFP expression (shaded histograms) in NIH3T3 cells 48 h following infection with viral supernatants. Numbers refer to the percentage of transduced cells. Uninfected NIH3T3 cells are also shown as a control (open histograms) (b). Detection of FLAG-tagged Evi-1 mutants in NIH3T3 cells 48 h following retroviral transduction. Evi-1a, Evi-1c and Evi-1 mutants are detected with the anti-FLAG antibody by Western blot analysis (arrow head) (c).

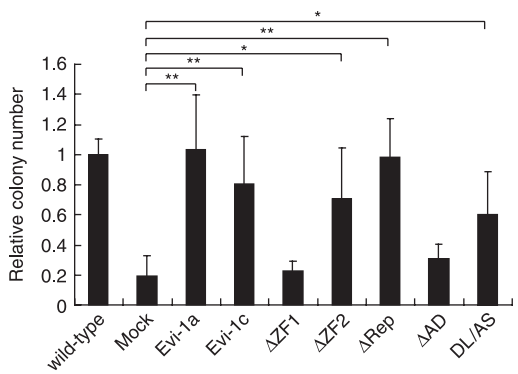


Fig. 3. The first zinc finger domain and the acidic domain of ecotropic viral integration site-1 (Evi-1) are required for hematopoietic proliferation. *Evi-1*-deficieint P-Sp cells were infected with retroviruses containing the Evi-1 mutants, and their colony forming capacity was evaluated. Relative numbers of colonies generated by mutant-transduced cells compared with that of empty vector-transduced *Evi-1*-deficieint P-Sp cells were shown. Evi-1c, ΔZF2, ΔRe,p and DL/AS retain the ability to recover the hematopoietic defect of *Evi-1*-deficieint P-Sp cells, whereas other mutants do not. Error bars indicate SEM of more than three independent experiments, each in duplicate. $*P < 0.05$. $**P < 0.005$.

We further assessed possible interactions between GATA-2 and TGF-β signaling pathways. In order to examine whether LY application or DNSmad3 transduction induces GATA-2 up-regulation, we performed quantitative RT-PCR analysis using wild-type P-Sp cells. Figure 5e shows that neither LY application nor DNSmad3 transduction could up-regulate GATA-2 mRNA expression, indicating that repression of TGF-β does not inhibit GATA-2 expression at a transcriptional level. We then carried out TGF-β-mediated transcriptional response assay to examine the effect of GATA-2 on TGF-β pathways. Interestingly, GATA-2 repressed TGF-β-induced p3TP-Lux activation in HepG2 cells as efficiently as Evi-1,[16] (Fig. 5f), suggesting that the up-regulated GATA-2 expression also contributes to repression of TGF-β signaling by Evi-1.

Discussion

Hematopoiesis is regulated by a large number of key transcriptional factors. As for Evi-1, the recent study showed that Evi-1 regulates HSC proliferation at embryonic stage through GATA-2 up-regulation.[22] In this study, we extended the previous analysis and showed that Evi-1 promotes early hematopoietic proliferation through both the first zinc-finger

doi: 10.1111/j.1349-7006.2008.00842.x

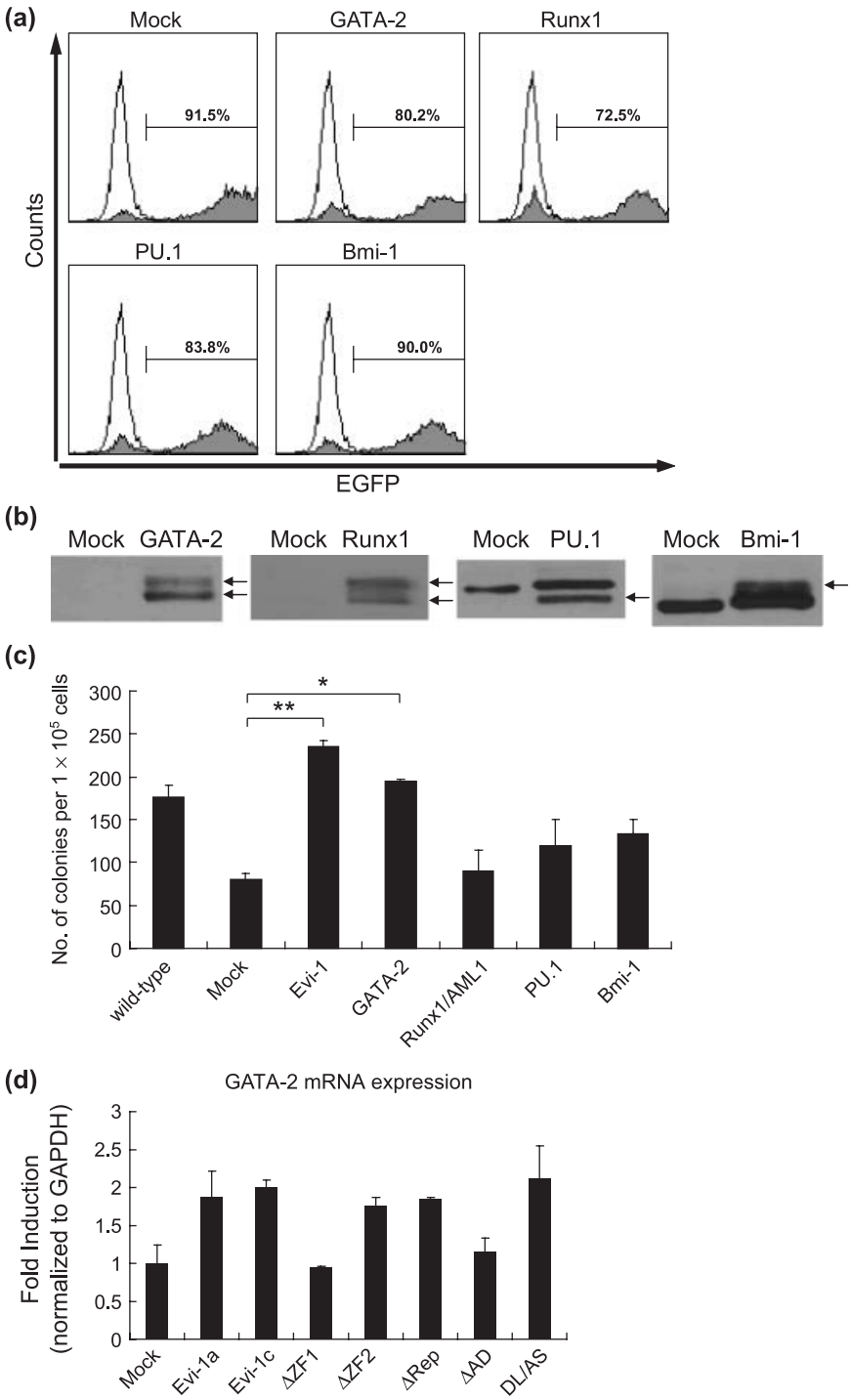


Fig. 4. GATA-2 is one of related transcription factors for ecotropic viral integration site-1 (Evi-1)–mediated P-Sp hematopoiesis. The efficiency of retrovirus-mediated gene transfer of *GATA-2*, *Runx1/AML1*, *PU.1* or *Bmi-1* was estimated by infecting NIH3T3 cells. Retrovirus-infected cells were evaluated by the expression of GFP (shaded histograms). Uninfected NIH3T3 cells are also shown as a control (open histograms) (a). Expression of individual proteins was confirmed by Western blotting (arrow) (b). *Evi-1*-deficieint P-Sp cells were infected with mock retrovirus or retroviruses containing *GATA-2*, *Runx1/AML1*, *PU.1* or *Bmi-1*, and their colony-forming capacity was evaluated. GATA-2-overexpressed *Evi-1*-deficieint P-Sp cells could generate obviously more hematopoietic colonies than mock-infected *Evi-1*-deficieint P-Sp cells. Error bars indicate SEM of two independent experiments, each in duplicate (c). $*P < 0.05$. $**P < 0.005$. Wild-type (*Evi-1*+/+) P-Sp cells were retrovirally transduced with Evi-1 mutants and cultured for 3 days on OP9 stromal cells. The mRNA levels of GATA-2 in these cells were examined. The ability of Evi-1 mutants to up-regulate GATA-2 expression was partly related to its hematopoietic activity. Error bars indicate SEM of two independent experiments, each in triplicate (d).

domain and the acidic domain in the OP9 culture system. These domains are partly related to up-regulation of GATA-2 in P-Sp cells. Furthermore, we demonstrated that enforced expression of GATA-2 and repression of TGF-β signaling could restore the colony-forming capacity of the *Evi-1*-deficient P-Sp cells.

Although Evi-1 has several distinct domains with defined biochemical functions, physiological contributions of these domains have not been elucidated. Using P-Sp culture system, here we have demonstrated that the first zinc-finger domain and the acidic domain of Evi-1 are required to overcome the defective hematopoiesis in the *Evi-1*-deficient P-Sp culture, while the second zinc-finger domain, the repression domain or the CtBP-binding domain, is dispensable. We also found that Evi-1 mutants that retain hematopoietic activity have a greater tendency to increase GATA-2 expression in P-Sp cells than the mutants lacking hematopoietic activity, although the difference was not statistically significant. In addition, among several genes involved in hematopoiesis, only GATA-2 could recover efficiently the hematopoietic defect of *Evi-1*-deficient P-Sp cells. Thus, GATA-2 up-regulation seems important in Evi-1-mediated hematopoiesis.

Interestingly, Evi-1c, a PR-domain-containing form of Evi-1, is as potent as Evi-1a in hematopoietic recovery of *Evi-1*-deficient

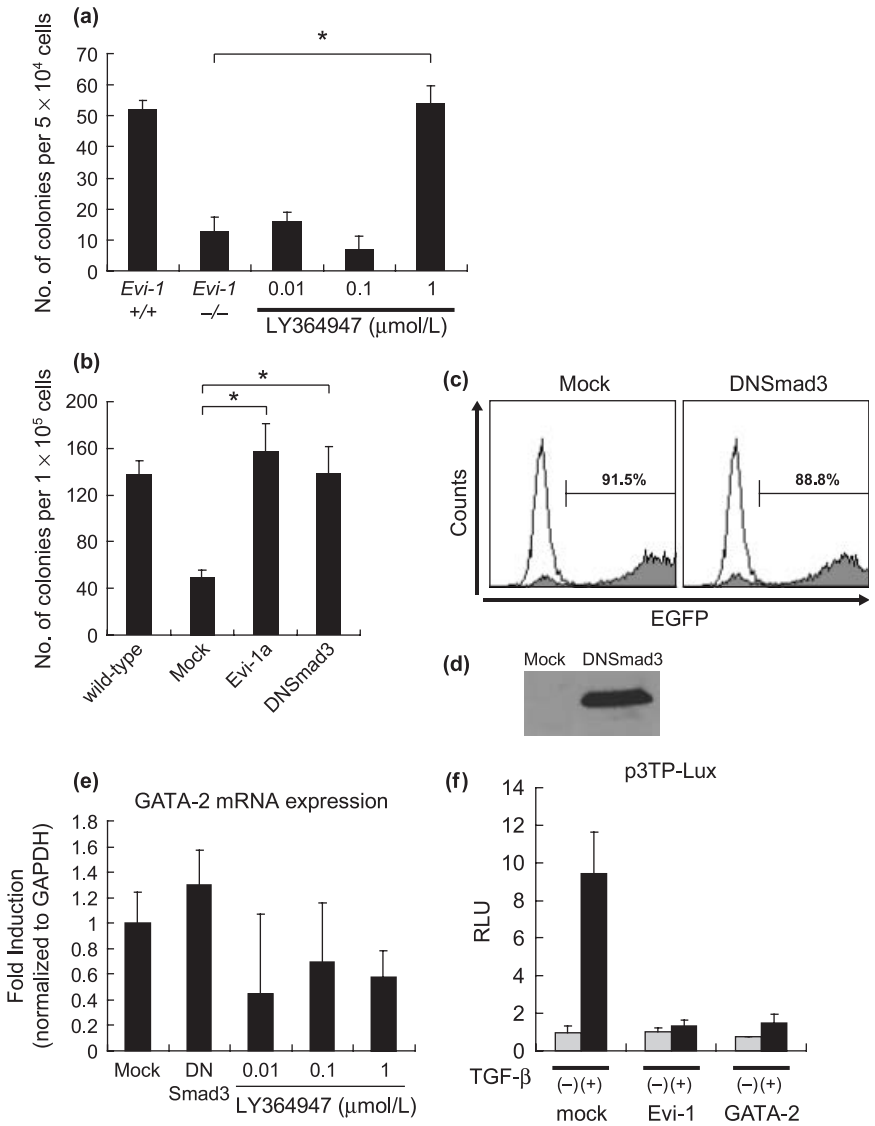


Fig. 5. Repression of transforming growth factor-β (TGF-β) signaling can overcome the defective colony formation capacity of ecotropic viral integration site-1 (*Evi-1*)-deficient P-Sp region. *Evi-1–/–* P-Sp cells were cultured in the absence or presence of LY364947 (LY) and seeded in M3434 media. Application of 1 μmol/L LY restored the colony-forming capacity of *Evi-1*-deficieint P-Sp cells. Error bars indicate SEM of two independent experiments, each in duplicate (a). $*P < 0.05$. Retrovirally transduced dominant-negative-type Smad3 also restored colony forming capacity of *Evi-1*-deficieint P-Sp cells. Error bars indicate SEM of two independent experiments, each in duplicate (b). $*P < 0.05$. FACS analysis shows EGFP expression (shaded histogram) in NIH3T3 cells infected with viral supernatants. Uninfected NIH3T3 cells are also shown as a control (open histogram) (c). Flag-tagged DNSmad3 protein is detected by Western blotting (d). LY364947-treated or DNSmad3-transduced wild-type P-Sp cells were harvested on day 3 of culture and GATA-2 mRNA expression in these cells was examined by quantitative real-time polymerase chain reaction. LY application or DNSmad3 transduction has little effect on up-regulation of GATA-2 expression. Error bars indicate SEM of two independent experiments, each in triplicate (e). Luciferase assays showed that TGF-β-mediated transcriptional responses are repressed by GATA-2. p3TP-Lux was cotransfected into HepG2 cells with empty vector (mock), Evi-1 or GATA-2 in the absence (gray bars) or presence (black bars) of 200 pmol/L TGF-β. Error bars indicate SEM of two independent experiments, each in duplicate (f).

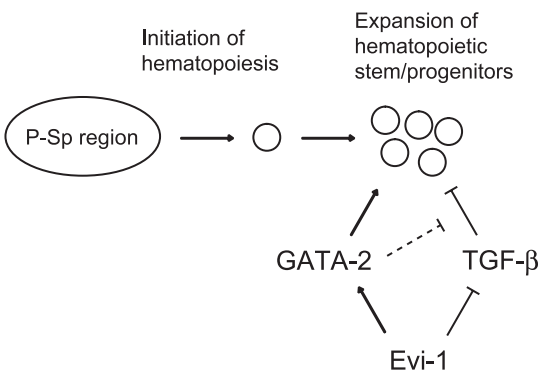


Fig. 6. A scheme for ecotropic viral integration site-1 (Evi-1)–mediated early hematopoiesis. In early hematopoiesis, Evi-1 acts as a key factor to regulate the expansion of hematopoietic stem/progenitor cells through GATA-2 up-regulation and repression of transforming growth factor-β (TGF-β) signaling.

P-Sp cells. Although available evidence suggests that Evi-1c on its own does not exert an oncogenic effect on hematopoietic cells, our finding indicates that Evi-1 proteins have a redundant role in early hematopoietic development. The mutant mice selectively deficient in Evi-1c will be useful to clarify the role of Evi-1c in normal hematopoietic development.

TGF-β is known to inhibit hematopoietic colony formation,[32] and Evi-1 has been shown to antagonize the growth-inhibitory effects of TGF-β by interacting with Smad3.[16] We therefore investigated the role of TGF-β signaling in P-Sp hematopoiesis using ALK5 inhibitor or dominant-negative type Smad3. Blocking of TGF-β signaling with these methods could recover the defective hematopoiesis in the *Evi-1*-deficient P-Sp culture, suggesting that excessive TGF-β signaling is responsible for the reduced colony forming capacity of *Evi-1*-deficient P-Sp cells. However, it should be noted that the hematopoietic potential of Evi-1 mutants is not strictly correlated with its ability of TGF-β inhibition. Evi-1c, Evi-1ΔRep, and Evi-1-DL/AS have the reduced repression ability of TGF-β signaling while they possess normal hematopoietic activity. Conversely, Evi-1ΔZF2 and Evi-1ΔAD can repress TGF-β signaling, but they showed reduced hematopoietic activity. This discrepancy is probably due to the involvement of other pathways, including the GATA-2 pathway, in Evi-1-mediated hematopoiesis, or due to the extent of TGF-β inhibition.

From these findings, we consider that Evi-1 promotes early hematopoietic stem/progenitor expansion through two pathways: up-regulation of GATA-2 and repression of TGF-β signaling (Fig. 6). These two pathways may interact with

doi: 10.1111/j.1349-7006.2008.00842.x

each other. Indeed, we showed that GATA-2 could repress TGF-β signaling by a luciferase assay. In addition, it is possible that TGF-β alters GATA-2 activity at a post-transcriptional level,[33,34] although our data indicate that repression of TGF-β did not induce GATA-2 expression in P-Sp cells at a transcriptional level. Thus, both of these pathways regulated by Evi-1 might cooperatively contribute to early hematopoietic development during normal embryogenesis. Transgenic expression of GATA-2 under the control of an appropriate promoter in *Evi-1*-deficient background, or mice with a combined deficiency of Evi-1 and Smad3, may reveal further the *in vivo* mechanism underlying *Evi-1*-mediated hematopoiesis.

Acknowledgments

The authors thank T Kitamura (The Institute of Medical Science, University of Tokyo, Japan) for the Plat-E packaging cells and pMYs/IRES-EGFP retrovirus vector; Kirin Brewery Pharmaceutical Research Laboratory for cytokines; H Nakauchi (The Institute of Medical Science, University of Tokyo, Japan) for pGCsam expression vector; T Nakano (Osaka University) for stromal cell line OP9; A Iwama (Chiba University) for Bmi-1; M Kano and K Miyazono (Department of Molecular Pathology, University of Tokyo, Japan) for LY364947, and R Derynck (University of California at San Francisco) for Smad3ΔSSVS. This work was supported by a Grant-in Aid for Scientific Research from the Japan Society for the Promotion of Science, and by Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare.

References

1 Mucenski ML, Taylor BA, Ihle JN *et al*. Identification of a common ecotropic viral integration site, Evi-1, in the DNA of AKXD murine myeloid tumors. *Mol Cell Biol* 1988; **8**: 301–8.
2 Ogawa S, Mitani K, Kurokawa M *et al*. Abnormal expression of Evi-1 gene in human leukemias. *Hum Cell* 1996; **9**: 323–32.
3 Ogawa S, Kurokawa M, Tanaka T *et al*. Structurally altered Evi-1 protein generated in the 3q21q26 syndrome. *Oncogene* 1996; **13**: 183–91.
4 Suzukawa K, Parganas E, Gajjar A *et al*. Identification of a breakpoint cluster region 3′ of the ribophorin I gene at 3q21 associated with the transcriptional activation of the EVI1 gene in acute myelogenous leukemias with inv (3) (q21q26). *Blood* 1994; **84**: 2681–8.
5 Nucifora G. The EVI1 gene in myeloid leukemia. *Leukemia* 1997; **11**: 2022–31.
6 Buonamici S, Chakraborty S, Senyuk V, Nucifora G. The role of EVI1 in normal and leukemic cells. *Blood Cells Mol Dis* 2003; **31**: 206–12.
7 Pintado T, Ferro MT, San Roman C, Mayayo M, Larana JG. Clinical correlations of the 3q21;q26 cytogenetic anomaly. A leukemic or myelodysplastic syndrome with preserved or increased platelet production and lack of response to cytotoxic drug therapy. *Cancer* 1985; **55**: 535–41.
8 Hirai H. The transcription factor Evi-1. *Int J Biochem Cell Biol* 1999; **31**: 1367–71.
9 Buyse IM, Shao G, Huang S. The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein. *Proc Natl Acad Sci USA* 1995; **92**: 4467–71.
10 Huang S. Blimp-1 is the murine homolog of the human transcriptional repressor PRDI-BF1. *Cell* 1994; **78**: 9.
11 Huang S. Histone methyltransferases, diet nutrients and tumour suppressors. *Nat Rev Cancer* 2002; **2**: 469–76.
12 Jiang GL, Huang S. The yin-yang of PR-domain family genes in tumorigenesis. *Histol Histopathol* 2000; **15**: 109–17.
13 Lugthart S, Drunen EV, Norden YV *et al*. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. *Blood* 2008; **111**: 4329–37.
14 Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL *et al*. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. *Blood* 2003; **101**: 837–45.
15 Nucifora G, Laricchia-Robbio L, Senyuk V. EVI1 and hematopoietic disorders. history and perspectives. *Gene* 2006; **368**: 1–11.
16 Kurokawa M, Mitani K, Irie K *et al*. The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* 1998; **394**: 92–6.
17 Kurokawa M, Mitani K, Yamagata T *et al*. The evi-1 oncoprotein inhibits c-Jun N-terminal kinase and prevents stress-induced cell death. *EMBO J* 2000; **19**: 2958–68.
18 Tanaka T, Nishida J, Mitani K, Ogawa S, Yazaki Y, Hirai H. Evi-1 raises AP-1 activity and stimulates c-fos promoter transactivation with dependence on the second zinc finger domain. *J Biol Chem* 1994; **269**: 24 020–6.
19 Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H. The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling. *Blood* 2001; **97**: 2815–22.
20 Nitta E, Izutsu K, Yamaguchi Y *et al*. Oligomerization of Evi-1 regulated by the PR domain contributes to recruitment of corepressor CtBP. *Oncogene* 2005; **24**: 6165–73.
21 Hoyt PR, Bartholomew C, Davis AJ *et al*. The Evi1 proto-oncogene is required at midgestation for neural, heart, and paraxial mesenchyme development. *Mech Dev* 1997; **65**: 55–70.
22 Yuasa H, Oike Y, Iwama A *et al*. Oncogenic transcription factor Evi1 regulates hematopoietic stem cell proliferation through GATA-2 expression. *EMBO J* 2005; **24**: 1976–87.
23 Goyama S, Yamaguchi Y, Imai Y *et al*. The transcriptionally active form of AML1 is required for hematopoietic rescue of the AML1-deficient embryonic para-aortic splanchnopleural (P-Sp) region. *Blood* 2004; **104**: 3558–64.
24 Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 1994; **265**: 1098–101.
25 Kumano K, Chiba S, Shimizu K *et al*. Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. *Blood* 2001; **98**: 3283–9.
26 Nakagawa M, Ichikawa M, Kumano K *et al*. AML1/Runx1 rescues Notch1-null mutation-induced deficiency of para-aortic splanchnopleural hematopoiesis. *Blood* 2006; **108**: 3329–34.
27 Choy L, Skillington J, Derynck R. Roles of autocrine TGF-beta receptor and Smad signaling in adipocyte differentiation. *J Cell Biol* 2000; **149**: 667–82.
28 Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000; **7**: 1063–6.
29 Kurokawa M, Tanaka T, Tanaka K *et al*. Overexpression of the AML1 proto-oncoprotein in NIH3T3 cells leads to neoplastic transformation depending on the DNA-binding and transactivational potencies. *Oncogene* 1996; **12**: 883–92.
30 Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. *Blood* 2000; **96**: 2022–36.
31 Kano MR, Bae Y, Iwata C *et al*. Improvement of cancer-targeting therapy, using nanocarriers for intractable solid tumors by inhibition of TGF-{beta} signaling. *Proc Natl Acad Sci USA* 2007; **104**: 3460–65.
32 Sitnicka E, Ruscetti FW, Priestley GV, Wolf NS, Bartelmez SH. Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. *Blood* 1996; **88**: 82–8.
33 Towatari M, May GE, Marais R *et al*. Regulation of GATA-2 phosphorylation by mitogen-activated protein kinase and interleukin-3. *J Biol Chem* 1995; **270**: 4101–7.
34 Hayakawa F, Towatari M, Ozawa Y, Tomita A, Privalsky ML, Saito H. Functional regulation of GATA-2 by acetylation. *J Leukoc Biol* 2004; **75**: 529–40.