

in pediatric steroid-refractory aGVHD in Canada and New Zealand as a cell-based medicine [24].

We confirmed that third party-derived bone marrow MSCs are safe and effective for patients with steroid-resistant aGVHD. In our study, only MSCs were given to patients with steroid-refractory GVHD as soon as possible after the diagnosis of steroid-refractory GVHD. Since the cell dose of infused MSCs was constant and the number of MSC infusions was strictly scheduled, our results are reliable to estimate the effects of MSCs on steroid-resistant aGVHD. A high CR rate and good OS were obtained. Of note, gut aGVHD comprised 71 % (10 of 14 patients) of our patients and all the patients except one showed CR. These results are consistent with others, i.e., MSCs have a favorable clinical effect on gut aGVHD [7, 12, 23]. Our trials did not include patients with grade IV aGVHD. Therefore, the good results in our studies may have been overestimated. As in other reports, no apparent adverse effects associated with MSC therapy were observed in short-term and long-term observations.

The presence of fetal bovine serum is necessary for standard conditions for MSC expansion [25]. However, it is better not to use animal products to avoid unknown infections and other complications. von Bonin et al. and Lucchini et al. showed the usefulness of platelet-lysate-expanded bone marrow MSCs for steroid-refractory aGVHD [13, 15]. Alternatively, MSC donor serum can be used for MSC expansion. Arima et al. and Pérez-Simon et al. successfully treated steroid-refractory aGVHD with bone marrow MSCs expanded in a medium supplemented with autologous serum [16, 18]. It is not known which MSC culture is the best in terms of the safety and growth of MSCs. Ideally, a serum-free culture of MSCs should be introduced in a clinical setting [26].

After the completion of the JCR-031-201 and JCR-031-202 trials, we started a phase III trial using JR-031 focusing on steroid-refractory grade III or IV aGVHD. In the near future, the results of this study will be published.

**Conflict of interest** K. Muroi and K. Ozawa received payment for consultancy from JCR Pharmaceuticals Co., Ltd. Other authors declare no conflicts of interest.

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# The Satb1 Protein Directs Hematopoietic Stem Cell Differentiation toward Lymphoid Lineages

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## SUMMARY

How hematopoietic stem cells (HSCs) produce particular lineages is insufficiently understood. We searched for key factors that direct HSC to lymphopoiesis. Comparing gene expression profiles for HSCs and early lymphoid progenitors revealed that *Satb1*, a global chromatin regulator, was markedly induced with lymphoid lineage specification. HSCs from *Satb1*-deficient mice were defective in lymphopoietic activity in culture and failed to reconstitute T lymphopoiesis in wild-type recipients. Furthermore, *Satb1* transduction of HSCs and embryonic stem cells robustly promoted their differentiation toward lymphocytes. Whereas genes that encode *Ikaros*, *E2A*, and *Notch1* were unaffected, many genes involved in lineage decisions were regulated by *Satb1*. *Satb1* expression was reduced in aged HSCs with compromised lymphopoietic potential, but forced *Satb1* expression partly restored that potential. Thus, *Satb1* governs the initiating process central to the replenishing of lymphoid lineages. Such activity in lymphoid cell generation may be of clinical importance and useful to overcome immunosenescence.

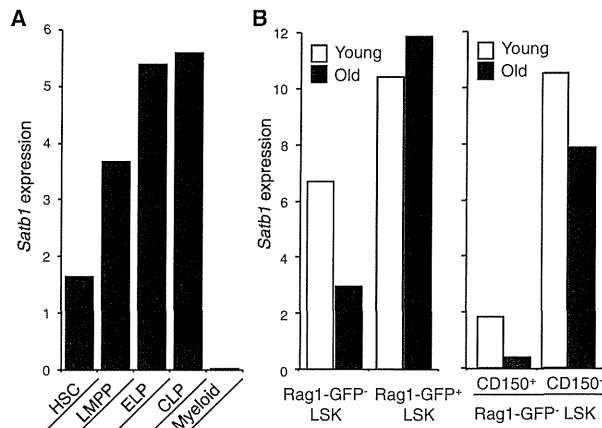
## INTRODUCTION

To maintain the immune system, hematopoietic stem cells (HSCs) differentiate to lymphoid-primed multipotent progenitors (LMPPs) and then to lymphoid-specified progenitors in a process accompanied by the loss of erythroid-megakaryocyte and myeloid potential (Adolfsson et al., 2005; Lai and Kondo, 2008). Accumulating evidence has suggested that combinations of transcription factors coordinately and sequentially

regulate lymphopoiesis. Five transcription factors, PU.1, *Ikaros*, *E2A*, *EBF*, and *Pax5* are hierarchically involved in the early steps of B-lineage differentiation (Medina et al., 2004). Whereas *EBF* and *Pax5* specifically act in B-lineage-determined progenitors, PU.1 and *Ikaros* are expressed in earlier hematopoietic progenitors and involved in multiple lineage decision processes (Scott et al., 1997; Yoshida et al., 2006). *E2A*, an indispensable factor for B lymphopoiesis, can also affect T lymphocyte formation by regulating *Notch1* expression (Ikawa et al., 2006). Furthermore, recent reports have shown that *E2A* proteins are expressed in primitive hematopoietic progenitors and play a critical role in early lymphoid specification (Dias et al., 2008; Yang et al., 2008; Semerad et al., 2009). However, whether the initiation of lymphoid differentiation is regulated entirely by transcription factors in a hierarchical manner remains unclear.

The immune system changes qualitatively and quantitatively with ontogeny and age (Miller and Allman, 2005; Montecino-Rodriguez and Dorshkind, 2006). Indeed, lymphocyte progenitors expand substantially in the fetal liver (FL), but their production shifts to bone marrow (BM) and becomes stable after birth. With age, replenishment of the adaptive immune system declines (Rossi et al., 2005; Sudo et al., 2000). Qualitative changes in lymphopoietic activity of HSCs are reflected in *in vitro* cell-culture experiments. If key inducers in early lymphoid lineages can be identified, they will be useful for expanding lymphocytes in culture for clinical purposes. Additionally, manipulating the expression of relevant genes might boost the immune system of immunocompromised and elderly people.

We have developed a method to sort early lymphoid progenitors (ELPs) from Rag1-GFP reporter mice (Igarashi et al., 2002; Yokota et al., 2003a). ELPs expressing Rag1 are present in the Sca1<sup>+</sup>c-kit<sup>hi</sup> HSC-enriched fraction; they displayed high B and T lymphopoietic potential, but limited myeloerythroid potential and self-renewal ability. In contrast, Rag1<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>hi</sup> HSCs effectively reconstitute and sustain the lymphohematopoietic system for long periods in lethally irradiated recipients. We conducted gene array comparisons between those two fractions



**Figure 1. *Satb1* Expression Levels Change with Differentiation and Aging of HSCs**

HSCs, LMPP, ELP, CLP, and the myeloid progenitor-enriched fractions were sorted from BM of 8- to 10-week-old Rag1-GFP knockin or WT mice according to cell surface markers and GFP expression (see Experimental Procedures), and transcripts for *Satb1* were quantitatively evaluated with real-time RT-PCR. (B) The LSK Rag1-GFP<sup>-</sup> and LSK Rag1-GFP<sup>+</sup> fractions (left panel) or the CD150<sup>+</sup> LSK Rag1-GFP<sup>-</sup> and CD150<sup>-</sup> LSK Rag1-GFP<sup>-</sup> fractions (right panel) were sorted from 6-week-old or 2-year-old Rag1-GFP knockin mice, respectively. Then *Satb1* expression was evaluated with real-time RT-PCR. The *Satb1* expression values were normalized by *Gapdh* expression and shown in each panel. Each data represents two independent examinations that showed essentially the same results (Figure 1; see also Figure S1 and Table S1).

with the goal of discovering molecules involved in the transition of HSCs to lymphoid lineages.

Herein, we showed that special AT-rich sequence binding 1 (*Satb1*), a nuclear architectural protein that organizes chromatin structure, plays an important role in lymphoid lineage specification. In parallel with or ahead of key transcription factors, the expression of *Satb1* increased with early lymphoid differentiation. In functional assays, lymphopoietic activity was compromised in *Satb1*-deficient hematopoietic cells, but the induced expression of *Satb1* strongly enhanced lymphocyte production from HSCs. Furthermore, exogenous *Satb1* expression primed lymphoid potential even in embryonic stem cell (ESC)-derived mesoderm cells and aged BM-derived HSCs. Global analysis of potential *Satb1* target genes identified a number that may have critical roles in early lymphopoiesis. The findings demonstrate that the earliest steps in lymphopoiesis are regulated by an epigenetic modifier and indicate how modulation of the process might be used to induce or rejuvenate the immune system.

## RESULTS

### Profiling Gene Expression of Rag1<sup>+</sup> ELP in Fetal Liver

We sorted the Rag1<sup>lo</sup> c-kit<sup>hi</sup> Sca1<sup>+</sup> ELP fraction and the Rag1<sup>-</sup> c-kit<sup>hi</sup> Sca1<sup>+</sup> HSC-enriched fraction with high purity from E14.5 FL of Rag1-GFP knockin heterozygous embryos and performed gene arrays. We found that transcripts of *Trbv14* and *Ighm* genes were upregulated even in very early lymphoid progenitors (see Table S1 available online). Furthermore, we detected increased expression of *Il7r*, *Notch1*, and *Flt3* genes encoding cell surface

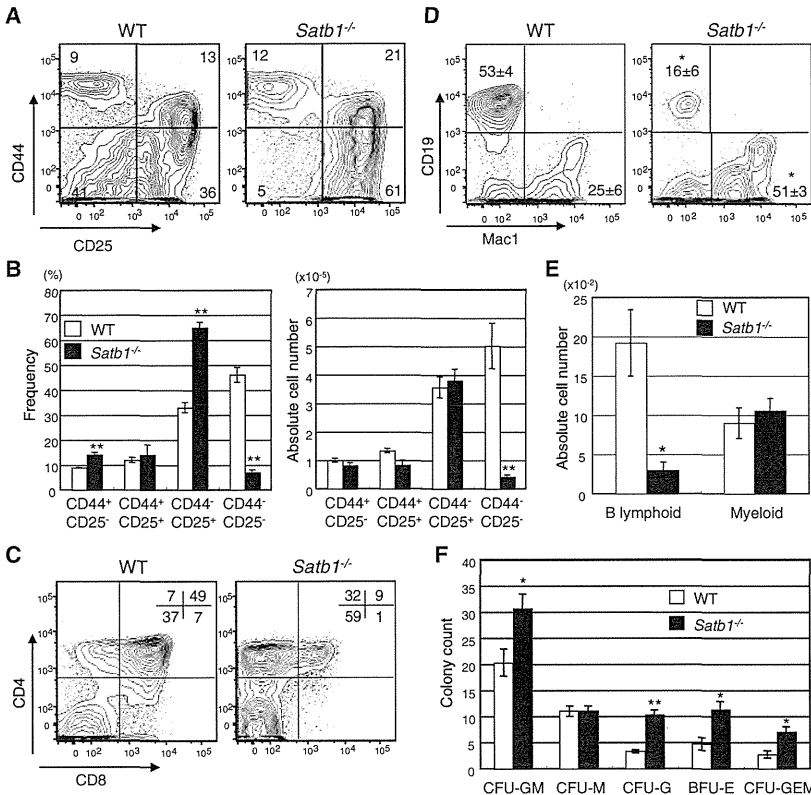
receptors important for B or T lymphocyte differentiation in the ELP fraction. In addition to discovering many signal transduction kinases with unknown functions in lymphopoiesis, our search identified *Lck* and *Xlr4b* genes as being involved in lymphoid differentiation signals. Transcripts for some of these lymphoid-related genes had already been detected in the Rag1<sup>-</sup> HSC-enriched fraction (see the microarray data; accession number CBX73). These results suggest that lymphoid-lineage specification begins even before the emergence of Rag1<sup>lo</sup> ELP. Additionally, the microarray data identified new candidate genes that might be important for early lymphoid development.

### Expression of *Satb1* Increases with Early Lymphoid Specification and Declines with Age

Our major goal was to find key genes involved in the specification of lymphoid fates. Because the microarray data showed that expression of various lymphoid-related genes was activated before the ELP stage, we hypothesized the existence of a modulator that synchronously regulates multiple genes. Among the list in Table S1, *Satb1* attracted attention because it was originally identified as a protein binding to the enhancer region of the *Igh* gene and later shown to play a critical role in T cell development (Alvarez et al., 2000; Dickinson et al., 1992). Additionally, recent studies had demonstrated that it serves as a master regulator for many genes, including cytokines, cytokine receptors, and transcription factors (Cai et al., 2006; Han et al., 2008; Notani et al., 2010; Yasui et al., 2002).

To explore possible relationships between *Satb1* and early lymphopoiesis, we examined its expression in primitive hematopoietic progenitors. The HSC-enriched Rag1-GFP<sup>-</sup> Flt3<sup>-</sup> lineage marker-negative (Lin<sup>-</sup>) Sca1<sup>+</sup> c-kit<sup>hi</sup> (LSK) fraction, the LMPP-enriched fraction, the ELP-enriched fraction, the common lymphoid progenitor (CLP)-enriched fraction, and the myeloid progenitor-enriched Lin<sup>-</sup> c-kit<sup>hi</sup> Sca1<sup>-</sup> fraction were sorted from BM of 8- to 10-week-old mice. Transcripts for *Satb1* were then quantitatively evaluated with real-time RT-PCR. *Satb1* expression increased substantially when HSC differentiated into LMPP and ELP (Figure 1A). This trend matched that of other early lymphoid lineage-related genes including those that encode PU.1 (*Sfp1*), Ikaros (*Ikzf1*), E2A (*Tcf3*), and Notch1 (Figure S1). Importantly, in contrast to its expression in the lymphoid lineage, *Satb1* expression was shut off when HSC differentiated to committed myeloid progenitors. These results suggest that *Satb1* is potentially involved in early lymphoid differentiation.

Lymphopoietic activity becomes compromised during aging. Accumulating evidence suggests that the earliest lymphoid progenitor pools proximal to HSC are deficient in aged BM (reviewed by Miller and Allman, 2005). Indeed, the Rag1<sup>+</sup> ELP population markedly decreases with age (data not shown). The downregulation of genes mediating lymphoid specification and function is likely a major cause (Rossi et al., 2005). Because *Satb1* has been listed in microarray panels as a downregulated gene in aged HSC (Chambers et al., 2007; Rossi et al., 2005), we sorted Rag1-GFP<sup>-</sup> LSK and ELP-enriched Rag1-GFP<sup>+</sup> LSK from BM of 6-week-old or 2-year-old Rag1-GFP heterozygous mice and examined their expression. In agreement with previous studies, our real-time RT-PCR identified an approximate 50% reduction of *Satb1* transcripts in aged Rag1-GFP<sup>-</sup> LSK cells (Figure 1B, left panel). The few ELP recovered from aged mice



**Figure 2. *Satb1* Deficiency Alters Lymphoid and Myeloid Activities of Hematopoietic Stem/Progenitor Cells in Culture**

Lin<sup>-</sup> cells were isolated from FL of E14.5 *Satb1*<sup>-/-</sup> embryos or their WT littermates.

(A–C) Cells were cocultured with OP9-DL1 stromal cells for evaluation of T-lineage differentiation. (A) Flow cytometry results are shown for cells recovered on day 14 and stained for CD44 and CD25/IL-2R $\alpha$ . (B) Frequencies and absolute numbers of each phenotype were calculated. (C) A similar analysis was performed for CD4 and CD8 $\alpha$  bearing cells recovered on day 18.

(D and E) The same cell suspensions were cocultured with MS5 stromal cells to assess B and myeloid lineage potentials and representative data are shown for day 7 of culture.

(F) In parallel, the Lin<sup>-</sup> cells were evaluated with methylcellulose colony assays. Each dish contained 1,000 sorted cells and colony counts were performed on day 10. The bars indicate numbers of CFU-GM, CFU-M, CFU-G, BFU-E, or CFU-GEM scored per dish. The results are shown as mean  $\pm$  SE. Statistically significant differences between WT and *Satb1*<sup>-/-</sup> cells are marked with asterisks (\* $p$  < 0.05, \*\* $p$  < 0.01) (Figure 2; see also Figure S2).

expressed amounts of *Satb1* comparable to those in ELP from young mice. Recent purification methods for HSC with CD150, a SLAM family receptor that marks HSC even in aged BM (Yilmaz et al., 2006), identified an approximate 80% reduction in *Satb1* transcripts in aged HSC compared with ones from young mice (Figure 1B, right panel). These observations suggest that *Satb1* may be a key molecule related to immunosenescence.

**Satb1 Deficiency Reduces the Lymphopoietic Activity of Hematopoietic Stem and Progenitor Cells**

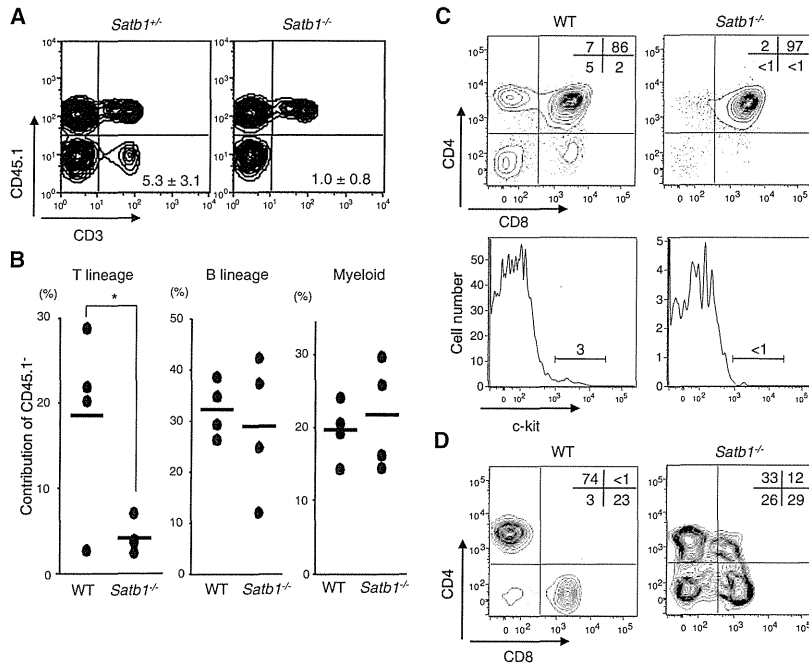
T cell development in the thymus is impaired in *Satb1*<sup>-/-</sup> mice (Alvarez et al., 2000). Although the profile of B220, immunoglobulin M (IgM), and IgD expression appears to be unaffected in the *Satb1*<sup>-/-</sup> spleen, the total number of B cells is reportedly reduced to approximately 25% of wild-type (WT) at 2 weeks of age (Alvarez et al., 2000). We have determined that the number and frequency of cells that can be recovered from lymphoid organs were reduced in E18.5–19.5 *Satb1*<sup>-/-</sup> fetuses. Body sizes of *Satb1*<sup>-/-</sup> fetuses were not different from WT and heterozygous littermates (Figures S2A and S2B).

We then sorted Lin<sup>-</sup> cells from FL of *Satb1*<sup>-/-</sup> mice or their WT littermates and cultured them with stromal cells that support lymphopoiesis. T cell differentiation can be recapitulated in vitro with hematopoietic cells cultured with OP9 expressing the Notch ligand Delta-like 1 (OP9-DL1). Under these coculture conditions, the differentiation patterns of WT and *Satb1*<sup>-/-</sup> Lin<sup>-</sup> cells differed significantly (Figures 2A and 2B). The majority of *Satb1*<sup>-/-</sup> cells were arrested in the CD44<sup>-</sup>CD25<sup>+</sup> stage and did not differentiate

into CD44<sup>-</sup>CD25<sup>-</sup> cells. Reduction of IL-7 from culture media normally induces maturation of the CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) into CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) cells and subsequently into either the CD4<sup>+</sup> or the CD8<sup>+</sup> single-positive cells. However, more than half of the *Satb1*<sup>-/-</sup> cells were arrested in DN stages even after the IL-7 reduction, and their differentiation to the DP stage was aberrantly skewed toward CD4<sup>+</sup>CD8<sup>-</sup> (Figure 2C).

Substantial differences were also observed in B-lineage cell production. In coculture with MS5, which supports B and myeloid lineages in the presence of SCF, Flt3-ligand, and IL-7, *Satb1*<sup>-/-</sup> progenitors exhibited significant reductions in B-lymphopoietic potential (Figures 2D and 2E). Coculture with OP9, which originated with M-CSF-deficient mice and supported the B lineage predominantly, also yielded reduced B/myeloid ratios with *Satb1*<sup>-/-</sup> progenitors (Figure S2C). Essentially the same results were obtained when cultures were initiated with LSK Flt3<sup>-</sup>, more stringently purified HSC (Figure S2D, 2E). In addition, B cell lineage output was also reduced when *Satb1*<sup>-/-</sup> LMPP or CLP were cultured (Figure S2F). In contrast, the myeloid potential was retained in *Satb1*<sup>-/-</sup> progenitors (Figures 2D and 2E). Indeed, the Lin<sup>-</sup> fraction of E14.5 *Satb1*<sup>-/-</sup> FL contained more myeloid-erythroid progenitors than that of the WT control (Figure 2F).

In transplantation experiments, we observed that CD45.2<sup>+</sup> *Satb1*<sup>-/-</sup> HSC sorted from 2-week-old BM did not effectively reconstitute CD3<sup>+</sup> T-lineage cells in lethally irradiated CD45.1<sup>+</sup> WT recipients (Figure 3A). Peripheral blood CD3<sup>+</sup> T-lineage recoveries from *Satb1*<sup>-/-</sup> HSC were decreased approximately 90% compared with that from WT HSC (Figure 3B). Conversely, we observed varied amounts of reconstitution of the B lineage and no reduction in reconstitution of the myeloid lineage



**Figure 3. Defective T Lymphopoiesis from Transplanted *Satb1*<sup>-/-</sup> HSC**

(A and B) One thousand stem-cell-enriched Flt3<sup>-</sup> LSK cells were sorted from BM of 2-week-old *Satb1* deficient or littermate mice (CD45.2). They were then mixed with  $4 \times 10^5$  adult BM cells obtained from WT (CD45.1) mice and were transplanted into lethally irradiated WT CD45.1 mice. At 8 weeks after transplantation, peripheral blood cells of the recipients were identified with anti-CD45.1 and anti-CD3. Numbers in each panel of (A) represent percentages of CD3<sup>+</sup> CD45.1<sup>+</sup> cells among the total leukocytes and are shown as averages with SD (n = 4 in each). Chimerisms of CD45.1<sup>-</sup> cells in the CD3<sup>+</sup> T-lineage, the CD45R/B220<sup>+</sup> B lineage, or the Gr1<sup>+</sup> myeloid lineage were determined. Statistical significance is \*p < 0.05.

(C and D) One thousand Flt3<sup>-</sup> LSK cells sorted from E14.5 FL of *Satb1* homozygous or their WT littermates (CD45.2) were transplanted into lethally irradiated WT CD45.1 mice. At 8 weeks after transplantation, T-lineage reconstitution in the thymus and the spleen was analyzed. The CD4 and CD8 profiles of CD45.2<sup>+</sup> thymocytes (C, upper panels) and the c-kit expression of CD45.2<sup>+</sup> CD3<sup>-</sup> CD4<sup>-</sup> CD8<sup>-</sup> CD44<sup>+</sup> CD25<sup>-</sup> thymocytes (C; lower panels) are shown. (D) Representative CD4 and CD8 profiles are shown for CD45.2<sup>+</sup> CD3<sup>+</sup> cells in recipient spleens.

resulted from *Satb1* ablation (Figure 3B). Compromised T cell lineage contributions of *Satb1*<sup>-/-</sup> HSC were also evident in the thymus and spleen (Figures 3C and 3D). Although T lymphopoiesis in the thymus was replaced by either WT or *Satb1*<sup>-/-</sup> donor cells when FL HSCs were transplanted, thymocytes were reduced in the *Satb1*<sup>-/-</sup> recipients and their differentiation was affected. Besides apparent stagnation at the DP stage and marked reduction of the DN population (Figure 3C, upper panels), c-kit<sup>hi</sup> cells in the CD44<sup>+</sup>CD25<sup>-</sup> DN1 stage were rare in *Satb1*<sup>-/-</sup> recipients (Figure 3C, lower panels). The reduced contribution of *Satb1*<sup>-/-</sup> cells was also evident in CD3<sup>+</sup> splenic T lymphocytes. Interestingly, T cells in the spleens of *Satb1*<sup>-/-</sup> recipients contained substantial percentages of DP and DN cells. Such T cell lineage cells are extremely rare in normal mouse spleens (Figure 3D).

Taken together, these results demonstrate that *Satb1* is indispensable for normal T lymphopoiesis, but not for myelopoiesis. The factor may normally have a lesser role in B-lineage differentiation. Furthermore, our data indicate that abnormalities of lymphoid development observed in *Satb1*<sup>-/-</sup> mice are intrinsic to *Satb1*<sup>-/-</sup> hematopoietic cells.

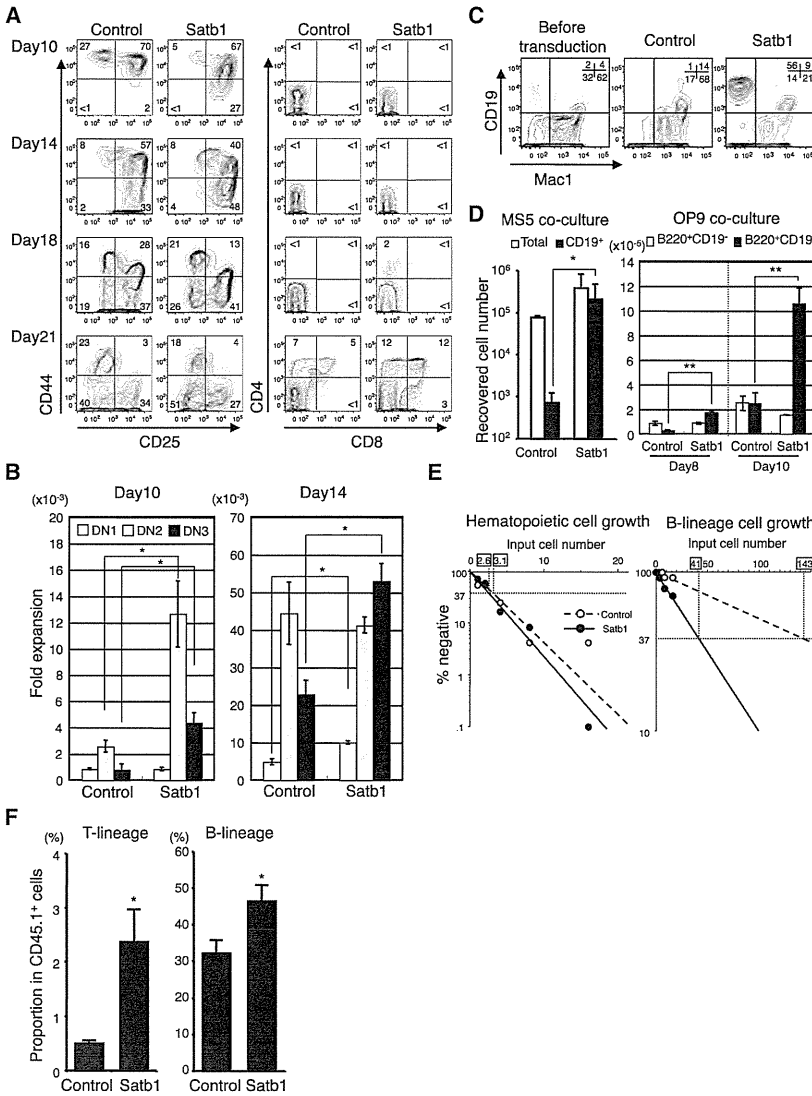
#### Forced Expression of *Satb1* in HSC Induces Lymphopoiesis

Next we conducted overexpression experiments to define the role of *Satb1* in lineage-fate decisions of HSCs. LSK Flt3<sup>-</sup> cells were sorted from BM of adult WT mice and then retrovirally transduced with either a fluorescence-activating protein (FAP) expressing control or a native *Satb1* construct combined with a GFP-expressing vector. Successfully transduced cells were sorted according to GFP expression. Real-time RT-PCR and immunoblots revealed that *Satb1*-transduced cells expressed more than 10-fold

*Satb1* transcripts and *Satb1* proteins compared to control cells (Figure S3A).

The sorted cells were cultured with stromal cells that supported lymphopoiesis. Results from these experiments complemented the observations with *Satb1*<sup>-/-</sup> cells. *Satb1* transduction enhanced T cell lineage growth in OP9-DL1 cocultures (Figures 4A and 4B). By day 10 of the culture, cells had been increased more than 5-fold by *Satb1*-transduction, and a majority of the recovered cells had progressed to the DN2 and DN3 stages. Differentiation to the DP stage was also advanced by the *Satb1*-transduction (Figure 4A). The kinetics of cell differentiation and expansion in the B cell lineage showed more changes. Whereas both control and *Satb1*-transduced cells produced substantial numbers of B-lineage cells, the latter produced B220<sup>+</sup>CD19<sup>+</sup> cells more quickly and efficiently (Figure S3B). Specifically, the *Satb1* transduction resulted in approximately 50- to 300-fold and 5-fold greater recovery of B220<sup>+</sup>CD19<sup>+</sup> cells on day 10 in the MS5 and OP9 cocultures, respectively (Figures 4C and 4D). Notably, *Satb1* transduction negatively influenced the output of myeloid cells, particularly Mac1<sup>lo</sup>Gr1<sup>+</sup> granulocytes (Figure S3C). In addition, CFU-GM formation of HSC was decreased by *Satb1* transduction (Figure S3D).

In stromal-free cultures containing SCF, Flt3-ligand, and IL-7, *Satb1* expression strongly induced CD19<sup>+</sup> cell production from the LSK fraction (Figure S3E). When calculated on a per-cell basis, one LSK cell with *Satb1* overexpression produced approximately 450 CD19<sup>+</sup> cells, whereas only 50 cells with this B-lineage marker were produced from control progenitors. As for other hematopoietic lineages, DX5<sup>+</sup>CD3e<sup>-</sup> NK cells emerged when IL-15 was added to the stromal cell-free cultures. Coexpression of NK1.1 and/or CD94 confirmed the NK-lineage, and



**Figure 4. Satb1 Overexpression Promotes Lymphopoiesis**

LSK  $Flt3^{-}$  cells obtained from WT BM were retrovirally transduced with either a fluorescence-activated cell sorting (FACS) alone expressing control or a native Satb1 combined with GFP expressing vector. Successfully transduced cells were cultured, and their differentiation and proliferation were analyzed at the indicated period.

(A and B) Time-course analyses were performed for T-lineage cell generation in the OP9-DL1 coculture. Absolute numbers of recovered cells were divided by the numbers of transduced LSK  $Flt3^{-}$  cells used to initiate the cultures to obtain the fold expansion values. Data are shown as mean  $\pm$  SE.

(C) CD19 and Mac1 profiles are shown for cells recovered from MS5 cocultures on day 10. The left panel shows data obtained from fresh LSK  $Flt3^{-}$  cells that did not undergo the retroviral infection.

(D) The absolute numbers of total recovered cells and B-lymphoid cells in the MS5 coculture (left panel). The output of  $B220^{+} CD19^{-}$  or  $B220^{+} CD19^{+}$  B-lineage cells was evaluated in the OP9 coculture (right panel). Cultures were established in triplicate. Data are shown as mean  $\pm$  SE. Statistical significance is \* $p < 0.05$ , \*\* $p < 0.01$ .

(E) Limiting-dilution analyses were performed to determine the frequencies of hematopoietic progenitors that could give rise to  $CD19^{+}$  B-lineage cells. Input cell numbers corresponding to each 37% negative value are shown in rectangles.

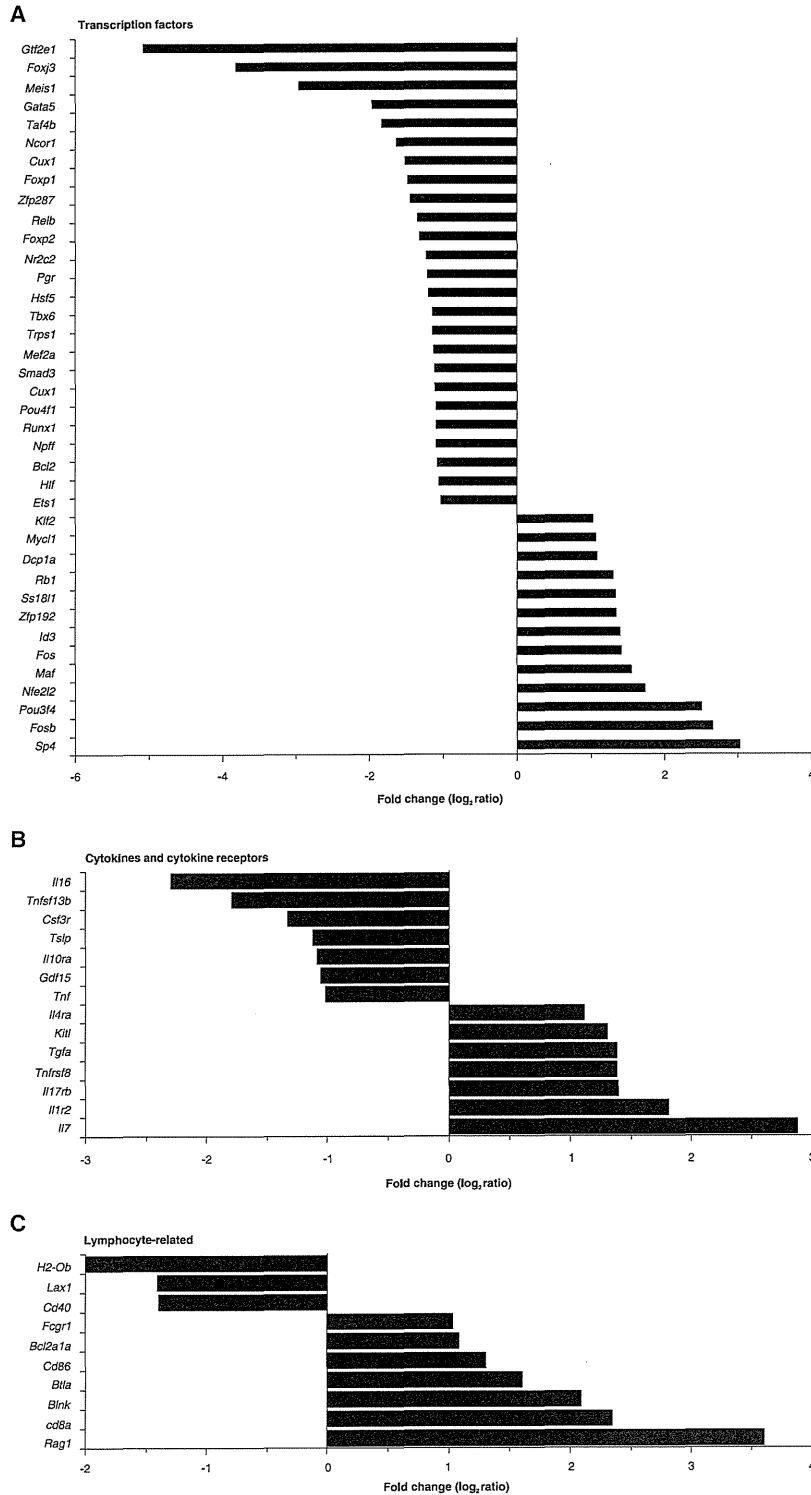
(F) One thousand LSK  $Flt3^{-}$  cells ( $CD45.1$ ) transduced with either Satb1-expressing or control vectors were transplanted to lethally irradiated WT mice ( $CD45.2$ ) with  $1 \times 10^5$  adult BM cells ( $CD45.2$ ). Two weeks after transplantation, peripheral blood was collected to determine the proportion of  $CD4/CD8^{+}$  T lineage and  $CD19^{+}$  B lineage in  $CD45.1^{+}$  cells. Data are shown as mean  $\pm$  SE. Statistical significance is \* $p < 0.05$ . ( $n = 5$  in each group) (Figure 4, see also Figure S3).

their numbers were also enhanced by Satb1 overexpression (Figure S3F). Interestingly, the same Satb1-transduced LSKs differentiated to neither conventional nor plasmacytoid dendritic cells (Figure S3G).

The results from in vitro bulk cultures and assessment of lymphoid lineage cell numbers might reflect enhanced survival of lymphoid progenitors rather than priming or expansion of lymphoid potential in individual clones. Notably, no obvious increase in apoptotic cells occurred in any tested cultures with *Satb1*<sup>-/-</sup> cells or *Satb1*<sup>-/-</sup> lymphopoietic organs (data not shown and Figure S3H). Additionally, Satb1 overexpression conferred growth advantages to hematopoietic progenitors without influencing their viability in any of the cultures we used (data not shown). To investigate further the mechanisms through which Satb1 exerts its effect on early progenitors, we performed limiting dilution assays. On average, 1 in 3.1 control cells and 1 in 2.6 Satb1-transduced cells gave rise to blood cells, indicating that both are highly potent progenitors for hematopoietic cell

growth (Figure 4E, left). Nevertheless, we observed significant differences between them regarding the frequencies of progenitors with lymphopoietic potential. While 1 in 41 Satb1-transduced  $Flt3^{-}$  LSK cells produced B cells, only 1 in 143 control cells were lymphopoietic under these conditions (Figure 4E, right). In the same experiment, fresh  $Flt3^{-}$  LSK cells without retroviral transfection produced hematopoietic cells and B cells at a frequency of 1 in 6.7 cells and 1 in 61 cells, respectively (data not shown).

These results suggest that Satb1 expression affects early lineage decisions in individual HSC and expands the growth and differentiation of lymphoid cells in vitro. To evaluate whether these findings were of practical value, we performed in vivo transplantation experiments with SATB1-transduced LSK  $Flt3^{-}$  cells. We observed enhanced contribution of the SATB1-transduced cells to both T and B lineages in short-term engraftment (Figure 4F). To assess whether the overexpression of SATB1 induces tumors, we evaluated long-term and short-term



**Figure 5. Genes Affected by Satb1 Expression**

A microarray expression experiment was performed to compare gene expression in Satb1 and control-transduced LSK Flt3<sup>-</sup> cells. Upregulation in Satb1-transduced cells is shown as positive in each figure. (A) Transcription factors, (B) cytokine and cytokine receptors, and (C) other lymphoid lineage-related genes are summarized (Figure 5; see also Tables S2 and S3).

**Satb1 Regulates Lymphoid Lineage-Related Genes in HSC**

During early lymphocyte differentiation, several transcription factors have been shown to play roles in a hierarchical manner. To identify the target genes of Satb1, we first examined whether the exogenous expression of Satb1 influences the expression of lineage-relevant transcription factors in LSK Flt3<sup>-</sup> cells. Although high Satb1 expression was achieved, no significant upregulation was observed in the expression of *Sfp1*, *Ikzf1*, *Tcf3*, or *Notch1* (data not shown). The expression of *Cebpa*, which is important for myeloid differentiation, was also not significantly affected (data not shown).

Next, to find candidate genes involved in the Satb1 induction of lymphopoiesis, we performed a microarray comparing gene expression between Satb1- and control-transduced LSK Flt3<sup>-</sup> cells (Table S2). In accordance with the results described above, the data showed no significant changes in the expression of *Sfp1*, *Ikzf1*, *Tcf3*, *Notch1*, or *Cebpa*. However, several transcription factors involved in lymphoid differentiation, *Sp4*, *Maf*, *Fos*, and *Id3*, were upregulated in Satb1-transduced cells (Figure 5A). Cytokines such as *Il7* and *Kitl*, which are critical for lymphocyte differentiation and generally believed to be stromal cell products, were induced in hematopoietic progenitor cells themselves by ectopic expression of Satb1 (Figure 5B). While receptors for IL-4 or IL-17 were induced, *Csf3r*, encoding the G-CSF receptor, was downregulated. Interestingly, among lymphoid-related genes, *Rag1*, which is indispensable for both T and B cell differentiation, was strongly induced by Satb1 (Figure 5C).

lymphohematopoiesis after transplantation. In eight transplanted mice, SATB1-overexpressing cells did not induce tumors, at least during 3 months of observation.

Expression of the CD86 gene that correlates with lymphoid competency (Shimazu et al., 2012) was also significantly elevated.



As a complementary experiment, we performed a set of microarray analyses comparing gene expression signatures between WT and *Satb1*<sup>-/-</sup> cells (Table S3). We again observed no direct correlations between *Satb1* expression and *Ikzf1*, *Tcf3*, or *Notch1*, but confirmed that the expression of numerous lineage-related genes was influenced. The expression of *Ilf7* and *Kitl* was detectable in WT hematopoietic progenitors, and their levels were significantly lower in the *Satb1*<sup>-/-</sup> progenitors. Of note, *Satb2*, which is a homolog of *Satb1*, as well as *Bright*, which codes a B cell-specific AT-rich sequence binding protein (Herrscher et al., 1995), were upregulated in *Satb1*<sup>-/-</sup> HSC. In addition, the *Satb1*<sup>-/-</sup> HSC aberrantly expressed *Rag1* and *Pax5*, whose levels decreased with differentiation to LMPP. These results indicate that *Satb1* expression globally influences many genes involved in lineage-fate decisions during the specification of HSC toward lymphoid lineages.

### Satb1 Induces Lymphopoiesis in ESCs

Next, we examined whether the exogenous expression of *Satb1* is sufficient to promote lymphopoiesis in ESCs. In the OP9 coculture system (Nakano et al., 1994), ESCs can produce mesoderm cells in 4.5 days, which have potential to become hematopoietic and endothelial cells. After a short period of retroviral transduction with the control-GFP or the *Satb1*-GFP vector, ES-derived mesoderm cells were cultured with OP9 in the presence of SCF, Flt3-ligand, and IL-7. As shown in Figure 6A, although both control- and *Satb1*-transfected cells contained substantial numbers of GFP<sup>+</sup> cells, the latter produced CD45<sup>+</sup> hematopoietic cells efficiently. Further phenotype revealed that most of the CD45<sup>+</sup> GFP<sup>+</sup> cells produced from the *Satb1*-transfected cells expressed B220 and CD19 (Figure 6A, right panels). Notably, those cells were also positive for AA4.1, CD11b, and CD5, suggesting that they were likely B1-B-lineage cells (Figure 6B).

Next, we established ESC clones, which can be induced to express *Satb1*-GFP on removal of tetracycline (Tet) from the culture medium. Eight days after Tet deprivation (day 12.5; Figure 6C), approximately 15% of the recovered cells were GFP<sup>+</sup> (data not shown). Thirty-five percent of these cells expressed CD45 and included substantial numbers of AA4.1<sup>+</sup> CD19<sup>+</sup> B-lineage cells (Figure 6D, right panels). Conversely, in the presence of Tet, the proportions of AA4.1<sup>+</sup> and CD19<sup>+</sup> cells among the CD45<sup>+</sup> fraction were very low (Figure 6D, left panels). A majority of the CD19<sup>+</sup> cells among the *Satb1*-GFP<sup>+</sup> ES-derived cells were positive for Mac1 or CD5, again indicating a preference for the B1-B lineage (Figure 6E). In cytospin preparations, many of the ES-derived cells cultured with Tet showed myelomonocytic morphology, whereas *Satb1*/GFP<sup>+</sup> cells exhibited lymphocyte-like morphology (Figure 6F). Finally, a PCR-based *Igh* rearrangement assay confirmed D<sub>H</sub>-J<sub>H</sub> recombination in the *Satb1*-GFP<sup>+</sup> ES-derived cells (Figure 6G).

To test T-lineage potential, we transduced the control-GFP or the *Satb1*-GFP vector to ES-derived mesoderm cells and cultured them with OP9-DL1 cells. The *Satb1*-transduced cells effectively produced CD4<sup>+</sup> CD8<sup>+</sup> DP cells with rapid kinetics (Figures 6H and 6I). Substantial numbers of ES-derived T-lineage cells expressed TCR-γδ or TCR-β, and *Satb1*-transduced cells were advanced in this regard (data not shown). Taking these results together, we conclude that *Satb1* expression directs even ES-derived cells toward lymphoid lineages.

### Ectopic Satb1 Expression in Aged HSC Restores Lymphopoietic Potency

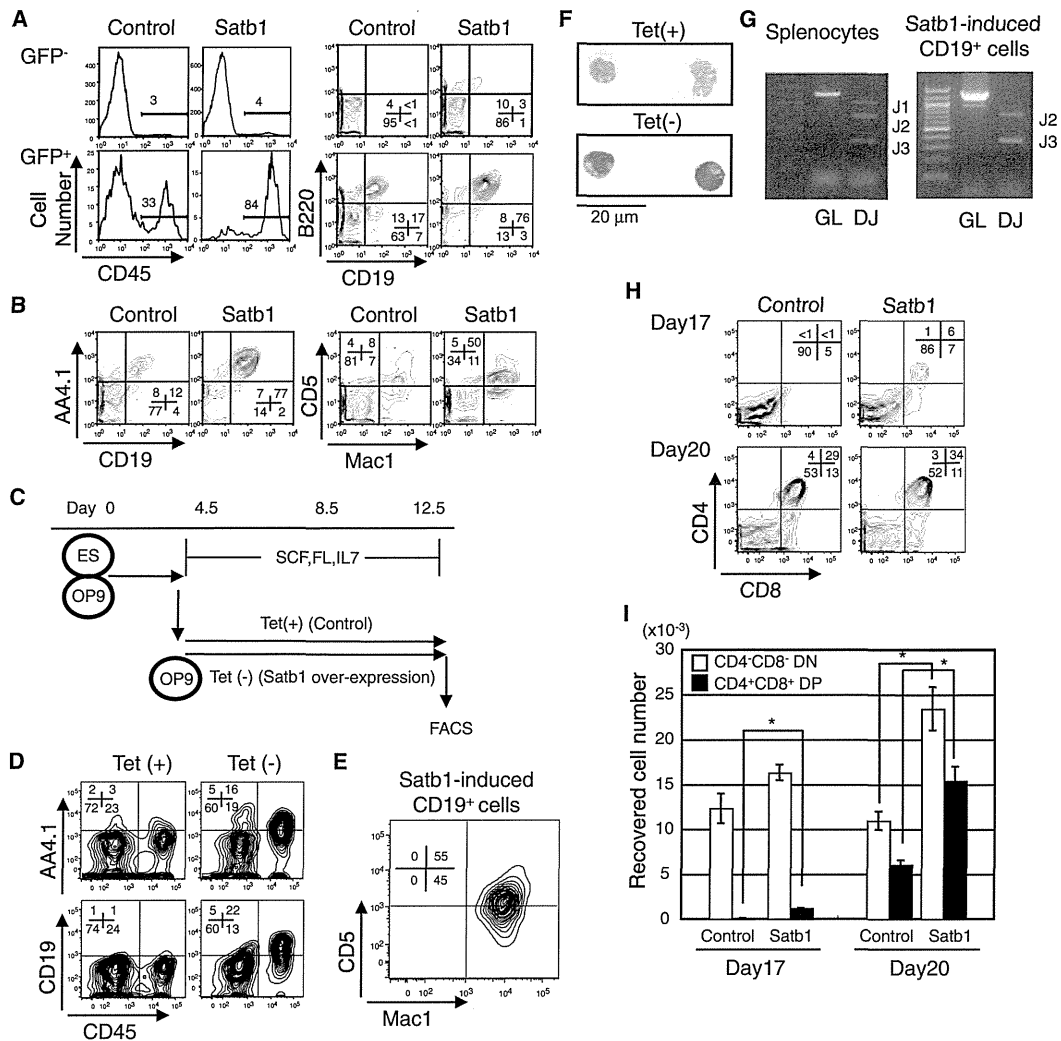
As shown in Figure 1B, the *Satb1* expression in HSC declines with age. This decline might be correlated with the age-dependent impairment of lymphopoiesis. Therefore, we examined whether *Satb1* expression restores the lymphopoietic activity of progenitors from aged mice. Rag1-GFP<sup>-</sup> LSK cells of 2-year-old mice were transduced with control or *Satb1*-DsRed vectors. After 72 hr of transduction, DsRed<sup>+</sup> cells were sorted and cultured on OP9 in the presence of SCF, Flt3-ligand, and IL-7. The *Satb1*-transduced cells produced a percentage of Rag1-GFP<sup>+</sup> B220<sup>+</sup> cells that was significantly higher than that of control cells (Figure 7A). Indeed, most of the aged Rag1-GFP<sup>-</sup> LSK cells were prone to differentiate into Rag1-GFP<sup>+</sup> cells as a result of exogenous *Satb1* expression. With respect to the recovered B-lineage cell counts, approximately 3-fold more B220<sup>+</sup> Rag1-GFP<sup>+</sup> Mac1<sup>-</sup> cells were obtained through *Satb1* overexpression (Figure 7B).

Conversely, fewer B-lineage cells were generated from aged ELP than from young ELP despite their similar expression of *Satb1* (Figure 1B; Figure S4A). B-lineage differentiation of aged ELP also showed decreased Rag1 expression (Figure S4B). Nonetheless, aged ELP showed substantial lymphopoietic activity in MS5 cocultures, in which aged HSC scarcely produced B-lineage cells (Figure S4A). These results suggest that the downregulation of *Satb1* expression is involved in the compromised lymphopoietic potential of aged HSC and that ectopic induction of *Satb1* can at least partially restore the activity.

### DISCUSSION

Despite accumulating evidence that multiple transcription factors support lymphocyte differentiation, ones that specifically direct HSC to the lymphoid lineage have remained elusive. One aim of this study was to describe molecular signatures of early stages of lymphopoiesis by comparing gene expression patterns between HSC and ELP. While we observed that many genes specific for the lymphoid lineage including *Tcr*, *Igh* and *Ilf7r* were highly induced at the ELP stage, some lymphoid genes were already expressed at low levels in the HSC-enriched fraction. Among them, we were particularly interested in chromatin modifiers because of their ability to control spatial and temporal expression of essential genes. Our screen identified *Satb1*, whose expression was previously linked to T lymphocyte differentiation (Alvarez et al., 2000). We show that *Satb1* plays a critical role in directing HSC to lymphoid lineages.

*Satb1* was originally identified as a protein that binds specifically to genomic DNA in a specialized DNA context with high base-unpairing potential (termed base-unpairing regions; BURs) (Dickinson et al., 1992). *Satb1* is predominantly expressed in the thymus and subsequent studies revealed critical roles in thymocyte development (Alvarez et al., 2000), T cell activation (Cai et al., 2006), and Th2 differentiation (Notani et al., 2010). In thymocyte nuclei, *Satb1* has a cage-like distribution and tethers BURs onto its regulatory network, thus organizing 3-dimensional chromatin architecture (Cai et al., 2003). By recruiting chromatin modifying and remodeling factors, *Satb1* establishes region-specific epigenetic status at its target gene loci and regulates a large number of genes (Yasui et al., 2002;



**Figure 6. Satb1 Promotes Lymphoid Differentiation from ES-Derived Cells**

E14tg2a ESCs were deprived of leukemia inhibitory factor and seeded onto OP9 cells. After 4.5 days, the differentiated mesoderm cells were infected with retroviral supernatants containing control-GFP or Satb1-GFP expressing vectors. Subsequently, the cells were cultured on OP9 for 8 days. At the end of culture, all cells were harvested and stained with the antibodies indicated in each panel.

(A) Total recovered cells were divided according to GFP expression (left panels). The percentages of CD45<sup>+</sup> cells in GFP<sup>-</sup> (upper panels) and GFP<sup>+</sup> populations (lower panels) are shown. CD45R/B220 and CD19 profiles of the CD45<sup>+</sup> cells corresponding to the left panels (right panels) are shown.

(B) Representative AA4.1 and CD19 or Mac1 and CD5 profiles of the GFP<sup>+</sup> CD45<sup>+</sup> cells recovered from control or Satb1-transduced culture.

(C) The experimental design used with a Tet-off system (upper panel). ESCs, which inducibly express Satb1 by Tet deprivation, were established. After 4.5 days of culture without leukemia inhibitory factor in the presence of Tet, the differentiated cells were reseeded onto new OP9 stromal cells with or without Tet. Subsequently, FACS analysis was performed after 8 days of culture (day 12.5).

(D) Tet (+) indicates profiles of GFP<sup>-</sup> cells cultured with Tet (left panels). Tet (-) panels show profiles of Satb1/GFP<sup>+</sup> cells cultured without Tet (right panels).

(E) Mac1 and CD5 expression on the Satb1/GFP<sup>+</sup> CD19<sup>+</sup> cells grown without Tet.

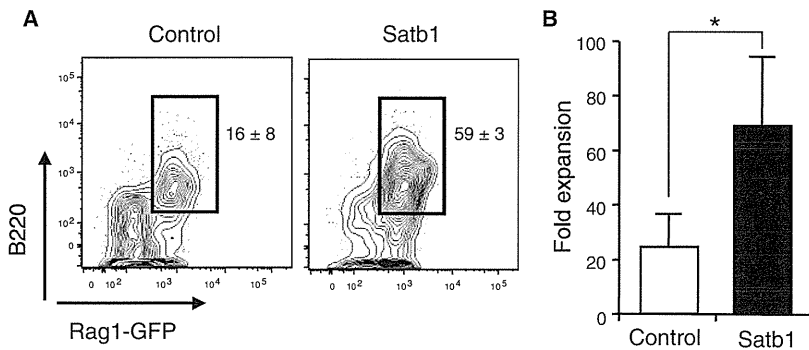
(F) Morphology of ES-derived hematopoietic cells on day 12.5.

(G) DNA PCR assays of germline (GL) or D<sub>H</sub>-J<sub>H</sub> rearranged *Igh* chain (DJ) genes were performed with the Satb1/GFP<sup>+</sup> CD19<sup>+</sup> cells recovered without Tet (right panel). Splenocytes were used as a positive control for the D<sub>H</sub>-J<sub>H</sub> recombination (left panel). On each gel, a size marker was loaded in the left lane.

(H and I) E14tg2a ESCs were differentiated to mesoderm cells for 4.5 days and then infected with the retroviral supernatant containing control-GFP or Satb1-GFP expressing vectors for 3 days. Subsequently, the cells were cultured on OP9-DL1 and T-lineage output was evaluated on the indicated days. Data are shown as mean ± SE. Statistical significance is \*p < 0.05.

Cai et al., 2003). Increased Satb1 expression in hematopoietic progenitors compared with HSC has been observed by others (Forsberg et al., 2005; Ng et al., 2009); however, no study has

been conducted concerning the role of Satb1 in differentiation of HSC to either lymphoid or myeloid progenitors. Our results clearly show a tight association of Satb1 expression with



**Figure 7. Satb1 Overexpression Restores Lymphopoietic Activity of Aged HSC**

(A) Rag1/GFP<sup>-</sup> LSK cells were sorted from 2-year-old mice and retrovirally transduced with control or Satb1-DsRed vectors. Successfully transfected cells were cultured on OP9 cells. Cultures were established in triplicate. Numbers in each panel indicate the frequency of Rag1/GFP<sup>+</sup> CD45R/B220<sup>+</sup> cells.

(B) Yields of CD45R/B220<sup>+</sup> Rag1/GFP<sup>+</sup> Mac1<sup>-</sup> B-lineage cells per 1 input control- or Satb1-transduced Rag1/GFP<sup>-</sup> LSK cells were calculated and given as averages with SD bars. Statistical significance is \**p* < 0.05 (Figure 7; see also Figure S4).

lymphoid lineages even at the earliest stages. In addition, *Satb1*<sup>-/-</sup> HSCs are hindered in producing lymphocytes in vitro and in vivo that are consistent with the phenotypes originally described in *Satb1*<sup>-/-</sup> mice, suggesting an indispensable role of Satb1 in physiological lymphopoiesis.

Although we have previously identified molecules regulating early lymphoid differentiation, information about ones that initiate the process has been elusive (Oritani et al., 2000; Yokota et al., 2003b, 2008). The present study demonstrates that ectopic expression of Satb1 strongly induces differentiation toward lymphoid lineages and promotes lymphocyte growth from primitive progenitors, even when they are derived from aged BM or ESCs. We believe that these findings are important because they reveal that the earliest step of lymphopoiesis is affected by a global chromatin organizer. In addition, our results suggest that Satb1 expression could be a useful biomarker of aging and be manipulated to reverse immunosenescence.

Lymphoid-fate decisions are not necessarily determined by a few transcription factors or cytokines that positively regulate the differentiation in a hierarchical manner. The process should involve “closed windows” and “open opportunities.” Gene array studies comparing HSC and ELP have shown that various lymphoid-related genes appear to be synchronously upregulated in ELP, whereas stem cell-related or myeloid-related ones are downregulated. From these observations, we speculated that a master regulator is present and involved in the synchronicity along with the hierarchical factors; further, we focused on the function of SATB1 in this process. Our results show that once Satb1 is substantially expressed in HSCs, it regulates hundreds of genes, including *Rag1*, *Ii7*, *kitl*, and *Csf3r*, which together determine the lymphoid lineage fate. Satb1 itself has the determinant role in regulating a set of genes to exhibit the phenotype that we observed in vitro and in vivo experiments.

Increasing Satb1 beyond physiologic levels in HSCs and ESCs strongly augmented B lymphopoiesis, while depleting Satb1 from HSC dominantly impaired T lymphopoiesis in vivo. Satb1 overexpression in HSCs by itself induces an expression profile that favors B cell production. Conversely, Satb1 deficiency might have disrupted the delicate balance of Satb1 and other BUR-binding proteins such as Satb2 or Bright. We detected minimum levels of *Satb2* and *Bright* expression in WT HSC, and their expression levels significantly increase with B-lineage differentiation (data not shown). Interestingly, both genes were aberrantly induced in Satb1-deficient HSC (Table S2). Satb2 has a binding

specificity similar to that of Satb1, and its expression is more predominant in the B lineage than in the T lineage (Dobrev et al., 2003). In ESCs, Satb2 function is antagonistic to Satb1 in regulating some target genes (Savarese et al., 2009). Whether these BUR-binding proteins are antagonistic or sometimes function synergistically, depending on cell differentiation or lineage remains unknown. Further studies of their functional correlation could yield important information about gene regulation in T and B lymphopoiesis.

Although our data provide evidence of a lymphocyte-inductive role of Satb1, an important question remains; that is, what regulates Satb1 expression? Depletion of long-lived mature B cells rejuvenates B-lymphopoiesis in old mice, suggesting that age-associated accumulation of aged B cells seems to be sensed by HSCs or early progenitors in BM (Keren et al., 2011). It will be interesting to learn whether such environmental cues influence Satb1 expression in HSCs. New strategies for boosting lymphocyte regeneration or protecting this capability during aging might emerge from studies of Satb1-related molecular mechanisms.

## EXPERIMENTAL PROCEDURES

### Animals

Animal studies were performed with the approval of the Institutional Review Board of Osaka University. Rag1-GFP knockin mice were previously described (Kuwata et al., 1999). *Satb1*<sup>-/-</sup> mice were also previously established (Alvarez et al., 2000). WT C57BL/6 mice and the congenic C57BL/6SJL strain (CD45.1 alloantigen) were obtained from Japan Clea (Shizuoka, Japan) and The Jackson Labs (Bar Harbor, ME), respectively. To obtain mouse fetuses, we considered the morning of the day of vaginal plug observation as E0.5.

### Flow Cytometry and Cell Sorting

Cells were stained with Abs indicated in each experiment and analyzed with FACScanto or FACSaria (BD Bioscience). Adult BM cells from Rag1-GFP heterozygotes were used to isolate Lin<sup>-</sup> c-kit<sup>hi</sup> Sca-1<sup>+</sup> Flt3<sup>-</sup> Rag1-GFP<sup>-</sup> IL-7R $\alpha$ <sup>-</sup> (HSC-enriched), Lin<sup>-</sup> IL-7R $\alpha$ <sup>-</sup> c-kit<sup>hi</sup> Sca-1<sup>+</sup> Flt3<sup>+</sup> Rag1-GFP<sup>-</sup> (LMPP-enriched), Lin<sup>-</sup> IL-7R $\alpha$ <sup>-</sup> c-kit<sup>hi</sup> Sca-1<sup>+</sup> Flt3<sup>+</sup> Rag1-GFP<sup>+</sup> (ELP-enriched), Lin<sup>-</sup> c-kit<sup>lo</sup> Sca-1<sup>lo</sup> Flt3<sup>+</sup> Rag1-GFP<sup>+</sup> IL-7R $\alpha$ <sup>+</sup> (CLP-enriched), and Lin<sup>-</sup> c-kit<sup>hi</sup> Sca-1<sup>-</sup> IL-7R $\alpha$ <sup>-</sup> myeloid progenitors (Adolfsson et al., 2005; Igaraishi et al., 2002; Kondo et al., 1997). For culture experiments, we also sorted a HSC-enriched fraction from WT C57BL/6 or *Satb1*<sup>-/-</sup> mice according to the cell surface phenotype of Lin<sup>-</sup> c-kit<sup>hi</sup> Sca-1<sup>+</sup> Flt3<sup>-</sup>.

### Stromal Cell Coculture

Murine stromal cell lines MS5 and OP9 were generous gifts from Dr. Mori (Niigata University) and Dr. Hayashi (Tottori University), respectively. Freshly

isolated or transduced cells were cocultured with stromal cells in  $\alpha$ -MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL), and rm IL-7 (1 ng/mL). The cultures were fed twice a week and maintained for the indicated periods in each experiment. OP9-DL1 cells originated by Dr. Kawamoto (Riken, Japan) were obtained from Riken Cell Bank (Tsukuba, Japan) and used to produce T-lineage cells. In this case, cells were cultured in the presence of rm Flt3-ligand (5 ng/mL) and rm IL-7 (1 ng/mL) for 14 days, and rm Flt3-ligand (5 ng/mL) alone thereafter. At the end of culture, cells were counted and analyzed by flow cytometry.

#### Competitive Repopulation Assay

The CD45.1/CD45.2 system was adapted to a competitive repopulation assay. One thousand Flt3<sup>-</sup> LSK cells sorted from FL or BM of WT, Satb1 heterodeficient, or Satb1 homozygous-deficient mice (CD45.2) were mixed with  $4 \times 10^5$  unfractionated adult BM cells obtained from WT C57BL/6-Ly5.1 (CD45.1) mice and were transplanted into C57BL/6-Ly5.1 mice lethally irradiated at a dose of 920 rad. At 8 weeks after transplantation, engraftment of CD45.2 cells was evaluated by flow cytometry.

#### Retrovirus Transfection

Murine Satb1 expression vector was purchased from OriGene (Rockville, MD). A retrovirus expression vector for Satb1 was generated by subcloning into the pMYs-IRES-GFP or DsRed vector (a gift from Dr. Kitamura, University of Tokyo). Conditioned medium containing high titer retrovirus particles was prepared as reported previously (Sato et al., 2008). Sorted HSC were cultured in D-MEM containing 10% FBS, rm SCF (100 ng/ml), rm TPO (100 ng/ml), and rm Flt3-ligand (100 ng/ml) for 24 hr. Then, the cells were seeded into the culture plates coated with Retronectin (Takara Bio, Shiga, Japan) and cultured with conditioned medium containing retrovirus. After 24 hr, cells were washed and performed second transfection by the same condition. After 48 hr from the second transfection, GFP or DsRed-positive cells were sorted by FACSaria.

#### Limiting Dilution Assays

The frequencies of lymphohematopoietic progenitors were determined by plating cells in limiting dilution assays by using 96-well flat-bottom plates. Pre-established MS5 layers were plated with 1, 2, 4, 8, or 16 cells each by using the Automated Cell Deposition Unit of the FACSaria. Cells were cultured in  $\alpha$ -MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL), and rm IL-7 (1 ng/mL). At 10 days of culture, wells were inspected for the presence of hematopoietic clones. Positive wells were harvested and analyzed by flow cytometry for the presence of CD45<sup>+</sup> hematopoietic cells and CD45R/B220<sup>+</sup> CD19<sup>+</sup> Mac1<sup>-</sup> B-lineage cells. The frequencies of progenitors were calculated by linear regression analysis on the basis of Poisson distribution as the reciprocal of the concentration of test cells that gave 37% negative cultures.

#### Lymphocyte Development from Murine ESCs

To induce differentiation toward hematopoietic cells, we deprived E14tg2a ESCs of leukemia inhibitory factor and seeded onto OP9 cells in 6-well plates at a density of  $10^4$  cells per well in  $\alpha$ -MEM supplemented with 20% FBS (Nakano et al., 1994). After 4.5 days, the cells were harvested and whole-cell suspensions were transferred into a new 10 cm dish and incubated in 37°C for 30 min to remove adherent OP9 cells. The collected floating cells were infected with the retroviral supernatant in Retronectin-coated plates by 2 hr spinoculation (1100 g) (Kitajima et al., 2006). Subsequently, the cells were cultured on OP9 or OP9-DL1.

#### Tetracycline-Regulated Inducible Expression of Satb1 in ESCs

To inducibly express Satb1 in ESCs, we utilized a Tet-off system as reported previously (Era and Witte, 2000), in which transcription of the target gene is initiated by the removal of Tet from the culture medium. Briefly, we initially introduced pCAG20-1-tTA and pUHD10-3-puro by electroporation and selected one clone designated E14 by culture with 1  $\mu$ g/ml of Puro and/or 1  $\mu$ g/ml of Tet. We further transfected pUHD10-3-Satb1-GFP, which can inducibly express Satb1 and GFP as a single mRNA through the internal ribosome entry site in response to the Tet removal, together with the neomycin-resistant plasmid pcDNA3.1-neo. After the culture with G418, we selected clones that can inducibly express GFP in response to the Tet deprivation.

#### DNA PCR Assays for Igh Rearrangement

DNA PCR assays were performed as reported previously (Schlissel et al., 1991). PCR was performed by using genomic DNA extracted from splenocytes or ES-derived cells as a template. D<sub>H</sub>-J<sub>H</sub> recombination was detected as amplified fragments of 1,033 bp, 716 bp, and 333 bp by using a primer D<sub>H</sub>L(5') and J3(3'). Germline alleles were detected as an amplified fragment of 1,259 bp by using a primer Mu0(5') and J3(3'). The sequence of primers are as follows: D<sub>H</sub>L(5'), GGAATTCG(AorC)TTTTTGT(CorG)AAGGGATCTACTA CTGTG; Mu0(5'), CCGCATGCCAAGGCTAGCCTGAAAGATTACC; and J3(3'), GTCTAGATTCTCACAAAGTCCGATAGACCCCTGG.

#### Statistical Analyses

Unpaired, two-tailed t test analyses were used for intergroup comparisons, and p values were considered significant if they were less than 0.05.

#### ACCESSION NUMBERS

The microarray data in Tables S2 and S3 has been deposited in NCBI GEO database under the accession numbers GSE45566 and GSE45299.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.05.014>.

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# Increased plasma thrombopoietin levels in patients with myelodysplastic syndrome: a reliable marker for a benign subset of bone marrow failure

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## ABSTRACT

Although myelodysplastic syndromes are heterogeneous disorders comprising a benign subset of bone marrow failure similar to aplastic anemia, no laboratory test has been established to distinguish it from bone marrow failures that can evolve into acute myeloid leukemia. Plasma thrombopoietin levels were measured in 120 patients who had myelodysplastic syndrome with thrombocytopenia ( $< 100 \times 10^9/L$ ) to determine any correlation to markers associated with immune pathophysiology and outcome. Thrombopoietin levels were consistently low for patients with refractory anemia with excess of blasts, while patients with other myelodysplastic syndrome subsets had more variable results. Patients with thrombopoietin levels of 320 pg/mL and over had increased glycosylphosphatidylinositol-anchored protein-deficient blood cells (49.1% vs. 0%), were more likely to have a low International Prognostic Scoring System (IPSS) score ( $\leq 1.0$ , 100% vs. 65.5%), a higher response rate to immunosuppressive therapy (84.2% vs. 14.3%), and a better 5-year progression-free survival rate (94.1% vs. 63.6% for refractory cytopenia with unilineage dysplasia; 100.0% vs. 44.4% for refractory cytopenia with multilineage dysplasia). In conclusion, increased plasma thrombopoietin levels were associated with a favorable prognosis of bone marrow failure and could, therefore, represent a reliable marker for a benign subset of myelodysplastic syndrome.

## Introduction

Myelodysplastic syndromes (MDS) are characterized by peripheral cytopenia and morphological abnormalities in mature and immature blood cells. Although MDS were originally defined as clonal hematopoietic disorders with a propensity to become acute myeloid leukemia (AML), they also comprise benign bone marrow (BM) failure which may benefit from immunosuppressive therapy (IST), as acquired aplastic anemia (AA) does.<sup>1,3</sup> MDS with thrombocytopenia are often difficult to differentiate from non-severe AA because diagnoses rely on a subjective judgment of blood cell morphological abnormalities, and BM cellularity is often inconsistent among the sites examined.<sup>4,5</sup> Laboratory markers such as glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP<sup>-</sup>) cells and HLA-DRB1\*1501 may differentiate between benign and pre-leukemic MDS subtypes.<sup>6,7</sup> Diagnostic values of these markers have not been established, however, due to conflicting results in the prediction of IST response<sup>8</sup> and cumbersome procedures in the detection of minor GPI-AP<sup>-</sup> cell populations.<sup>9,10</sup>

Thrombocytopenia is a symptom common to AA and a subset of MDS. Previous studies showed that plasma levels of thrombopoietin (TPO), a critical regulator of thrombopoiesis, were elevated in patients with AA<sup>11</sup> but remained low in patients with MDS despite the presence of severe thrombocytopenia.<sup>12-14</sup> The low TPO levels in MDS patients have been connected to an increase in megakaryocytes or TPO receptors on megakaryocytes,<sup>15</sup> but no studies have

focused on the possibility of different TPO levels among MDS subtypes. Patients with low-risk MDS could have high TPO levels and respond to IST as AA patients do. To test these hypotheses, plasma TPO was measured in patients with various types of MDS and correlated to IST response, patient prognosis and an increase in the percentage of GPI-AP<sup>-</sup> cells.

## Design and Methods

### Patients' characteristics

TPO plasma concentration was measured in 50 healthy volunteers (23 male, 27 female) aged 18-82 years old (median 41 years) and 191 patients diagnosed with thrombocytopenia from 2005 to 2010 at Kanazawa University Hospital, hospitals participating in the Study Group of Intractable Hematopoietic Disorders in Japan, or other affiliated institutions. There were 120 patients with MDS, including 37 with refractory cytopenia with unilineage dysplasia (RCUD), 40 with refractory cytopenia with multilineage dysplasia (RCMD), 22 with refractory anemia with excess of blasts (RAEB), and 21 with unclassified MDS (MDS-U), as well as 47 patients with AA and 24 with immune thrombocytopenia (ITP). Characteristics of these participants are summarized in Table 1. Patients dependent on platelet transfusions or with a liver dysfunction were excluded from the study. AA and MDS were classified according to diagnostic criteria from the International Agranulocytosis and Aplastic Anemia Study Group and World Health Organization 2008, respectively. Prognostic scores of MDS patients were calculated according to the International Prognostic Scoring System (IPSS). AA severity was

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determined by criteria from Camitta *et al.*<sup>15</sup> All patients with MDS were judged to have 30% or more marrow cellularity by pathologists. MDS-U patients with abnormal karyotypes included 15 with del(13q), 2 with del(1;7), one with del(11q), one with t(4;21)(q11;q11), one with trisomy 8, and one with del(20q). The patients with trisomy 8 and del(20q) were diagnosed with MDS-U by the presence of bicytopenia. The ethics committee of Kanazawa University Graduate School of Medical Science approved the study protocol, and all patients provided their informed consent prior to sampling.

### Measurement of TPO levels

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood was drawn from patients at diagnosis, and plasma was separated by centrifugation at 1000 g for 10 min for storage at -20°C. Plasma TPO was measured with a commercially available assay kit (Quantikine™ Human TPO Immunoassay, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Corrected TPO values were calculated by dividing TPO concentration by platelet count ( $\times 10^9/L$ ).

### Detection of GPI-AP<sup>+</sup> blood cells

EDTA-anticoagulated peripheral blood was drawn from each patient. To detect GPI-AP<sup>+</sup> granulocytes, erythrocytes were lysed in buffer (8.26 g/L NH<sub>4</sub>Cl, 1.0 g/L KHCO<sub>3</sub>, and 0.037 g/L EDTA-4Na). After a saline wash, 50  $\mu$ L of leukocyte suspension was incubated with 4  $\mu$ L of phycoerythrin (PE)-labeled anti-CD11b mAb (Becton Dickinson, Franklin Lakes, NJ, USA) and 6  $\mu$ L of Alex Fluor 488-labeled inactive toxin aerolysin (FLAER; Pinewood Scientific Services, Victoria, BC, Canada). To detect GPI-AP<sup>+</sup> erythrocytes, fresh blood was diluted to 3% in phosphate buffered saline (PBS), and 50  $\mu$ L was then incubated with 4  $\mu$ L of PE-labeled anti-glycophorin A mAb (clone JC159; DAKO, Glostrup, Denmark), fluorescein-isothiocyanate (FITC)-labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA, USA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen). At least  $1 \times 10^5$  CD11b<sup>+</sup> granulocytes and glycophorin A<sup>+</sup> red blood cells (RBCs) were analyzed within each corresponding gate of a FACSCanto II® (Becton Dickinson) flow cytometer.<sup>9</sup> A significant increase in the percentage of GPI-AP<sup>+</sup> cells was defined as greater than 0.003% aerolysin-CD11b<sup>+</sup> granulocytes and/or greater than 0.005% glycophorin A<sup>+</sup> RBCs as determined from the peripheral blood of 50 healthy individuals. Careful handling of samples with elaborate gating could lower the cut-off values to these levels without producing false positive results.<sup>9,16,17</sup>

### IST

Horse anti-thymocyte globulin (ATG) 15 mg/kg/d (Genzyme, Cambridge, MA, USA) was administered for five days in combination with cyclosporine A (CsA) 6 mg/kg/d (Novartis, Basel, Switzerland). CsA was also given at 5-6 mg/kg/d for at least three months as monotherapy. Hematologic improvements, partial response, and complete response were defined according to the International MDS Working Group Criteria.<sup>18</sup>

### Human androgen receptor assay (HUMARA)

The androgen receptor gene was amplified from the genomic DNA of 8 female patients, as previously described by Ishiyama *et al.* with some modifications.<sup>19</sup> To correct an inequality of amplification efficiency between the 2 alleles, we determined the ratios of both allele areas before (lower allele/higher allele: A/B) and after (lower allele/higher allele:A'/B') HhaI digestion using a C value calculated by (A/B)/(A'/B') as a marker of skewing in granulocytes (CG) and T lymphocytes (CL). Skewing was judged evident when the absolute values of log (CG/CL) (S value) were more than 0.4.

### Statistical analysis

Plasma TPO concentrations were compared between subgroups with different clinical characteristics using the Mann-Whitney U test. Receiver operating characteristic (ROC) curves were calculated from the TPO levels and corrected TPO values of AA patients (representing benign BM failure) and RAEB patients (representing pre-leukemic BM failure) to determine thresholds of plasma TPO levels and define patients with high TPO levels. ROC curves were also calculated from the TPO levels of patients with early stage MDS who responded to IST and those who did not respond, and of patients who progressed to AML/death and those who did not progress. The Kaplan-Meier method and Cox's proportional hazards model were performed to estimate time-to-event analysis. Differences between patients with TPO levels of 320 pg/mL or more (TPO<sup>high</sup>) and patients with TPO levels less than 320 pg/mL (TPO<sup>low</sup>) were assessed by the log rank test. The differences in the percentages of patients with low International Prognostic Scoring System (IPSS) scores and of patients requiring platelet transfusions between TPO<sup>high</sup> and TPO<sup>low</sup> patients were assessed by Fisher's exact probability test. All statistical analyses were performed using JMP version 5.0.1J software (SAS Institute, Cary, NC, USA).  $P < 0.05$  was considered significant.

## Results

### TPO levels for patients with thrombocytopenia

Plasma TPO levels ranged from 19.4 to 38.8 pg/mL (mean  $\pm$  standard deviation,  $54.5 \pm 21.1$  pg/mL) in healthy individuals with platelet counts higher than  $150 \times 10^9/L$ . Similar to previous reports, TPO levels for patients with AA ( $1254.6 \pm 551.1$  pg/mL) were noticeably higher than for patients with ITP ( $63.5 \pm 8.7$  pg/mL) or RAEB ( $44.7 \pm 85.2$  pg/mL) (Figure 1A). Corrected TPO values were also higher in AA patients than RAEB patients (Figure 1B). ROC curves set the threshold between benign and pre-leukemic BM failure at 320 pg/mL for TPO levels and 157 pg/mL/10<sup>10</sup> L for corrected TPO values (Figure 2A). All patients with TPO levels of 320 pg/mL or over also had corrected TPO values over 157 pg/mL/10<sup>10</sup>L, except one with 155 pg/mL/10<sup>10</sup>L, indicating that the difference between TPO<sup>high</sup> and TPO<sup>low</sup> patients is not due to platelet

Table 1. Characteristics of study participants with thrombocytopenia and healthy volunteers.

	N. of patients	Sex, (M/F)	Age (years), (range)	Platelet count ( $\times 10^9/L$ ) (median $\pm$ SE)
MDS	120	60/49	70 (19-91)	54.0 $\pm$ 2.7
RCUD	37	17/20	65 (46-85)	53.7 $\pm$ 4.3
RCMD	40	23/17	69 (33-91)	43.5 $\pm$ 8.2
RAEB	22	16/6	74 (43-85)	49.1 $\pm$ 2.7
MDS-U	21	10/11	72 (19-76)	62.2 $\pm$ 2.1
AA	47	25/22	58 (12-93)	24.9 $\pm$ 7.3
Severe	23	12/11	56 (28-93)	11.6 $\pm$ 6.9
Moderate	24	13/11	58 (12-83)	43.9 $\pm$ 5.4
ITP	24	14/10	62 (35-82)	23.5 $\pm$ 7.9
Healthy volunteers	50	23/27	41 (18-59)	304.3 $\pm$ 8.6

MDS: myelodysplastic syndrome; RCUD: refractory cytopenia with unilineage dysplasia; RCMD: refractory cytopenia with multilineage dysplasia; RAEB: refractory anemia with excess of blasts; MDS-U: unclassified myelodysplastic syndromes; AA: aplastic anemia; ITP: immune thrombocytopenia; HV: healthy volunteers.

count. TPO levels were highly variable in patients with MDS subtypes other than RAEB, with the proportion of TPO<sup>high</sup> patients at 40.5% for RCUD, 32.5% for RCMD, and 77.3% for MDS-U.

#### Prevalence of increased GPI-AP<sup>+</sup> cells, clonality, and IPSS scores for patients with MDS

Small populations of GPI-AP<sup>+</sup> granulocytes and/or erythrocytes were detected in some MDS patients, at 29.7% for RCUD, 7.5% for RCMD, 62.9% for MDS-U, and 0% for RAEB. Figure 3A shows the percentage of GPI-AP<sup>+</sup> granulocytes in 55 TPO<sup>high</sup> (25 with RCUD, 14 with RCMD, and 16 with MDS-U) and 65 TPO<sup>low</sup> (12 with RCUD, 26 with RCMD, 5 with MDS-U, and 22 with RAEB) patients. The prevalence of increased GPI-AP<sup>+</sup> granulocytes and/or erythrocytes in each group was 45.8% and 0% ( $P=0.003$ , Figure 3B). Eight female patients (4 with RCMD and 4 with MDS-U) underwent the HUMARA. All 4 TPO<sup>low</sup> patients showed S values over 0.4 (1.23, 1.39, 1.14, and 0.71) compatible with the presence of clonal hematopoiesis while none of the 4 TPO<sup>high</sup> patients showed such high S values (0.06, 0.16, 0.08, and 0.09,  $P=0.002$ , Online Supplementary Figure S1). When correlation of TPO levels and IPSS scores was examined for each MDS patient, none of the TPO<sup>high</sup> patients had an IPSS score above 1.5 (Figure 3C). In fact, the percentages of 0,

0.5, or 1.0 scores in TPO<sup>high</sup> and TPO<sup>low</sup> patient groups were 100.0% and 65.5%, respectively ( $P<0.001$ ).

#### Response of patients with MDS to IST

Forty-five TPO<sup>high</sup> MDS patients were treated with IST. Sixteen patients (7 with RCUD and 9 with MDS-U) were treated with a combination of ATG and CsA, while 29 patients (9 with RCUD, 10 with RCMD, and 10 with MDS-U) were treated with CsA alone. Response rates to ATG+CsA and CsA were 81.2% and 85.3%, respectively. In contrast, 7 TPO<sup>low</sup> patients received CsA monotherapy that produced only one case of improved anemia (from 5.4 g/dL to 8.1 g/dL). The cumulative rate of response to IST in TPO<sup>high</sup> patients was significantly higher than that in TPO<sup>low</sup> patients ( $P=0.002$ ), (Online Supplementary Figure S2A), while there was no significant difference either between patients with or without increased GPI-AP<sup>+</sup> granulocytes ( $P=0.38$ ), (Online Supplementary Figure S2B) or between TPO<sup>high</sup> patients with or without increased GPI-AP<sup>+</sup> granulocytes ( $P=0.28$ ), (Online Supplementary Figure S2C). ROC analysis revealed the threshold TPO level affecting the response to IST to be 286.7 pg/mL, with a sensitivity of 0.580 and a specificity of 0.850 (Figure 2B).

After 6-17 months of IST, TPO levels were measured again in 4 patients who achieved a platelet recovery above  $100 \times 10^9/L$ . The corrected TPO levels before and after IST were 718.7/14.2 pg/mL/ $10^{10} L$ , 266.8/10.8 pg/mL/ $10^{10} L$ , 330.6/8.75 pg/mL/ $10^{10} L$ , and 170.6/8.8 pg/mL/ $10^{10} L$ .

#### Correlation of high TPO levels with the prognosis of patients with MDS

The 5-year overall survival rates for RCUD, RCMD, MDS-U, and RAEB patients were 53.6%, 50.8%, 53.1%, and 37.4%, respectively (Figure 4A). The 5-year progression-free survival (PFS) rates for RCUD, RCMD, and MDS-U patients were 60.9%, 60.6%, and 71.4%, respectively (Figure 4B). The 5-year PFS rates, defined as whichever came first between: a) time from the first day of diagnosis until progression to AML; or b) disease-related death, was significantly higher for TPO<sup>high</sup> patients than TPO<sup>low</sup> patients with RCUD (94.1% vs. 60.6%;  $P=0.03$ ) or RCMD (100.0% vs. 44.4%;  $P=0.006$ ), while there was no significant difference between the two groups in patients with MDS-U (82.3% vs. 80.0%;  $P=0.22$ , Figure 4C-E). An ROC analysis revealed the threshold TPO level affecting

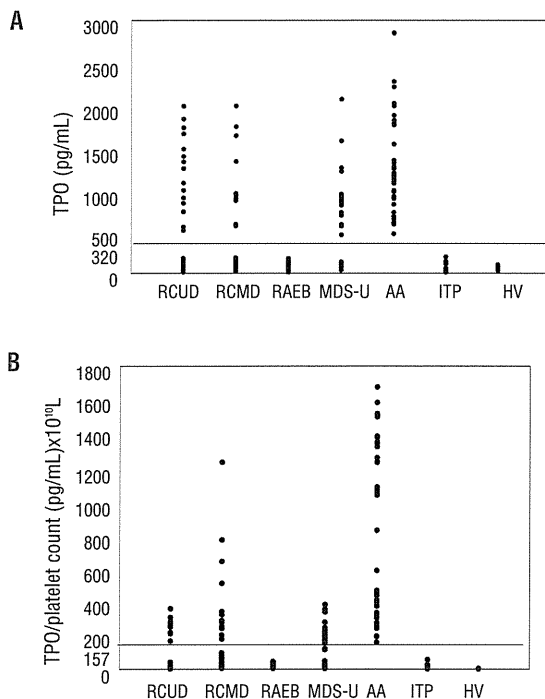


Figure 1. Plasma thrombopoietin levels in patients with thrombocytopenia and healthy volunteers. (A) Plasma thrombopoietin (TPO) levels for each patient group. (B) Corrected TPO values for each patient group. The horizontal line in each figure represents the cut-off value that potentially separates benign and pre-leukemic bone marrow failure. RCUD: refractory cytopenia with unilineage dysplasia; RCMD: refractory cytopenia with multilineage dysplasia; RAEB: refractory anemia with excess of blasts; MDS-U: unclassified myelodysplastic syndromes; AA: aplastic anemia; ITP: immune thrombocytopenia; HV: healthy volunteers.

Table 2. Multivariate analysis of factors negatively affecting progression-free survival of patients with myelodysplastic syndrome.

	Hazard ratio	P
TPO (pg/mL)		
<320 vs. $\geq 320$	0.05	0.009
Age (years old)		
<60 vs. $\geq 60$	1.87	0.262
Gender		
female vs. male	0.12	0.009
IPSS		
0 vs. 0.5 and 1.0	0.34	0.158
Transfusion		
yes vs. no	1.21	0.752
Abnormal karyotype		
yes vs. no	0.93	0.916



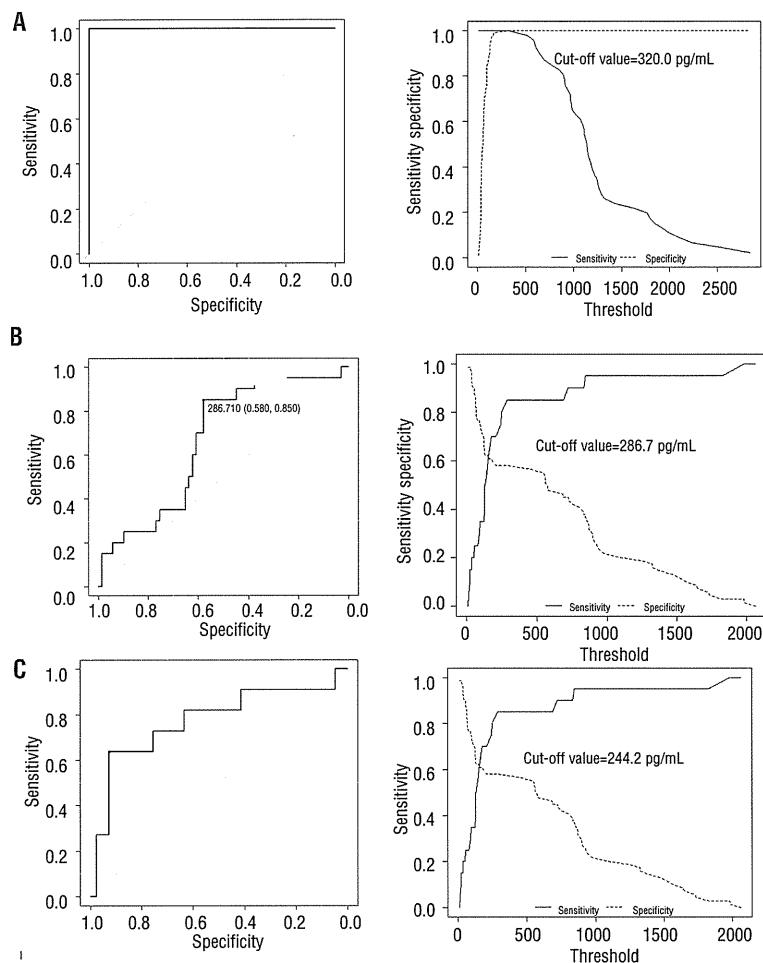


Figure 2. Receiver operating characteristic (ROC) curves to determine thresholds of plasma TPO levels discriminating benign (aplastic anemia)/pre-leukemic BM failure (RAEB, A), response/no response to immunosuppressive therapy (IST, B), and progression/no progression to AML/death (C).

progression to AML to be 244.2 pg/mL with a sensitivity of 0.927 and a specificity of 0.636 (Figure 2C). A multivariate analysis on patients with RCUD, RCMD and MDS-U revealed TPO<sup>low</sup> and patient gender to be significant factors associated with progression to AML (Table 2). In contrast to previous analyses,<sup>20</sup> red blood cell transfusion requirements were not identified as a significant prognostic factor in this study cohort.

This may reflect the fact that we selected patients with thrombocytopenia. There was no significant difference in the percentages of patients requiring platelet transfusions among TPO<sup>high</sup> (8/52, 4.3%) and TPO<sup>low</sup> (2/46, 15.3%;  $P=0.09$ ) patients. TPO levels were serially recorded for one patient as AA evolved into MDS-RAEB 20 months after IST. The TPO concentration was 1960 pg/mL at the onset of AA and 151 pg/mL when thrombocytopenia recurred with the emergence of atypical blasts in peripheral blood (Online Supplementary Figure S3).

## Discussion

Consistent with previous reports, the present study revealed an increase in the TPO levels of patients with AA, a decrease in those with ITP, and relatively low TPO levels

in patients with MDS.<sup>13,14</sup> While patients with RAEB had consistently low TPO levels, patients with low-risk MDS (RCUD, RCMD, and MDS-U) produced highly variable results. Feng *et al.* recently demonstrated similar variability among patients with hypoplastic MDS and reported the usefulness of TPO in distinguishing hypoplastic MDS from AA,<sup>21</sup> however, the significance of high TPO levels in a subset of patients with low-risk MDS was not the focus of the authors' attention. Our study is the first to demonstrate that a high concentration of TPO is associated with benign BM failure in patients with MDS as seen by the higher prevalence of increased GPI-AP<sup>+</sup> cells, lower incidence of clonal hematopoiesis, lower IPSS score, and better PFS in TPO<sup>high</sup> patients compared to TPO<sup>low</sup> patients. The negative prognostic impact of TPO levels below 320 pg/mL was confirmed by a multivariate analysis which also identified patient gender to be a poor prognostic factor as demonstrated by previous studies.<sup>22,23</sup> Plasma TPO levels are negatively regulated by platelets and megakaryocytes through their binding of TPO.<sup>24,25</sup> Some studies show an inverse correlation between TPO levels and BM megakaryocyte count,<sup>26,27</sup> but the TPO levels of patients with RAEB were consistently low in our study despite the low megakaryocyte content (*data not shown*). Low TPO levels in MDS patients with a poor prognosis may be

explained by an increased turnover of platelets produced by abnormal megakaryocytes.<sup>28</sup> We previously demonstrated that the aberrant increase in the proportion of immature platelets is frequently seen in MDS patients with chromosomal abnormalities associated with poor prognosis.<sup>28</sup> Increased production and destruction of platelets may accelerate TPO consumption in patients with RAEB, leading to a decrease in plasma TPO levels. Although one report demonstrated increased TPO receptors on the megakaryocytes of MDS patients using flow cytometry,<sup>14</sup> we were unable to reproduce their results (*data not shown*).

Small populations of GPI-AP<sup>-</sup> cells have been detected in a subset of patients with refractory anemia as defined by French-American-British classification and are associated with a favorable response to IST and good future prognosis.<sup>2</sup> Patients with increased GPI-AP<sup>-</sup> cells (PNH<sup>+</sup> patients) are characterized by predominant thrombocytopenia and no increase in BM megakaryocytes,<sup>6,9</sup> which agrees with our findings of high TPO levels in all PNH<sup>+</sup> patients with MDS and no PNH<sup>+</sup> patients with low TPO levels. These results show no need to examine peripheral blood for GPI-AP<sup>-</sup> cells in patients with BM failure if their TPO levels are less than 320 pg/mL.

The response rate to ATG+CsA therapy or CsA monotherapy for TPO<sup>high</sup> MDS patients was as high as or higher than the response for AA patients.<sup>29</sup> When we previously assessed the efficacy of CsA therapy for patients with thrombocytopenia and decreased BM megakaryocytes, more than 70% improved regardless of the presence or absence of GPI-AP<sup>-</sup> cells (Seiki *et al.*, unpublished observation, 2013). High TPO levels may have a stronger association with the immune pathophysiology of BM failure in a subset of MDS than the presence of increased GPI-AP<sup>-</sup> cells. However, the impact of the increased percentage of GPI-AP<sup>-</sup> granulocytes on PFS could not be analyzed by our multivariate analysis because all patients with increased GPI-AP<sup>-</sup> granulocytes were sorted into the TPO<sup>high</sup> group. When we conducted another multivariate analysis using increased GPI-AP<sup>-</sup> granulocytes, rather than TPO levels, as an independent factor, the hazard ratio of the increased GPI-AP<sup>-</sup> granulocytes was 0.64, with a *P* value of 0.69. Thus, the impact of increased GPI-AP<sup>-</sup> granulocytes on PFS appears to be much smaller than high TPO levels.

Previous reports documented the important role of DRB1\*1501 as a predictor of response to IST in patients with MDS.<sup>8</sup> This factor may also have affected PFS in our patient cohort. Unfortunately, since only a limited number of patients underwent HLA typing, its impact on PFS could not be determined in this study. However, we previously examined various factors, including GPI-AP<sup>-</sup> cells and DRB1\*1501, for their association with good response to IST in 140 patients with AA using multivariate analysis and found that only the presence of increased GPI-AP<sup>-</sup> cells significantly predicts favorable response to IST.<sup>30,31</sup> Thus, it is unlikely that DRB1\*1501 affects PFS of patients with MDS more strongly than increased GPI-AP<sup>-</sup> cells or high TPO levels. Cytogenetic abnormality was not identified as a poor risk factor by the multivariate analysis. This is probably because 15 of 21 patients with cytogenetic abnormalities had del(13q), which represents benign BM failure with immune pathophysiology.<sup>32</sup>

Although the exact mechanism for decreased TPO in MDS patients with a poor prognosis remains unclear,

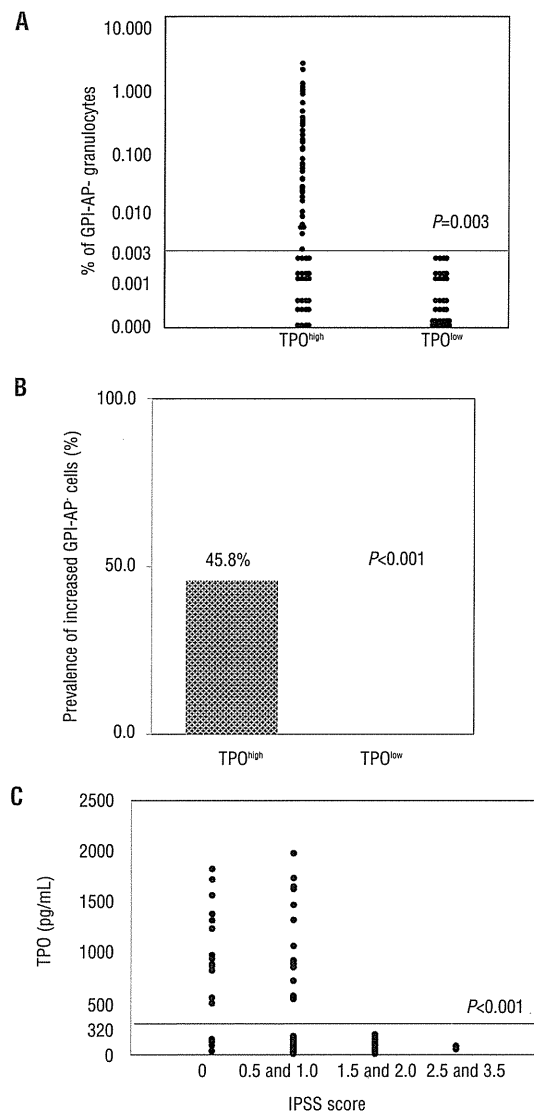


Figure 3. Relationship between thrombopoietin levels and other prognostic markers for patients with myelodysplastic syndrome. (A) Percentage of GPI-AP<sup>-</sup> granulocytes in TPO<sup>high</sup> (n=55) and TPO<sup>low</sup> (n=65) patients. The horizontal line represents the cut-off value that separates significant and non-significant increases in GPI-AP<sup>-</sup> granulocyte percentage. (B) The prevalence of increased GPI-AP<sup>-</sup> granulocytes and/or erythrocytes in TPO<sup>high</sup> (n=55) and TPO<sup>low</sup> (n=65) patients. (C) TPO levels for MDS patients classified by IPSS score. GPI-AP<sup>-</sup> cells, glycosylphosphatidylinositol-anchored protein-deficient cells; TPO<sup>high</sup>, thrombopoietin levels  $\geq 320$  pg/mL; TPO<sup>low</sup>, thrombopoietin levels <320 pg/mL; RCMD: refractory cytopenia with multilineage dysplasia; MDS-U: unclassified MDS; AA: aplastic anemia; IPSS: International Prognostic Scoring System.

measurement of its concentration could play a significant role in managing MDS with thrombocytopenia. A patient with TPO levels of 320 pg/mL or over is considered to have a good prognosis with a small likelihood of developing AML, and IST can be considered rather than radical treatments such as chemotherapy and stem cell transplantation. Recent National Comprehensive Cancer Network guidelines recommend hypomethylating agents as a treatment for MDS patients with thrombocytopenia,<sup>33</sup> but this

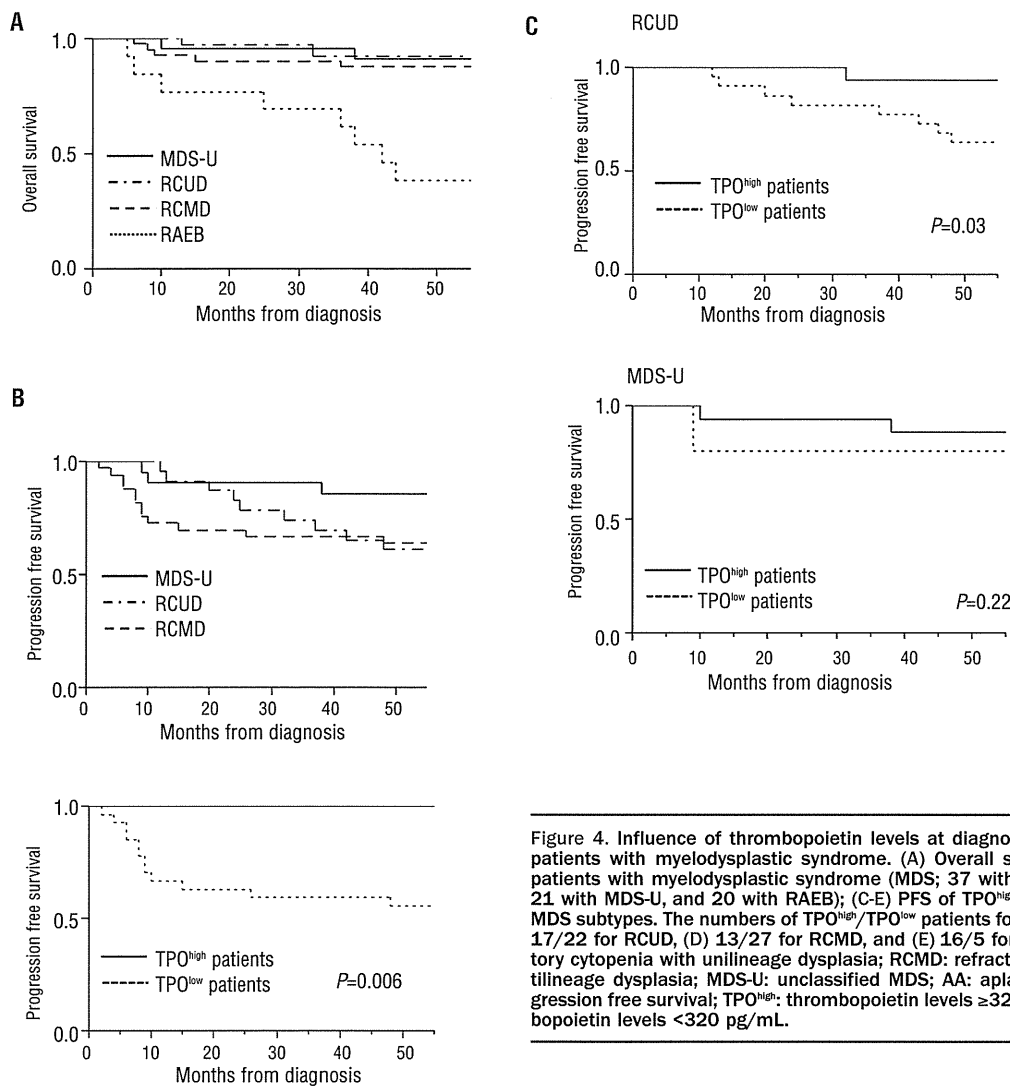


Figure 4. Influence of thrombopoietin levels at diagnosis on survival rates of patients with myelodysplastic syndrome. (A) Overall survival and (B) PFS of patients with myelodysplastic syndrome (MDS; 37 with RCUD, 40 with RCMD, 21 with MDS-U, and 20 with RAEB); (C-E) PFS of TPO<sup>high</sup> and TPO<sup>low</sup> patients by MDS subtypes. The numbers of TPO<sup>high</sup>/TPO<sup>low</sup> patients for each subtype were (C) 17/22 for RCUD, (D) 13/27 for RCMD, and (E) 16/5 for MDS-U. RCUD: refractory cytopenia with unilineage dysplasia; RCMD: refractory cytopenia with multilineage dysplasia; MDS-U: unclassified MDS; AA: aplastic anemia; PFS: progression free survival; TPO<sup>high</sup>: thrombopoietin levels  $\geq 320$  pg/mL; TPO<sup>low</sup>: thrombopoietin levels  $< 320$  pg/mL.

option may be hazardous to TPO<sup>high</sup> patients because their BM failure is not based on abnormal stem cells with pre-leukemic features. Measurement of TPO levels is, therefore, recommended for patients with low-risk MDS and thrombocytopenia so that the most appropriate therapy can be chosen. The exact role of TPO levels in the management of MDS will need to be evaluated in a prospective study.

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**Authorship and Disclosures**

Information on authorship, contributions, and financial and other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

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