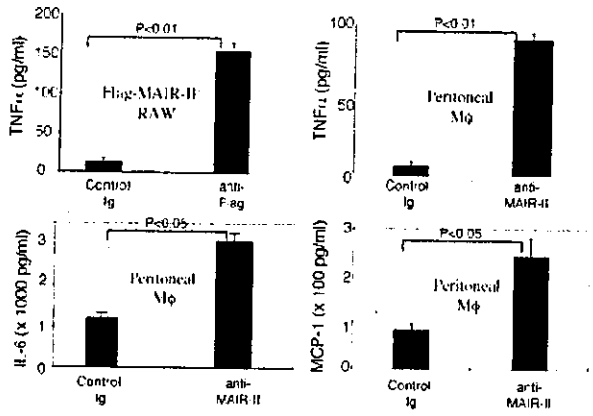

Figure 7. MAIR-II stimulates cytokines and chemokines secretion from macrophages. RAW cells transfected with *Flag-tagged MAIR-II* cDNA at the NH_2 terminus and peritoneal macrophages were pretreated with anti-CD16/32 (Fcγ receptors) to block Fc binding of mAbs, stimulated with plastic-coated control Ig, anti-Flag, or anti-MAIR-II, and cultured for 48 h. Culture supernatants were harvested, and cytokine's and chemokine's concentrations were measured by ELISA.

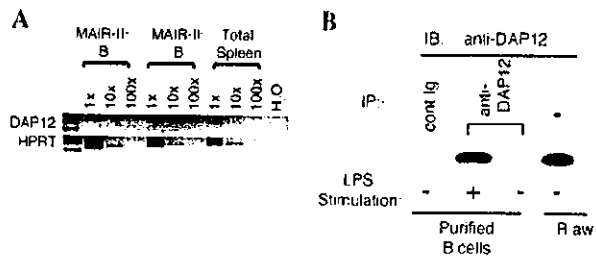

Figure 8. Expression of DAP12 in B cells. (A) Spleen cells were stained with biotin-conjugated anti-MAIR-II and PE-conjugated B220, followed by allophycocyanin-conjugated streptavidin. MAIR-II$^+$B220$^+$ and MAIR-II$^-$B220$^+$ cells were purified by repeating sorting twice on flow cytometry (>99.8% purity). The RNA was extracted from the fractionated and total spleen cells and was subjected to semiquantitive RT-PCR (30 cycles) for DAP12 and HPRT, according to template dose by dilution. (B) B cells were purified from spleen cells by repeating positive selection twice with B220$^+$ MACS-beads (>99.0% purity). 5×10^6 purified B cells were stimulated or not with 10 μg/ml LPS for 24 h, lysed in 1% NP-40 lysis buffer, and immunoprecipitated with control Ig or anti-DAP12. Immunoprecipitates or lysates of 10^6 raw cells were immunoblotted with anti-DAP12.

Expression of DAP12 in B Cells. Although human primary B cells and a mouse B cell line also express *DAP12* transcript (35, 36), there has been no paper reporting that primary mouse B cells express DAP12. To investigate the functional role of MAIR-II on B cells, we examined whether spleen B cells express DAP12. B cells were highly fractionated from spleen cells stimulated with LPS according to MAIR-II expression by repeating sorting on flow cytometry (>99.8% purity, as determined by reanalysis by flow cytometry) and subjected to semiquantitative RT-PCR. As demonstrated in Fig. 8 A, both fractions of B cells express *DAP12* transcript. To further examine whether B cells express DAP12 protein, DAP12 was immunoprecipitated from purified B cells before or after stimulation with LPS for 24 h and immunoblotted with anti-DAP12. Although DAP12 protein was undetectable in primary resting B cells, we observed a significant up-regulation of DAP12 protein expression in stimulated B cells (Fig. 8 B). These results suggest that MAIR-II associates with DAP12 (also on B cells, at least in the activated state with LPS), resulting in MAIR-II–mediated signaling in B cells.

Discussion

Paired activating and inhibitory Ig-like receptors have attracted widespread interest because they are expressed on a variety of immune cell types and facilitate activation or deactivation of immune responses. Although most genes encoding these receptors are located on human chromosome 19q13.4 or its syntenic region on murine chromosome 7 (6), *MAIR-I* and *MAIR-II* genes are mapped to a unique location on murine chromosome 11E2, suggesting that the MAIR is a novel family of paired activating and inhibitory receptors.

The regulatory mechanism of paired activating and inhibitory receptor expressions on the cell surface is quite an important concept to be clarified; these receptors may play opposite roles in immune responses upon binding to their, sometimes common, ligand(s). However, their protein expression profiles on immune cells remain largely unknown, mainly because of lacking mAbs which distinguish between these paired receptors containing highly conserved extracellular domains each other. This is the case also in CMRF-35-H9 and CMRF-35 (31), possible human homologues of MAIR-I and MAIR-II, respectively.

In this paper, we have analyzed expressions of MAIR-I and MAIR-II using TX-8 and TX-13 mAbs that specifically react to MAIR-I and MAIR-II, respectively, demonstrating that MAIR-I is expressed on the majority of all the myeloid lineage cells and a subset of B cells, whereas MAIR-II is expressed only on subsets of B cells and peritoneal macrophages (Fig. 3, B, C, and D). This suggests that the MAIR-I inhibitory receptor is always dominantly expressed on these cells. These findings were not in complete agreement with the results of transcript expression, which indicated that both MAIR-I and MAIR-II are similarly expressed in the multilineage immune cells (Fig. 2 B). However, we have demonstrated that MAIR-II protein expression on B cell surface is up-regulated after culture (Fig. 3 E). Similarly, MAIR-I expression was also up-regulated on NK cells after stimulation with IL-12. We have also shown the down-regulation of MAIR-I cell surface expression by the receptor internalization upon cross-linking (Fig. 4). Although physiological significances of the up-regulation of MAIR-I and MAIR-II expression and the down-regulation of MAIR-I expression on cell surface are uncertain at present, they may play a regulatory role in cell activation when these receptors encounter their ligand(s). Further studies on the regulatory mechanisms of MAIR-I and

MAIR-II expressions are required to clarify roles of these receptors in immune responses.

In the present work, we have shown that cross-linking MAIR-I and MAIR-II transduces inhibitory and activating signals, respectively. We have demonstrated the inhibition of IgE-mediated degranulation by cross-linking MAIR-I (Fig. 5). This situation is similar in murine PIR-B, an inhibitory type of paired Ig-like receptor, which, but not the activating type PIR-A, is also expressed on mast cells, where coligation of FcεRI and PIR-B induced inhibition of IgE-mediated mast cell activation (28). MAIR-I associates with the phosphatase SHIP, which should be involved in the inhibition of mast cell activation. However, we do not conclude which ITIM-like sequence in the cytoplasmic region interacts with SHIP to mediate an inhibitory signal. Although the aa sequences VEYSTL and LHYSSV fit the consensus sequence for ITIMs (I/V/L/SxYxxL/V), we cannot exclude a possibility that the YSTL and YSSV within these sequences consist of ITAM because of the appropriate aa distance for ITAM (eight aa) between the sequences (37). In fact, SHIP also associates with ITAM in several activating adaptors, such as the β and γ subunits of FcεRI, CD3 γ, δ, and ε chains as well as the T cell receptor ζ chain and Fcγ receptor via its SH2 domain (38–40), although the biological significance of these interactions has not yet been determined. Nonetheless, MAIR-I mediates an inhibitory signal and the concrete signaling pathways mediated by MAIR-I should be clarified in future papers.

On the contrary, MAIR-II associates with the ITAM-bearing adaptor DAP12 and mediates activating signals for secretion of inflammatory cytokines and chemokines, such as TNF-α and MCP-1, but not IL-12, in macrophages. TNF-α and MCP-1 are secreted from human macrophages upon initiation of DAP12-dependent pathways via TREM-1, whereas IL-6 and IL-12 are not (41). Presently, the discrepancy of IL-6 secretion mediated by mouse MAIR-II and human TREM-1 from macrophages, both of which are dependent on DAP12 pathways, is unclear. MAIR-II expression is also observed on a small subset of resting B cells and up-regulated on the majority of B cells after culture (Figs. 3, B and E). Because we have demonstrated that B cells express DAP12 (Fig. 8, A and B), MAIR-II may mediate activation signals via DAP12-dependent pathways in B cells. Future analyses are required to clarify MAIR-II–mediated signaling and the functional role of MAIR-II in B cells.

Finally, because the MAIR-II$^+$ cells coexpress MAIR-I simultaneously, a question should be raised how activation or deactivation of these cells is regulated by MAIR-I and MAIR-II if both receptors would bind the common ligand. To elucidate the regulatory roles of these receptors in cell signaling and immune responses, it is essentially required to identify and characterize the ligands for MAIR-I and MAIR-II and the receptor–ligand interactions.

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ELSEVIER

Self-renewal and lineage restriction of hematopoietic stem cells

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Over the past decade, the purification and characterization of hematopoietic stem cells have ascertained their presence at the clonal level although they had hitherto existed conceptually. Now we have begun to understand their functions in molecular terms. Several important works indicative of such a new era in stem cell biology have been published recently. In particular, Bmi1, which belongs to the Polycomb group of genes, has been implicated as one of the basic molecules to maintain the proliferation capacity in hematopoietic stem cells. We need to seek other similarly important molecules for their functions. Perhaps studying interactions among genes is one of the most exciting subjects in stem cell research.

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Abbreviations

CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRU	competitive repopulating unit
E	erythroblast
ES	embryonic stem
HSCs	hematopoietic stem cells
KO	knockout
KSL	Kit^+, $Sca\text{-}1^+$, Lin^-
m	macrophage
M	megakaryocyte
MAS	mean activity in stem cell
n	neutrophil
PcG	Polycomb group

Introduction

Hematopoietic stem cells (HSCs) are defined by their two functions: self-renewal and multilineage differentiation capabilities. The two major goals of HSC study are to understand these capacities at the molecular level and to clarify the mechanisms regulating self-renewal and lineage commitment. It is well known that serial transplantation eventually results in poor reconstitution. Our recent quantitative assessment of *in vivo* self-renewal at the clonal level has suggested that the self-renewal capacity of HSCs is extensive but also limited. Early work by Hayflick demonstrated that cultured normal human cells have a population doubling limit. Telomerase is a ribonucleoprotein enzyme consisting of a catalytic reverse transcriptase component (TERT) and an RNA template component. Cell senescence is associated with telomere shortening in human somatic cells where telomerase is not expressed. A low level of telomerase activity has been detected in an HSC population, but it is not sufficient for preventing progressive shortening of their telomeres after transplantation. It was expected that overexpression of TERT may lead to enhancement of their self-renewal ability. However, it has recently been shown that this is not the case [1•].

From our point of view, there are two research directions to address the limit of self-renewal capacity. One is a search for molecules responsible for this limitation. Enforced gene expression and gene knockout (KO) studies have provided great information on positive and negative regulators for proliferation ability at the level of HSCs as briefly summarized herein. The other is to study developmental hematopoiesis. The HSC pool is enormously expanded during development, but not much in adult bone marrow and spleen. Self-renewal and lineage restriction of HSCs should be spatially and temporarily regulated.

A simple explanation for *in vivo* HSC expansion would be that certain embryonic cells can do what adult HSCs cannot. Adult HSCs have been detected as long-term multilineage repopulating cells by transplantation assay using lethally irradiated mice as recipients. Novel assays would be needed to know the existence of embryonic HSCs with limitless self-renewal capacity, if they are in hematopoietic organs from embryonic though adult life. The recent discovery of multipotent adult progenitor cells [2] and observation of HSC expansion in long-term bone marrow culture with FGF-1 [3] have suggested that certain types of culture system can be applied to their detection. Alternatively, adult HSCs may acquire properties of embryonic ones as a result of epigenetic changes induced by *in vitro* culture.

Another possibility is that HSCs must stay in proper niches to self-renew: once they are removed from such sites, they may be destined to differentiate. To verify this attractive idea, the niche localization has to be specified in hematopoietic tissues and molecules interplaying between HSCs and their microenvironment should be identified.

Molecular basis for self-renewal and lineage commitment

To better understand the molecular basis of HSC self-renewal and lineage restriction, several candidate genes have recently been the focus of interest. Humphries and co-workers have studied members of the clustered homeobox gene family extensively. Some of these genes have already been shown to play roles in definitive hematopoiesis. In particular, Overexpression of *HoxB4* in HSCs resulted in a ~50-fold expansion of competitive repopulating units (CRUs) *in vivo* [4]. Furthermore, it has been described that there is a remarkable growth advantage in HoxB4-transduced HSCs over untransduced ones when cultured *in vitro* [5•]. Pbx1 encoding a TALE homeodomain transcription factor has been considered to interact with HoxB4. It has been reported that knock down of endogenous Pbx1 expression enhances *in vivo* expansion of HoxB4-transduced HSCs, suggesting that Pbx1 suppresses the effects of HoxB4 [6]. Despite a large increase in CRU number *in vivo* as well as *in vitro*, the quality of each CRU has never been evaluated. It is important to compare the amount of repopulating activity in each CRU regenerated with that in normal CRU before expansion. We have suggested the mean activity in stem cell (MAS, RU/CRU) be used for this purpose [7]. It is known that embryonic stem (ES) cells do not contribute to hematopoietic reconstitution when transplanted into myeloablated adult mice. Surprisingly, the ectopic expression of *HoxB4* has been shown to support the development of HSCs from ES cells *in vitro* [8••]. HoxB4 can be one of the important molecules regulating repopulating activity, but it is unclear how this gene works in HSC development from ES cells and their expansion in culture.

The Polycomb group (PcG) genes and the counteracting trithorax group genes have been implicated as upstream regulators of Hox genes. Among PcG proteins, Bmi1, Rae28, M33, Mph1, Ring1a and Ring1b together constitute a multimeric complex. Null mutation mice for these genes similarly display skeletal transformations and hematopoietic defects. Regarding the function of HSCs, Bmi1- or Rae28-deficient mice have exhibited the progressive hematopoietic defect with increase of age. This defect has appeared more severe in Bmi1 than in Rae28 [9,10••,11••]. Long-term repopulating activity in fetal liver and adult bone marrow cells of Bmi1-deficient mice was too low to be detected, indicating a crucial role of Bmi1 in maintenance of proliferation capacities of HSCs and progenitors [10••,12••]. In addition, the work has provided an excellent insight into how Bmi1 functions in leukemic stem cell emergence [10••].

β-catenin is a component of the Wnt signaling pathway. Its activating mutations have been implicated in tumorigenesis. Very recently, it has been demonstrated that overexpression of such a stable form of β-catenin expands the pool of HSCs *in vitro* [13••]. As β-catenin-expressing HSCs could grow consistently in culture with SCF alone for two months, special attention should be paid to their leukemic transformation. Purification of Wnt proteins has been difficult because of their inefficient secretion from trasfectants and insolubility. At last, however, several Wnt proteins have been successfully purified and Wnt3a protein has thus been shown to act on HSCs as a growth factor [14••]. The data suggest that it can induce the self-renewal of HSCs *in vitro*. Frizzled proteins are receptors for Wnt proteins. Certain members of the Frizzled family are expressed in HSCs. Their binding specificities to members of the Wnt family remain obscure. It is of interest to examine whether or not any particular members of Wnt proteins have different effects on HSCs through their specific receptor signaling.

HSCs reside in a small subpopulation of bone marrow cells termed the 'side population' when stained with the fluorescent dye Hoechst 33342. The side-population phenotype of HSCs is due to their expression of Bcrp1, an ATP binding cassette transporter that is responsible for dye efflux. The dye efflux property of HSCs does not seem directly linked with their self-renewal and differentiation capacities [15].

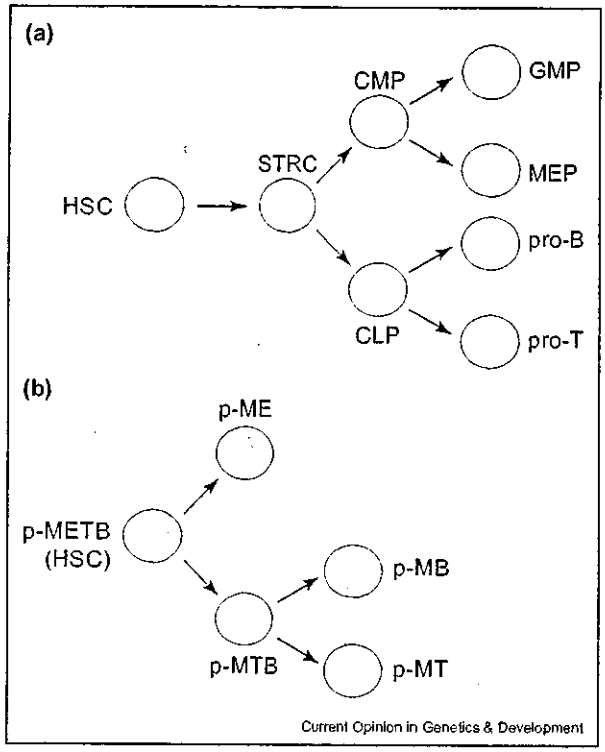
KO mouse studies have suggested that some transcriptional factors like SCL, GATA-2 and AML1 play essential roles in early development or specification of HSCs. As these mutant mice are embryonic lethal, the roles of these genes in the function of adult HSCs have not been elucidated. Conditional gene targeting is useful for addressing this problem. Studies of SCL conditional KO mice have revealed that continued expression of SCL is not required for HSC function but their differentiation along erythroid and megakaryocytic lineages is perturbed at the level of progenitors [16••,17•].

It is too complex to study the molecular basis of *in vivo* lineage commitment of HSCs. If we have an *in vitro* model system to work on, analysis of transgenic or KO mice would provide us more valuable information on molecules of interest.

The mode for hematopoietic stem cell differentiation

Two interesting models have recently been proposed to explain how lineage-restricted progenitors are generated from HSCs (Figure 1). Akashi, Weissman *et al.* have proposed that lineage commitment of HSCs first takes place when their progeny with multilineage differentiation potential give rise to common myeloid progenitors (CMPs) [18] and common lymphoid progenitors (CLPs) [19]. This model is based on phenotypic identification of CMPs and CLPs in adult mouse bone marrow. They have similarly shown the presence of CMPs and CLPs in the fetal liver [19,20]. This model seemed to have been

Figure 1



HSC differentiation models (a,b). Common myeloid progenitor (CMP), common lymphoid progenitor (CLP), megakaryocyte-erythrocyte-restricted progenitor (MEP), granulocyte-monocyte-restricted progenitor (GMP), short-term repopulating cell (STRC), myeloid, erythroid, T and B cell progenitor (p-METB), myeloerythroid progenitor (p-ME), myeloid, T and B cell progenitor (p-MTB), myeloid and B cell progenitor (p-MB), myeloid and T cell progenitor (p-MT).

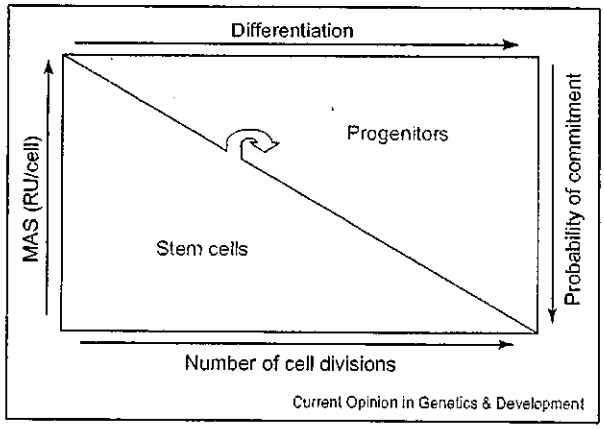
accepted by many researchers. However, in their proposal, a progenitor that gives rise to both CMP and CLP is still obscure. The 'short-term repopulating cell' is a candidate for such an intermediate cell, but the differentiation potential of this cell has not been clarified at the clonal level. Short-term repopulating cells likely represent a heterogeneous population in differentiation potential. Jacobsen and co-workers have found an interesting subpopulation in Kit^+, $Sca\text{-}1^+$, Lin^- (KSL) bone marrow cells [21]. KSL cells were subdivided into Flt-3-positive (FL^+KSL) and -negative (FL^-KSL) fractions. The majority of $CD34^-KSL$ cells highly enriched for HSCs were included in FL^-KSL fraction. FL^+KSL were able to give rise to CLP and neurrophil and macrophage (nm) progenitors, but not to erythroblasts (E) and megakaryocytes (M). These data are consistent with our own unpublished results. Moreover, T cell development occurring independently of CLP has been suggested recently [21]. We have obtained data showing that $CD34^-KSL$ cells asymmetrically give rise to nmEM and uni-, bi-, or tri-potent progenitors (H Ema, unpublished data). Thus, a progenitor for CMP and CLP may not lie at the main branching point for lineage commitment of HSCs.

Kawamoto, Katsura *et al.* [22•] have proposed another interesting model for HSC differentiation, as shown in Figure 1b. They have analyzed the differentiation potential of fractionated fetal liver cells. A unique culture called the multilineage progenitor assay has been used to detect myeloid, B- and T-cell lineage differentiation potentials. The multilineage progenitor assay has been invented by modifying fetal thymic organ culture. In addition to T cell progenitor (T), B cell (B), myeloid (M, namely nm) and E progenitors can be detected by this assay. They demonstrated the presence of clonogenic progenitor cells for METB, MTB, ME, MT and MB lineages in KSL fetal liver cells [22•]. They have claimed that CLPs have never been detected in the fetal liver, contradicting the observation by Akashi, Weissman *et al.* [19]. As it was reported that some fetal liver CLPs had macrophage differentiation potential, p-MTB can be equivalent to CLP. There are two unique points in their model. First, the lineage commitment of HSCs is defined by the appearance of progenitor cells for either the ME or MTB lineage (Figure 1b). Second, B- or T-cell differentiation potential is accompanied by myeloid differentiation potential in the early stages of lineage restriction. This model is only based on detection of a variety of distinct progenitor cells. To verify the model, each subset of progenitors should be prospectively separated and characterized. Of greater interest is a relationship between HSCs and their committed progeny.

The concept for self-renewal and lineage commitment

In general, HSCs are considered to make a choice of either self-renewing or committing to differentiation. Their fate decision may not be a simple as this. First,

Figure 2



Generation-age hypothesis proposed by Rosendaal, Hodgson and Bradley [23]. The authors have added the MAS to their model.

apoptosis should also be tightly regulated in HSCs. Second, HSCs show a variety of repopulating activity levels, suggesting their heterogeneity in nature. The generation–age hypothesis [23] is our preferred concept [24]. We have incorporated the MAS into this idea (Figure 2). As stem cells divide, the amount of repopulating activity per cell is progressively reduced. Simultaneously, the likelihood of lineage commitment increases. MAS level can be interpreted as an indicator of degree of self-renewal capacity [7]. In the strict sense, a stem cell may not regenerate daughter cells with the exact same activity. Therefore, HSCs should be measured quantitatively to evaluate self-renewal capacity. Lineage commitment in HSCs can be considered to occur when at least one of their differentiation potentials is lost. Dedifferentiation of progenitors has never been formally described despite its possibility [25].

Conclusions

On the basis of gene-expression studies of an HSC population, genes of interest have been selected and characterized mostly by using gene overexpression and targeting strategies. Because a number of genes expressed in HSCs have been known by now [26–28], more genes are likely to be found to play crucial roles in HSC functions. For a large-scale screening of functional genes, application of RNA interference may serve as a powerful tool. Sooner or later, the complexity of multiple gene expression in HSCs should be better understood. So far, signal transduction in HSCs is poorly understood despite the growing knowledge of various signaling molecules in many other cell types. This lack of understanding stems from the difficulty in obtaining the sufficient number of HSCs for such experiments, but this may be resolved through the use, for instance, of KO mice in which HSCs proliferate or *in vitro* culture. Epigenetic regulation of gene expression in HSCs may also be of great interest.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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This is a first definite report of *in vitro* generation of transplantable hematopoietic stem cells from mouse ES cells. Yolk sac and ES-derived cells were shown to be able to give rise to long-term repopulating stem cells in culture with OP-9 stromal cell line, SCF, VEGF and TPO following retroviral transduction with *HoxB4*. The efficiency of HSC generation and quality of generated HSCs are not clear. See also [12••].

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The numbers of repopulating stem cells and colony-forming cells in the fetal liver were compared between *rae28*$^{-/-}$ and *rae28*$^{+/+}$ mice. The number of colony-forming cells in the fetal liver of *rae28*$^{-/-}$ mice were significantly lowered by E18.5. The number of repopulating stem cells in the fetal liver of *rae28*$^{-/-}$ mice was reduced by a factor of 20 at E14.5. Compared with *Bmi1*$^{-/-}$ mice, similar but less severe phenotypes in *rae28*$^{-/-}$ mice enabled quantitative evaluation of stem and progenitor cells, providing useful information for interpreting data on *Bmi1*$^{-/-}$ mice. See also [10••,12••].

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See annotation [10••].

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Enhanced Self-Renewal of Hematopoietic Stem Cells Mediated by the Polycomb Gene Product Bmi-1

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Summary

The Polycomb group *(PcG)* gene *Bmi-1* has recently been implicated in the maintenance of hematopoietic stem cells (HSC) from loss-of-function analysis. Here, we demonstrate that increased expression of *Bmi-1* promotes HSC self-renewal. Forced expression of *Bmi-1* enhanced symmetrical cell division of HSCs and mediated a higher probability of inheritance of stemness through cell division. Correspondingly, forced expression of *Bmi-1*, but not the other *PcG* genes, led to a striking ex vivo expansion of multipotential progenitors and marked augmentation of HSC repopulating capacity in vivo. Loss-of-function analyses revealed that among *PcG* genes, absence of *Bmi-1* is preferentially linked with a profound defect in HSC self-renewal. Our findings define Bmi-1 as a central player in HSC self-renewal and demonstrate that Bmi-1 is a target for therapeutic manipulation of HSCs.

Introduction

Cell-type specific gene expression patterns are stabilized by changes in chromatin structure. Cellular memory of chromatin modifications can be faithfully maintained through subsequent cell divisions by the counteractions of transcriptional activators of the trithorax group (TrxG) proteins and repressors of the PcG (Jacobs and van Lohuizen, 2002; Orland, 2003). PcG proteins form multiprotein complexes that play an important role in the maintenance of transcriptional repression of target genes. At least two distinct PcG complexes have been identified and well characterized. One complex includes Eed, EzH1, and EzH2, and the other includes Bmi-1, Mel-18, Mph1/Rae28, M33, Scmh1, and Ring1A/B. Eed-containing complexes control gene repression through recruitment of histone deacetylase followed by local chromatin deacetylation and by methylation of histone H3 Lysine 27 by EzH2. In contrast, no enzymatic activity has yet been reported with regard to Bmi-1-containing complexes. However, Bmi-1 complexes antagonize chromatin remodeling by the SWI-SNF complex (Shao et al., 1999) and are recruited to methylated histone H3 Lysine 27 via M33 chromodomain to contribute to the static maintenance of epigenetic memory (Fischle et al., 2003). These two types of complexes coordinately maintain positional memory along the anterior-posterior axis by regulating *Hox* gene expression patterns during development (Jacobs and van Lohuizen, 2002; Orland, 2003). On the other hand, these two complexes play reciprocal roles in definitive hematopoiesis: negative regulation by the Eed-containing complex and positive regulation by Bmi-1-containing complex (Lessard et al., 1999).

The Bmi-1-containing complex has been implicated in the maintenance of hematopoietic and leukemic stem cells (HSC) (Ohta et al., 2002; Park et al., 2003; Lessard and Sauvageau, 2003). *Mph1/Rae28*$^{-/-}$ fetal liver contains 20-fold fewer long-term lymphohematopoietic repopulating HSCs than wild-type (wt) (Ohta et al., 2002). More importantly, although *Bmi-1*$^{-/-}$ mice show normal development of embryonic hematopoiesis, *Bmi-1*$^{-/-}$ HSCs have a profound defect in self-renewal capacity. They cannot repopulate hematopoiesis long term, leading to progressive postnatal pancytopenia (Park et al., 2003; van der Lugt et al., 1994). Notably, the self-renewal defect is not only confined to HSC but also applicable to leukemic stem cells and neuronal stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003). So far, the defective self-renewal of HSC has been attributed to derepression of Bmi-1 target genes $p16^{Ink4a}$ and $p19^{Arf}$, and deficiency of these genes partially reverses the self-renewal defect in *Bmi-1*$^{-/-}$ stem cells (Park et al., 2003; van der Lugt et al., 1994; Molofsky et al., 2003; Jacobs et al., 1999). More recently, Bmi-1 was reported also to be essential to the expansion of cerebellar granule cell progenitors, in which *Bmi-1* expression is reportedly regulated by the sonic hedgehog pathway (Leung et al., 2004). All of these findings have uncovered novel aspects of stem cell regulation exerted by epigenetic modifications. However, the defects in HSC in *Bmi-1*$^{-/-}$ mice has not yet been characterized in detail at the clonal level in vitro and in vivo. Furthermore, important questions remain, including the role of each component of the PcG complex in HSC and the impact of forced expression of *Bmi-1* on HSC self-renewal.

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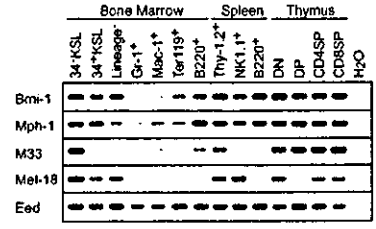
[6]These authors contributed equally to this work.

A



B

	E14.5 FL donor cells		Competitor Ly5.1 cells	PB chimerism 4 w	12 w	
Bmi-1	+/+	2x10^5 (n=10)	2x10^5	90.2 ± 1.2	95.0 ± 1.2	*
	+/–	2x10^6 (n=9)	2x10^5	86.1 ± 3.0	93.0 ± 2.4	
	–/–	2x10^6 (n=10)	2x10^5	0.7 ± 0.5	0.4 ± 0.4	
Mel-18	+/+	2x10^6 (n=19)	2x10^5	86.1 ± 4.3	88.9 ± 2.9	*
	–/–	2x10^6 (n=7)	2x10^5	62.1 ± 5.0	64.9 ± 9.0	
M33	+/+	2x10^6 (n=3)	2x10^5	94.0 ± 2.3	98.9 ± 0.7	
	–/–	2x10^6 (n=2)	2x10^5	90.3	99.2	

* p<0.0001

Figure 1. Role of Components of the Bmi-1-Containing Complex in HSC

(A) mRNA expression of mouse *PcG* genes in hematopoietic cells. Cells analyzed are bone marrow CD34$^-$c-Kit$^+$Sca-1$^+$Lineage marker$^-$ stem cells (CD34$^-$KSL), CD34$^+$KSL progenitors, Lineage marker$^-$ cells (Lin$^-$), Gr-1$^+$ neutrophils, Mac-1$^+$ monocytes/macrophages, TER119$^+$ erythroblasts, B220$^+$ B cells, spleen Thy-1.2$^+$ T cells, NK1.1$^+$ NK cells, B220$^+$ B cells, and thymic CD4$^-$CD8$^-$ T cells (DN), CD4$^+$CD8$^-$ T cells (DP), CD4$^+$CD8$^-$ T cells (CD4SP), and CD4$^-$CD8$^+$ (CD8SP).

(B) Competitive lymphohematopoietic repopulating capacity of *PcG* gene-deficient HSCs. The indicated number of E14 fetal liver cells from *Bmi-1*$^{-/-}$, *Mel-18*$^{-/-}$, or *M33*$^{-/-}$ mice (B6-Ly5.2) and B6-Ly5.1 competitor cells were mixed and injected into lethally irradiated B6-Ly5.1 recipient mice. Percent chimerism of donor cells 4 and 12 weeks after transplantation is presented as mean ± SD.

In this study, both loss-of-function and gain-of-function analysis revealed a central role for Bmi-1, but not the other components, in the maintenance of HSC self-renewal both in vitro and in vivo and in augmentation of HSC activity ex vivo. Our findings indicate that the expression level of Bmi-1 is the critical determinant for the self-renewal capacity of HSCs.

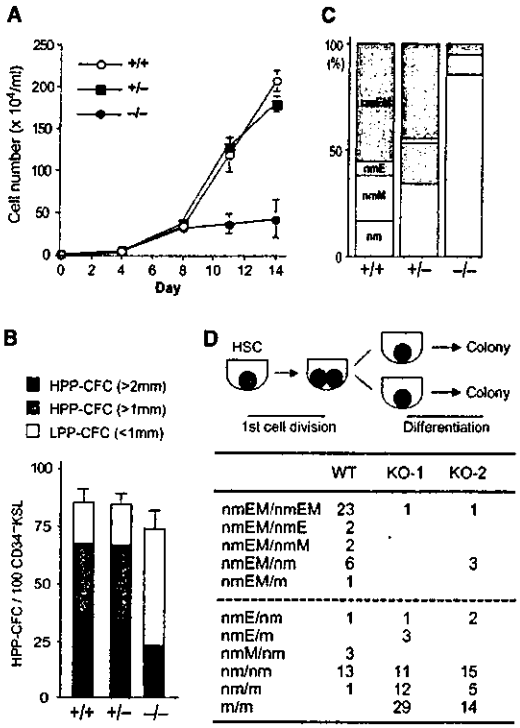
Results

The Role of Different Components of the Bmi-1-Containing Complex in HSC

Expression analysis of *PcG* genes in human hematopoietic cells has demonstrated that *Bmi-1* is preferentially expressed in primitive cells, whereas other *PcG* genes, including *M33*, *Mel-18*, and *Mph1/Rae-28*, are not detectable in primitive cells but upregulated along with differentiation (Lessard et al., 1998). Our detailed RT-PCR analysis of mouse hematopoietic cells, however, revealed that all *PcG* genes encoding components of the Bmi-1-containing complex, including *Bmi-1*, *Mph1/Rae-28*, *M33*, and *Mel-18*, are highly expressed in CD34$^-$KSL HSCs that comprise only 0.004% of bone marrow mononuclear cells (Osawa et al., 1996), and all are down regulated during differentiation in the bone marrow (BM) (Figure 1A). In contrast, *Eed*, whose product composes another PcG complex, was ubiquitously expressed. These expression profiles support the idea of positive regulation of HSC self-renewal by the Bmi-1-containing complex (Park et al., 2003; Lessard and Sauvageau, 2003). To evaluate the role of uncharacterized PcG components (Mel-18 and M33) in the maintenance of HSCs, we performed competitive repopulation assay with ten times more fetal liver cells from *Bmi-1*$^{-/-}$, *Mel-18*$^{-/-}$, or *M33*$^{-/-}$ mice than competitor cells. As reported, *Bmi-1*$^{-/-}$ fetal liver cells did not contribute at all to long-term reconstitution (Figure 1B). The profound defect of repopulating activity was confirmed in a radioprotection assay in which *Bmi-1*$^{-/-}$ hematopoietic cells failed to ensure long-term survival of lethally irradiated mice, although they prolonged the survival of recipients over 21 days, indicating that their short-term repopulation capacity is largely preserved (Supplemental Figure S1A available online at http://www.immunity.com/cgi/content/full/21/6/843/DC1/). Mel-18 is highly related to Bmi-1 in domain structure, particularly in their N-terminal Ring finger and helix-turn-helix domains. Unexpectedly, *Mel-18*$^{-/-}$ fetal liver cells showed a very mild deficiency in repopulating capacity when compared to *Bmi-1*$^{-/-}$ fetal liver cells (Figure 1B). Moreover, *M33*$^{-/-}$ fetal liver cells exhibited normal repopulating capacity in both primary (Figure 1B) and secondary recipients (chimerism after 3 months; wt 91.6 ± 2.0 versus *M33*$^{-/-}$ 92.2 ± 3.4, n = 4). As is the case with *Bmi-1*$^{-/-}$ fetal livers, both *Mel-18*$^{-/-}$ and *M33*$^{-/-}$ fetal livers did not show any gross abnormalities, including numbers of hematopoietic cells (data not shown). To examine the *Bmi-1*$^{-/-}$ hematopoietic microenvironment, wt BM cells were transplanted into sublethally irradiated *Bmi-1*$^{-/-}$ mice. Subsequent secondary transplantation exhibited that both *Bmi-1*$^{-/-}$ BM and spleen can support long-term lymphohematopoiesis, indicating again an intrinsic defect of *Bmi-1*$^{-/-}$ HSCs (Supplemental Figure S1B).

Defective Self-Renewal and Accelerated Differentiation of *Bmi-1*$^{-/-}$ HSCs

A progressive postnatal decrease in the number of Thy1.1^{low}c-Kit$^+$Sca-1$^+$lineage marker$^-$ HSC has been observed in *Bmi-1*$^{-/-}$ mice (Park et al., 2003). We also observed approximately 10-fold fewer total CD34$^-$KSL HSCs as measured by flow cytometry in 8-week-old *Bmi-1*$^{-/-}$ mice (data not shown). To evaluate the proliferative and differentiation capacity of *Bmi-1*$^{-/-}$ HSCs in BM, we purified the CD34$^-$KSL HSC fraction, which is highly enriched for long-term repopulating HSCs (Osawa et al., 1996). *Bmi-1*$^{-/-}$ CD34$^-$KSL cells showed comparable proliferation with wt and *Bmi-1*$^{+/-}$ cells for the first week of culture, but thereafter, they proliferated poorly (Figure 2A). Single cell growth assays demonstrated that *Bmi-1*$^{-/-}$ CD34$^-$KSL cells are able to form detectable colonies at a frequency comparable to *Bmi-1*$^{+/+}$ and *Bmi-1*$^{+/-}$ CD34$^-$KSL cells but contained 3-fold fewer high-proliferative-potential colony-forming cells (HPP-CFCs). Reduction of HPP-CFCs that gave rise to colonies larger than 2 mm in diameter was even more prominent (7-fold) (Figure 2B). All HPP colonies larger than 1 mm in diameter were evaluated morphologically. Surprisingly, most of the HPP colonies generated from *Bmi-1*$^{-/-}$ CD34$^-$KSL cells consisted of only neutrophils



	WT	KO-1	KO-2
nmEM/nmEM	23	1	1
nmEM/nmE	2		
nmEM/nmM	2		
nmEM/nm	6		3
nmEM/m	1		
nmE/nm	1	1	2
nmE/m		3	
nmM/nm	3		
nm/nm	13	11	15
nm/m	1	12	5
m/m		29	14

Figure 2. Defective Self-Renewal and Accelerated Differentiation of *Bmi-1*$^{-/-}$ HSCs

(A) Growth of *Bmi-1*$^{-/-}$ CD34$^-$KSL HSCs in vitro. Freshly isolated CD34$^-$KSL cells were cultured in the presence of SCF, IL-3, TPO, and EPO for 14 days. The results are shown as mean ± SD of triplicate cultures.

(B) Single cell growth assay. 96 individual CD34$^-$KSL HSCs were sorted clonally into 96-well microtiter plates in the presence of SCF, IL-3, TPO, and EPO. The numbers of high- and low-proliferative-potential colony-forming cells (HPP-CFC and LPP-CFC) were retrospectively evaluated by counting colonies at day 14 (HPP-CFC and LPP-CFC: colony diameter >1 mm and <1 mm, respectively). The results are shown as mean ± SD of triplicate cultures.

(C) Frequency of each colony type. Colonies derived from HPP-CFC were recovered and morphologically examined for the composition of colony-forming cells.

(D) Paired daughter assay. When a single CD34$^-$KSL HSC underwent cell division and gave rise to two daughter cells, daughter cells were separated by micromanipulation and were further cultured to permit full differentiation along the myeloid lineage. The colonies were recovered for morphological examination.

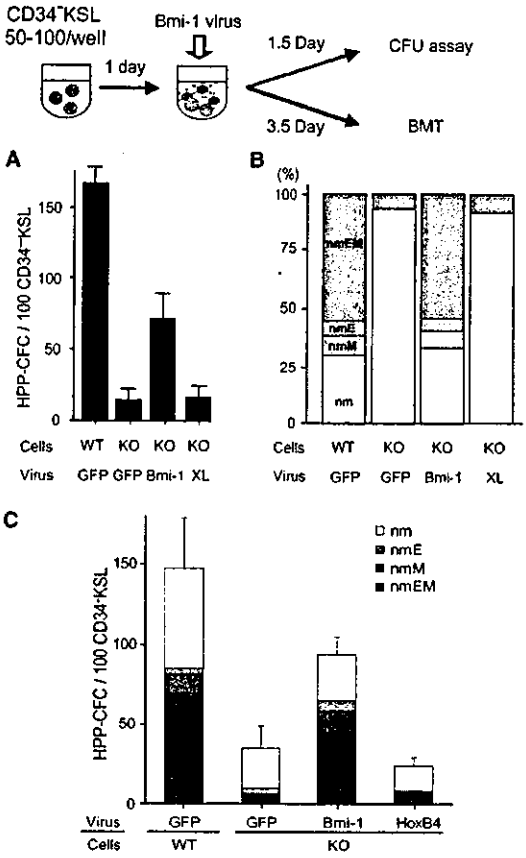
and macrophages. *Bmi-1*$^{-/-}$ CD34$^-$KSL cells presented a 9-fold reduction in their frequency of colony-forming unit-neutrophil/macrophage/erythroblast/megakaryocyte (CFU-nmEM), which retains multilineage differentiation capacity compared with *Bmi-1*$^{+/+}$ CD34$^-$KSL cells (Figure 2C). Failure of *Bmi-1*$^{-/-}$ CD34$^-$KSL cells to inherit multilineage differentiation potential through successive cell division was obvious in a paired daughter assay (Figure 2D). In most daughter cell pairs generated from wt CD34$^-$KSL cells, at least one of the two daughter cells inherited nmEM differentiation potential, whereas *Bmi-1*$^{-/-}$ CD34$^-$KSL cells showed accelerated loss of multilineage differentiation potential, leading to the limited differentiation and inefficient expansion of their progeny. In terms of differentiation, no apparent differentiation block has been observed in *Bmi-1*$^{-/-}$ lymphocytes despite their reduced numbers (Jacobs et al., 1999). Analysis of myeloid progenitors in BM did not detect any proportional deviations of common myeloid progenitors (CMP), granulocyte/macrophage progenitors (GMP), or megakaryocyte/erythroid progenitors (MEP) either (Supplemental Figure S2), indicating that abnormal hematopoiesis observed in *Bmi-1*$^{-/-}$ mice does not accompany any specific differentiation block in myeloid lineages.

These profound defects of *Bmi-1*$^{-/-}$ HSC function evoke the possibility that absence of Bmi-1 in HSCs causes additional epigenetic abnormalities that are irreversible, and CD34$^-$KSL cells do not retain stem cell properties anymore. Retroviral transduction of *Bmi-1*$^{-/-}$ CD34$^-$KSL cells with *Bmi-1*, however, completely rescued their defects in proliferation and multilineage differentiation potential in vitro (Figures 3A and 3B) and long-term repopulating capacity in vivo (Figure 3D). These findings suggest that execution of stem cell activity is absolutely dependent on Bmi-1. Because *Mel-18*$^{-/-}$ and *M33*$^{-/-}$ mice in a C57BL/6 background die during the perinatal period or soon after birth, we could not evaluate their roles in adult BM HSCs. We next asked if HoxB4, a well-characterized HSC regulator, could rescue *Bmi-1*$^{-/-}$ phenotypes. Real-time PCR analysis demonstrated that *HoxB4* expression is not significantly affected in freshly isolated *Bmi-1*$^{-/-}$ KSL cells (relative expression in *Bmi-1*$^{-/-}$ to wt KSL cells, 0.73 ± 0.21, n = 3, p = 0.4). Interestingly, expression of *HoxB4* did not rescue *Bmi-1*$^{-/-}$ HSC defects at all (Figures 3C and 3D), indicating that HoxB4 requires functional Bmi-1 to execute its activity in HSCs.

Given the reported involvement of derepression of *p16*^{Ink4a} and *p19*^{Arf} genes in the self-renewal defect in *Bmi-1*$^{-/-}$ HSCs (Park et al., 2003; Lessard and Sauvageau, 2003), we examined their expression in hematopoietic cells. As reported, both were significantly upregulated in *Bmi-1*$^{-/-}$ Lin$^-$ cells (Supplemental Figure S3A). Overexpression of *p16* inhibits G_1-S progression, and increased *p19* causes p53-dependent growth arrest and apoptosis (Jacobs and van Lohuizen, 2002; Park et al., 2003; Lessard and Sauvageau, 2003). However, cell cycle analysis of *Bmi-1*$^{-/-}$ BM cells, including KSL primitive progenitors (Supplemental Figure S3B), did not discriminate any difference between wt and *Bmi-1*$^{-/-}$ mice. Furthermore, in single cell assays, *Bmi-1*$^{-/-}$ CD34$^-$KSL HSCs underwent the first cell division in a fashion similar to that of wt control (Supplemental Figure S3C) and showed no detectable apoptotic cell death (data not shown), although total *Bmi-1*$^{-/-}$ BM cells presented a slight but significant increase in apoptotic cell percentage (Supplemental Figure S3D). In addition, retrovirally transduced *Bcl-xL* had no impact on *Bmi-1*$^{-/-}$ HSCs in vitro (Figures 3A and 3B). These findings indicate that derepression of *p16* and *p19* genes in *Bmi-1*$^{-/-}$ HSC does not largely affect the cell cycle or survival of HSCs.

Augmentation of HSC Activity by Forced *Bmi-1* Expression

An essential role of Bmi-1 in the maintenance of HSC self-renewal capacity prompted us to determine augmentation of HSC activity by *PcG* genes. CD34$^-$KSL



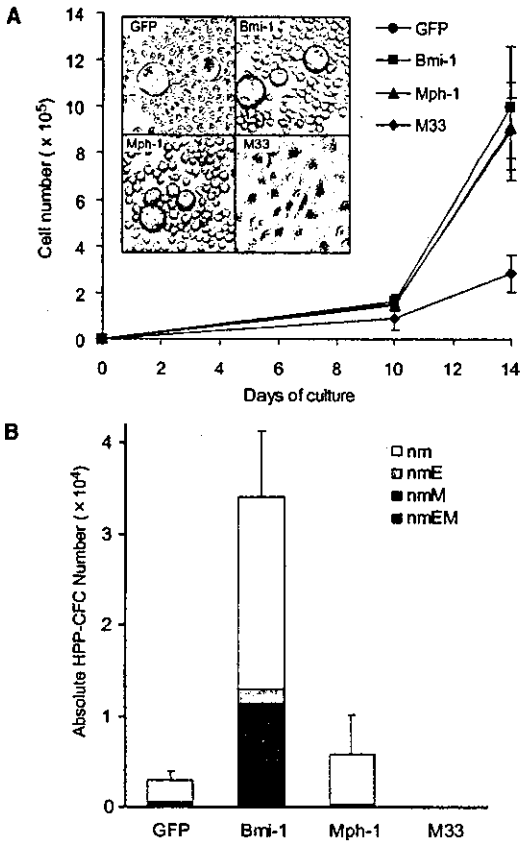
D

Virus	Donor cells Bmi-1$^{-/-}$	Competitor Ly5.1 cells	PB chimerism (%)
Bmi-1 (n=1)	80	2x10^5	73.0 (12 w)
Bmi-1 (n=1)	25	2x10^5	12.8 (12 w)
Bmi-1 (n=1)	25	2x10^5	13.9 (12 w)
Bmi-1 (n=4)	75	2x10^5	12.1±10.7 (6 w)
HoxB4 (n=4)	75	2x10^5	0.1±0.1 (6 w)

Figure 3. Rescue of Defective *Bmi-1*$^{-/-}$ HSC Function by Reexpression of *Bmi-1*

Wt and *Bmi-1*$^{-/-}$ CD34$^-$KSL cells were transduced with *GFP* control, *Bmi-1*, or *Bcl-xl* and plated in methylcellulose medium to allow colony formation 36 hr after the initiation of transduction. GFP$^+$ colonies larger than 1 mm in diameter, which were derived from HPP-CFCs, were counted at day 14 (A) and recovered for morphological analysis to evaluate frequency of each colony type (B). Wt and *Bmi-1*$^{-/-}$ CD34 KSL cells transduced with *HoxB4* were similarly processed (C). The results are shown as mean ± SD of triplicate cultures.

(D) Indicated numbers of *Bmi-1*$^{-/-}$ CD34$^-$KSL cells were transduced with either *Bmi-1* or *HoxB4*. After 3.5 days from the initiation of transduction, cells were injected into lethally irradiated Ly5.1 recipient mice along with Ly5.1 competitor cells. Repopulation by rescued *Bmi-1*$^{-/-}$ CD34$^-$KSL cells was evaluated by monitoring donor cell chimerism in peripheral blood at the indicated time points after transplantation.



C

Fold expansion of CFU-nmEM				
Exp.1	GFP	Bmi-1	Mph-1	M33
	8.6 ± 2.7	81.1 ± 17.3	5.4 ± 4.0	0 ± 0
Exp.2	GFP	Bmi-1	Mel-18	
	1.4 ± 0.3	86.1 ± 9.3	14.4 ± 5.1	
Exp.3	GFP	Bmi-1	HoxB4	
	1.5 ± 0.8	56.2 ± 33.4	37.9 ± 17.4	

Figure 4. Ex Vivo Expansion of CFU-nmEM by Forced Expression of *Bmi-1* in HSCs

(A) CD34$^-$KSL cells transduced with indicated *PcG* gene retroviruses were cultured in the presence of SCF and TPO, and their growth was monitored. Morphology of cultured cells at day 14 was observed under an inverted microscope (inset).

(B) At day 14 of culture, colony assays were performed to evaluate the content of HPP-CFC in culture. GFP$^+$ colonies derived from HPP-CFCs were examined on their colony types by morphological analysis.

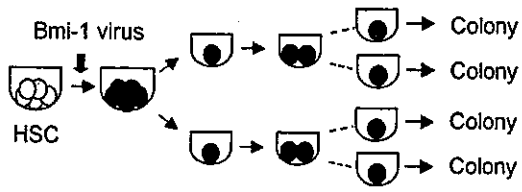
(C) Net expansion of CFU-nmEM during the 14 day culture period. The results are shown as mean ± SD of triplicate cultures.

HSCs were transduced with *Bmi-1*, *Mph1/Rae28*, or *M33* and then further incubated for 13 days (14 day ex vivo culture in total). Transduction efficiencies were over 80% in all experiments (data not shown). In the presence of stem cell factor (SCF) and thrombopoietin (TPO), which support expansion of HSCs and progenitors rather than their differentiation, forced expression of *Bmi-1* as well as *Mph1/Rae28* gave no apparent growth advantage in culture compared with the *GFP* control (Figure 4A). Notably, however, *Bmi-1*-transduced, but not *Mph1/Rae28*-transduced cells, contained numerous HPP-CFCs (Figure 4B). Morphological evaluation of the

colonies revealed significant expansion of CFU-nmEM by *Bmi-1*. Given that 60% of freshly isolated CD34$^-$KSL cells can be defined as CFU-nmEM, as shown in Figure 2D, there was a net expansion of CFU-nmEM of 56- to 80-fold over 14 days in the *Bmi-1* cultures (Figure 4C). Unexpectedly, expression of *M33* induced an adverse effect on proliferation and caused accelerated differentiation into macrophages that attached to the bottom of culture dishes (Figure 4A). In contrast to *Mph-1/Rae28* and *M33*, forced expression of *Mel-18*, a polycomb gene that is highly related to *Bmi-1* in domain structure, also showed a mild but significant effect on CFU-nmEM expansion, suggestive of functional redundancy between Bmi-1 and Mel-18 (Figure 4C). The effect of *Bmi-1* is comparable to that of *HoxB4*, a well-known HSC activator (Antonchuk et al., 2002) (Figure 4C). In addition, both *Bmi-1*- and *HoxB4*-transduced cells showed higher proliferative potential and generated much larger colonies compared with the *GFP* control (data not shown).

To determine the mechanism that leads to the drastic expansion of CFU-nmEM, which retains a full range of differentiation potential, we employed a paired daughter cell assay to see if overexpression of *Bmi-1* promotes symmetric HSC division in vitro. After 24 hr of prestimulation, CD34$^-$KSL cells were transduced with a *Bmi-1* retrovirus for another 24 hr. After transduction, single cell cultures were initiated by micromanipulation. When a single cell underwent cell division, the daughter cells were separated again and were allowed to form colonies. To evaluate the commitment process of HSCs while excluding committed progenitors from this study, we selected daughter cells retaining nmEM differentiation potential by retrospective inference. Expression of *Bmi-1* was assessed by GFP expression. As expected, forced expression of *Bmi-1* significantly promoted symmetrical cell division of daughter cells (Figure 5), indicating that Bmi-1 contributes to CFU-nmEM expansion by promoting self-renewal of HSCs.

We next performed competitive repopulation assays with 10 day ex vivo cultured cells corresponding to 20 initial CD34$^-$KSL cells per recipient mouse. After 3 months, mice that received *Bmi-1*-transduced HSCs demonstrated marked enhancement of multilineage repopulation whereas repopulation mediated by *GFP*-transduced HSCs was barely detectable (Figure 6A). The repopulating potential in a cell population can be quantitated by calculating repopulation units (RU) from the donor cell chimerism and the competitor cell number (Harrison et al., 1993). *Bmi-1*-transduced HSCs manifested 35-fold higher RU compared with *GFP* controls (Figure 6B). The competitive repopulation assay was similarly performed in parallel by using *p19*$^{-/-}$ HSCs. We expected a drop in *Bmi-1*-dependent enhancement of repopulation because *p19* is one of the targets negatively regulated by Bmi-1. Actually, expression of *p19* and another Bmi-1 target gene, *p16*, was completely repressed by *Bmi-1* in cultured cells (Figure 6C). Nonetheless, expression of *Bmi-1* in *p19*$^{-/-}$ HSC again enhanced multilineage repopulation compared with *p19*$^{-/-}$ *GFP* control cells (Figure 6A). A 15-fold increase in RU was obtained with *Bmi-1*-transduced *p19*$^{-/-}$ HSCs compared to *GFP*-transduced *p19*$^{-/-}$ HSCs (Figure 6B). This data suggest that *p19* is not the main target of Bmi-1 in HSCs. Expression of other cell cycle regulator genes



Colony pair	GFP virus	Bmi-1 virus
nmEM/nmEM	26 (53%)	40 (74%)*
nmEM/nmE	1	1
nmEM/nmM	3	6
nmEM/nm	19	7
Total	49	54

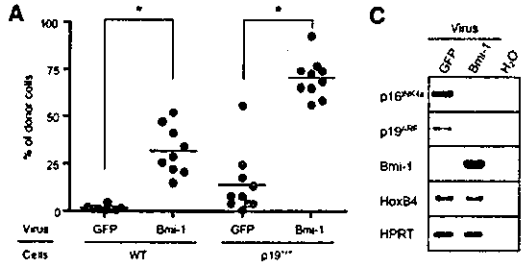
Figure 5. Forced Expression of *Bmi-1* Promotes Symmetrical Cell Division of HSCs

CD34$^-$ KSL HSCs were transduced with either *GFP* or *Bmi-1* retroviruses. After 24 hr of transduction, cells were separated clonally by micromanipulation. When a single cell underwent cell division, daughter cells were separated again by micromanipulation and were further cultured to permit full differentiation along the myeloid lineage. The colonies were recovered for morphological examination. Only the pairs whose parental cells should have retained neutrophil (n), macrophage (m), erythroblast (E), and megakaryocyte (M) differentiation potential were selected. The probability of symmetrical cell division of daughter cells transduced with *Bmi-1* was significantly higher than the control ($p < 0.044$).

such as *INK4* genes (*p15*^{INK4b}, *p18*^{INK4c}, and *p19*^{INK4d}) and *Cip/Kip* genes (*p21*, *p27*, and *p57*) was not grossly affected by *Bmi-1* expression in culture (data not shown). Analysis of percent chimerism of donor cells in each hematopoietic lineage revealed that *Bmi-1*-transduced HSCs retained full differentiation capacity along myeloid and lymphoid lineages (Figure 6B). As expected from in vitro data, HSCs transduced with *M33* did not contribute to repopulation at all (Figure 6B). We further asked whether Bmi-1 could confer long-term repopulating capacity on CD34$^+$KSL progenitor cells. We transduced *Bmi-1* or *HoxB4* into CD34$^+$KSL cells and carried out both in vitro and in vivo analyses. Unexpectedly, neither *Bmi-1* nor *HoxB4* enhanced colony-forming efficiency of CD34$^+$KSL progenitor cells or conferred long-term repopulation capacity on CD34$^+$KSL progenitor cells (Supplemental Figure S4).

Discussion

Loss-of-function analyses of the *PcG* genes *Bmi-1* and *Mph1/Rae-28* have established that they are essential for the maintenance of adult BM HSCs, but not for the development of definitive HSCs (Ohta et al., 2002; Park et al., 2003; Lessard and Sauvageau, 2003). Compared with *Mph1/Rae-28*$^{-/-}$ mice, however, hematopoietic defects are more severe in *Bmi-1*$^{-/-}$ mice and are attributed to impaired HSC self-renewal (Park et al., 2003; Lessard and Sauvageau, 2003). In this study, we observed normal development of definitive hematopoiesis also in *Mel-18*$^{-/-}$ and *M33*$^{-/-}$ fetal livers. Although both



B

Mice	Virus	Percent chimerism: All WBCs	Myeloid	T Lymphoid	B Lymphoid	RU
WT	GFP	1 6±1 3	0 1±0 1	1 1±1 3	4 5±3 3	0 03±0 03
	Bmi-1	31 9±12 7	30 6±19 1	29 6±14 7	33 9±16 6	1 04±0 66
	M33	0	0	0	0	0
p19-/-	GFP	14 0±16 4	14 6±24 2	9 4±8 1	16 0±11 3	0 45±0 83
	Bmi-1	70 5±10 4	76 1±20 7	51 2±9 4	69 6±10 8	6 68±7 70

Figure 6. Enhancement of Repopulation Activity of HSCs by *Bmi-1* Expression

(A) CD34$^-$KSL cells either from wt or *p19*$^{-/-}$ mice were transduced with indicated retroviruses and were further cultured in the presence of SCF and TPO. Competitive repopulation assays were performed by using cultured cells at day 10 corresponding to 20 initial CD34$^-$KSL cells per recipient mouse. Percent chimerism of donor cells 12 weeks after transplantation is plotted as dots and their mean values are indicated as bars. *$p < 0.001$.

(B) Percent chimerism in each lineage and RU of each population are presented.

(C) RT-PCR analysis was performed on the wt CD34$^-$KSL cells that were transduced with the indicated retrovirus and cultured for 14 days in the presence of SCF and TPO.

Mel-18 and *M33* genes are highly expressed in HSCs (Figure 1A), *Mel-18*$^{-/-}$ and *M33*$^{-/-}$ HSCs showed mild or no defects and retained long-term repopulating capacity (Figure 1B). Accordingly, overexpression of *PcG* genes in HSCs demonstrated that only *Bmi-1* enhances HSC function, whereas *M33* completely abolishes HSC function (Figures 4 and 6). All these findings clearly address a central role for Bmi-1 in the maintenance of HSC and suggest that the level of Bmi-1 protein is a critical determinant for the activity of the PcG complex in HSC. Bmi-1 may behave as a core component of the PcG complex in recruiting molecules essential for gene silencing or provide a docking site for DNA-binding proteins, such as Plzf on *HoxD* gene regulatory elements (Barna et al., 2002) and E2F6 that targets multimeric chromatin modifiers to E2F- and Myc-responsive genes (Trimarchi et al., 2001; Ogawa et al., 2002). On the other hand, the finding that M33 is dispensable in the maintenance of definitive HSC is surprising. Both *Bmi-1* and *M33* are involved in the maintenance of homeotic gene expression pattern through development, and strong dosage interactions between the two genes have been observed in this process (Bel et al., 1998). Our finding, however, presents a possibility that M33 does not contribute to the Bmi-1 PcG complex in HSC. M33 could be recruited to histone H3 Lysine 27 methylated by the Eed-containing complex and thereby mediate targeting of the Bmi-1-containing complex to PcG targets (Fischle et al., 2003). Thus, M33 is a key molecule for coordinated regulation of *Hox* genes by Eed- and Bmi-1-containing complexes. In contrast, the dispensable role of M33 in HSC correlated well to the reciprocal roles of the two complexes in definitive hematopoiesis (Lessard et al., 1999) and indicates that Bmi-1-containing complex has a silencing pathway of its own. The negative effect of overloaded M33 on HSCs could be due to squelching of PcG components by M33. In both loss-of-function and gain-of-function analyses, Mel-18 appeared to have a mild but significant biological function in HSCs. Given that Mel-18 shares domain structure with Bmi-1, there seems to be some functional redundancy between the two molecules in HSCs.

HSCs are maintained and expanded through self-renewal. HSC self-renewal secures its high repopulation capacity and multilineage differentiation potential through cell division. If HSCs fail to self-renew, they differentiate to lower orders of progenitors with limited proliferative and differentiation potential. Paired daughter cell assays that monitor the behavior of HSCs in vitro (Suda et al., 1984; Takano et al., 2004) demonstrated that Bmi-1 is essential for CD34$^-$KSL cells to inherit multilineage differentiation potential through successive cell divisions (Figure 2D). Notably, overexpression of *Bmi-1* in CD34$^-$KSL cells promoted their symmetrical cell division, indicating a higher probability of inheritance of stemness mediated by *Bmi-1* (Figure 5). This is evidence of successful genetic manipulation of HSC self-renewal in vitro. These clonal observations together with functional rescue of *Bmi-1*$^{-/-}$ HSC both in vitro and in vivo strongly support an essential role of Bmi-1 in HSC self-renewal.

The central role for Bmi-1 in HSC self-renewal was also demonstrated by overexpression experiments of *PcG* genes in HSC. The *Bmi-1*-mediated growth advantage was largely restricted to the primitive hematopoietic cells. During ex vivo culture, total cell numbers were almost comparable to the control while a net 56- to 80-fold CFU-nmEM expansion and 15- to 35-fold higher repopulation activity were obtained in the *Bmi-1* cultures (Figures 4 and 6). In agreement with these data, symmetrical cell division of HSC was promoted in the *Bmi-1* cultures (Figure 5), suggesting enhanced probability of HSC self-renewal and progenitor expansion mediated by *Bmi-1* overexpression. Importantly, transduction of *Bmi-1* into CD34$^+$KSL progenitor cells did not enhance their colony-forming efficiency or in vivo repopulating capacity at all (Supplemental Figure S4). These findings suggest that Bmi-1 preferentially promotes HSC self-renewal and MPP expansion but does not confer growth advantage or long-term repopulation capacity on CD34$^+$KSL progenitor cells with limited differentiation potential. Although *Bmi-1*-transduced HSC established higher repopulation in vivo, chimerism of *Bmi-1*-transduced HSC progenies reached its plateau between 2 and 3 months and never showed continuous growth advantages in vivo. This could be due to silencing of retroviral *Bmi-1* expression in vivo as suggested by a significant decrease in GFP intensity detected by flow cytometric analysis (data not shown). Actually, real-time PCR analyses demonstrated that the relative expression of *Bmi-1* in transduced cells to *GFP* control cells was 5.8 ± 1.3-fold in purified KSL cells from 14 day ex vivo culture and 1.3 ± 0.1-fold in KSL cells recovered from reconstituted recipients 6 months after transplantation. Thus, marked enhancement of HSC repopulating capacity might be obtained by enhanced HSC recovery after

ex vivo culture. Alternatively, increased expression of *Bmi-1* may not confer a growth advantage in steady-state hematopoiesis once HSC becomes quiescent in the niche. The comparable effect of *Bmi-1* to that of *HoxB4*, a well-known HSC activator (Antonchuk et al., 2002), is noteworthy. Recent findings indicated that genetic manipulation of *HoxB4* can support generation of long-term repopulating HSCs from ES cells (Kyba et al., 2002), and ex vivo expansion of HSCs can be obtained by direct targeting of HoxB4 protein into HSCs (Amsellem et al., 2003; Krosl et al., 2003). Similar to HoxB4, Bmi-1 could be a novel target for therapeutic manipulation of HSCs. Although PcG proteins regulate expression of homeotic genes including *HoxB4* during development (Takihara et al., 1997), deregulation of *Hox* genes in definitive hematopoietic cells has not yet been identified in mice deficient for *PcG* genes (Ohta et al., 2002; Park et al., 2003; Lessard and Sauvageau, 2003). In this study, *HoxB4* expression was not altered in *Bmi-1*$^{-/-}$ KSL cells or *Bmi-1*-overexpressing hematopoietic cells (Figure 6C). Moreover, forced expression of *HoxB4* failed to rescue defective *Bmi-1*$^{-/-}$ HSC function (Figures 3C and 3D). These findings are quite interesting and suggest that HoxB4 requires functional Bmi-1 to execute its function as a HSC activator. In this regard, *Bmi-1* could be epistatic to *HoxB4* or these two molecules may have some functional crosstalk in the regulation of HSC self-renewal. Actually, the enhancement of HSC activity by two genes is highly similar in many aspects. It will be intriguing to ask how these two molecules work as HSC activators.

The mechanism whereby Bmi-1 maintains HSC remains to be defined. Although derepression of the Bmi-1 target genes *p16* and *p19* has been attributed to defective HSC self-renewal, the cell cycle status of CD34$^-$KSL HSCs was not grossly altered in *Bmi-1*$^{-/-}$ mice (Supplemental Figure S3). In addition, apoptosis was not increased during observation of clonal HSC cultures, either. Therefore, a detailed analysis of *Bmi-1*$^{-/-}$*p16*$^{-/-}$ *p19*$^{-/-}$ HSCs will be necessary to define their roles in HSC. Nonetheless, *p19*$^{-/-}$ HSCs showed higher repopulating capacity than wt control (Figures 6A and 6B), and enhanced HSC repopulating capacity mediated by *Bmi-1* was correlated with repressed *p16* and p*19* expression in ex vivo cultured HSCs (Figure 6C). One attractive hypothesis is that derepression of *p16* and *p19* genes causes early senescence of primitive hematopoietic cells as reported in *Bmi-1*$^{-/-}$ mouse embryonic fibroblasts (Jacobs et al., 1999). In the case of multipotent hematopoietic cells, senescence could mean accelerated differentiation and early cell cycle exit as observed in *Bmi-1*$^{-/-}$ mice. In BM, HSCs reside in a niche in close contact with supporting cells like osteoblasts (Zhang et al., 2003; Calvi et al., 2003), in which most of the HSCs stay in the G_0 stage. The quiescence of HSCs has a critical biological importance in preventing premature HSC exhaustion (Cheng et al., 2000). Taken together, HSC stemness might be maintained by a fine regulation of the cell cycle machinery.

Additional mechanisms regulating self-renewal could be responsible for preventing differentiation (Wang and Lin, 2004). We found that forced expression of *Bmi-1* inhibits differentiation of an immature hematopoietic cell line (M.N., unpublished data). It is well recognized that HSCs express most myeloid genes at a low level (Miyamoto et al., 2002). Bmi-1 in HSC might be involved in repressing differentiation-related gene expression below the level of biological significance. In this regard, the role of Bmi-1 in the function of HSC derived from C/EBPα-deficient mice is of interest. C/EBPα is a transcription factor that is required for myeloid differentiation. Of note is that C/EBPα$^{-/-}$ HSCs demonstrate increased *Bmi-1* expression and enhanced repopulation capacity and self-renewal (P. Zhang, et al., 2004 [this issue of *Immunity*]). A 3.5-fold increase in *Bmi-1* transcript level in C/EBPα$^{-/-}$ KSL cells is comparable to a 5.8-fold increase in our transduced KSL cells and thus could be sufficient to enhance HSC self-renewal capacity. Therefore, C/EBPα-deficient mice represent the first mouse model of *Bmi-1* overexpression in HSCs that recapitulates our findings described here. The increase in Bmi-1 expression may mediate many, if not all, of the phenotypic changes in C/EBPα$^{-/-}$ HSCs and may also mediate some of the block in myeloid differentiation observed in C/EBPα$^{-/-}$ mice. Further analysis of the underlying mechanisms in *Bmi-1*$^{-/-}$ cells will be needed to unveil the relative contributions of Bmi-1 to self-renewal and/or differentiation. Finally, however, because disruption of C/EBPα has been described in a number of humans with acute myeloid leukemia, it will also be of interest to investigate whether *Bmi-1* is upregulated in the leukemic blasts, and whether such upregulation contributes to the self-renewal function of leukemic stem cells, which is defective in experimental models of leukemia in cells lacking *Bmi-1* (Lessard and Sauvageau, 2003).

Experimental Procedures

Mice

Bmi-1$^{-/-}$ mice (van der Lugt et al., 1994), *Mel-18*$^{-/-}$ mice (Akasaka et al., 1996), *M33*$^{-/-}$ mice (Katoh-Fukui et al., 1998), and *p19*$^{-/-}$ mice (Kamijo et al., 1997) that had been backcrossed at least eight times onto a C57BL/6 (B6-Ly5.2) background were used in this study. C57BL/6 (B6-Ly5.2) mice were purchased from Charles River Japan, Inc. Mice congenic for the Ly5 locus (B6 Ly5.1) were bred and maintained at the Animal Research Center of the Institute of Medical Science, University of Tokyo.

Purification of Mouse Hematopoietic Stem Cells

Mouse hematopoietic stem cells (CD34 KSL cells) were purified from bone marrow cells of 2-month-old mice. In brief, low-density cells were isolated on Lymphoprep (1.086 g/ml; Nycomed, Oslo, Norway). The cells were stained with an antibody cocktail consisting of biotinylated anti-Gr-1, Mac-1, B220, CD4, CD8, and Ter-119 mAbs (PharMingen, San Diego, CA). Lineage-positive cells were depleted with streptavidin-magnetic beads (M-280; Dynal Biotech, Oslo, Norway). The cells were further stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34, phycoerythrin (PE)-conjugated anti-Sca-1, and allophycocyanin (APC)-conjugated anti-c-Kit antibodies (PharMingen). Biotinylated antibodies were detected with streptavidin-Texas Red (Molecular Probes, Eugene, OR). Four-color analysis and sorting were performed on a FACS Vantage (Becton Dickinson, San Jose, CA).

Transduction of CD34$^-$KSL Cells

The murine *Bmi-1* and *Mph-1* cDNAs were FLAG-tagged at their amino terminus. The retroviral vector GCDNsam (pGCDNsam), with an LTR derived from MSCV, has intact splice donor and splice acceptor sequences for generation of subgenomic mRNA (Kaneko et al., 2001). Murine *Bmi-1*, *Mph-1*, *M33*, *Mel-18*, *Bcl-xL*, and human *HoxB4* cDNAs were subcloned into a site upstream of an *IRES-*

EGFP construct in pGCDNsam. To produce recombinant retrovirus, plasmid DNA was transfected into 293gp cells (293 cells containing the *gag* and *pol* genes but lacking an envelope gene) along with a VSV-G expression plasmid by $CaPO_4$ coprecipitation. Supernatants from transfected cells were concentrated by centrifugation at 6000 × g for 16 hr and then resuspended in α-MEM supplemented with 1% fetal calf serum (FCS) (1/200 of the initial volume of supernatant). Virus titers were determined by infection of Jurkat cells (a human T cell line). $CD34^-KSL$ cells were deposited into recombinant fibronectin fragment (Takara Shuzo, Otsu, Japan)-coated 96-well microtiter plates at 50–150 cells per well, and were incubated in α-MEM supplemented with 1% FCS, 100 ng/ml mouse stem cell factor (SCF), and 100 ng/ml human thrombopoietin (TPO) (Peprotech, Rocky Hill, NJ) for 24 hr. Then cells were transduced with a retrovirus vector at a multiplicity of infection (MOI) of 600 in the presence of protamine sulfate (5 μg/ml; Sigma, St. Louis, MO) for 24 hr. After transduction, cells were further incubated in S-Clone SF-O3 (Sanko Junyaku, Tokyo, Japan) supplemented with 1% FCS, 100 ng/ml SCF, and 100 ng/ml TPO and subjected to in vitro colony assay or competitive repopulation assay at the indicated time point. In all experiments, transduction efficiency was over 80% as judged from the GFP expression observed under a fluorescent inverted microscope.

Colony Assay

$CD34^-KSL$ cells transduced with indicated retroviruses were plated in methylcellulose medium (Stem Cell Technologies, Vancouver, BC) supplemented with 20 ng/ml mouse SCF, 20 ng/ml mouse IL-3 (Peprotech), 50 ng/ml human TPO, and 2 units/ml human erythropoietin (EPO) (Peprotech). The culture dishes were incubated at 37°C in a 5% CO_2 atmosphere. GFP^+ colony numbers were counted at day 14. Colonies derived from HPP-CFCs (colony diameter >1 mm) were recovered, cytospun onto glass slides, then subjected to May-Grünewald Giemsa staining for morphological examination.

Paired Daughter Cell Assay

$CD34^-KSL$ cells were clonally deposited into 96-well microtiter plates in S-Clone SF-O3 supplemented with 0.1% BSA, 100 ng/ml SCF, and 100 ng/ml TPO. When a single cell underwent cell division and gave rise to two daughter cells, the daughter cells were separated into different wells by micromanipulation techniques as previously described (Suda et al., 1984; Takano et al., 2004). Individual paired daughter cells were further incubated in S-Clone SF-O3 supplemented with 10% FCS, 20 ng/ml SCF, 20 ng/ml IL-3, 50 ng/ml TPO, and 2 units/ml EPO. The colonies generated from each daughter cell were recovered for morphological examination. To evaluate the effect of Bmi-1 on HSC fate, $CD34^-KSL$ cells were transduced with a *Bmi-1* retrovirus as described above. After 24 hr transduction, cells were separated clonally by micromanipulation into 96-well microtiter plates. When a single cell underwent cell division, daughter cells were separated again by micromanipulation and were processed as described above.

Competitive Repopulation Assay

Competitive repopulation assay was performed by using the Ly5 congenic mouse system. In brief, hematopoietic cells from B6-ly5.2 mice were mixed with bone marrow competitor cells (B6-Ly5.1) and were transplanted into B6-ly5.1 mice irradiated at a dose of 9.5 Gy. In the case of Ly5.1 hematopoietic cells, cells were mixed with bone marrow competitor cells (B6-Ly5.2) and were transplanted into B6-Ly5.2 mice. 4 and 12 weeks after transplantation, peripheral blood cells of the recipients were stained with PE-conjugated anti-Ly5.1 (A20) or biotinylated anti-Ly5.2 (104) (PharMingen). The cells were simultaneously stained with PE-Cy7-conjugated anti-B220 antibody and a mixture of APC-conjugated anti-Mac-1 and anti-Gr-1 antibodies or a mixture of APC-conjugated anti-CD4 and anti-CD8 antibodies (PharMingen). The biotinylated antibody was detected with streptavidin-Texas Red. Cells were analyzed on a FACS Vantage. Percentage chimerism was calculated as (percent donor cells) × 100/(percentage donor cells + percent recipient cells). When percent chimerism was above 1.0 with myeloid, B and T lymphoid lineages, recipient mice were considered to be multilineage reconstituted (positive mice). Repopulation unit (RU) was calculated with Harrison's method (Harrison et al., 1993) as follows: RU = (percent donor cells) × (number of competitor cells) × 10^{-5}/100 − (percent donor cells). By definition each RU represents the repopulating activity of 1 × 10^5 BM cells. In this study, the number of BM competitors was fixed as 2 × 10^5 cells. T/C ratio defined above was applied to Harrison's formula as follows: RU = T/C ratio × 2.

Semiquantitative RT-PCR and Real-Time PCR

Semiquantitative RT-PCR was carried out by using normalized cDNA by the quantitative PCR with TaqMan rodent GAPDH control reagent (Perkin-Elmer Applied Biosystem, Foster City, CA) as described before (Osawa et al., 2002). PCR products were separated on agarose gels and visualized by ethidium bromide staining. Real-time PCR was done with SYBR Green PCR master mix (Perkin-Elmer Applied Biosystem) according to the manufacturer's instruction. Primer sequences and amplification conditions are available from the authors on request.

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Roles of a conserved family of adaptor proteins, Lnk, SH2-B, and APS, for mast cell development, growth, and functions: *APS*-deficiency causes augmented degranulation and reduced actin assembly

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Abstract

Lnk, SH2-B, and APS form a conserved adaptor protein family. All of those proteins are expressed in mast cells and their possible functions in signaling through c-Kit or FcεRI have been speculated. To investigate roles of Lnk, SH2-B or APS in mast cells, we established IL-3-dependent mast cells from *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, and *APS*$^{-/-}$ mice. IL-3-dependent growth of those cells was comparable. Proliferation or adhesion mediated by c-Kit as well as degranulation induced by cross-linking FcεRI were normal in the absence of Lnk or SH2-B. In contrast, *APS*-deficient mast cells showed augmented degranulation after cross-linking FcεRI compared to wild-type cells, while c-Kit-mediated proliferation and adhesion were kept unaffected. *APS*-deficient mast cells showed reduced actin assembly at steady state, although their various intracellular responses induced by cross-linking FcεRI were indistinguishable compared to wild-type cells. Our results suggest potential roles of APS in controlling actin cytoskeleton and magnitude of degranulation in mast cells.

Keywords: Actin cytoskeleton; Adaptor protein; BMMC; c-Kit; Cytokine; Cytokine receptor; Degranulation; FcεRI; IgE; Signal transduction; Tyrosine kinase

Mast cells play critical roles in allergic and inflammatory responses. Mast cells express the high affinity IgE receptor FcεRI and cross-linking of IgE bound to FcεRI by antigens initiates a series of molecular events in mast cells, which lead to degranulation and release of a wide variety of chemical mediators such as histamine, arachidonic acid metabolites, and soluble proteins including neutral proteases and cytokines [1–3]. Even in the absence of antigen, binding of monomeric IgE to FcεRI induces cytokine production and cell survival [4]. Mast cells differentiate from hematopoietic progenitor cells. Stem cell factor (SCF), which is also known as mast cell growth factor, and IL-3 provide signals for their differentiation, proliferation, and survival mediated through c-Kit receptor tyrosine kinase and IL-3 receptor, respectively. SCF also regulates chemotaxis and adhesion of mature mast cells [1,5].

Lnk, SH2-B, and APS form a conserved family of adaptor proteins, whose members share a homologous N-terminal region with proline rich stretches, PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site [6–9]. Lnk plays a critical role in regulating production of B cell precursors and hematopoietic progenitor cells, and functions as a negative regulator of c-Kit-mediated signaling. We have shown that *lnk*$^{-/-}$ mice show enhanced B cell production because of the hypersensitivity of B cell precursors to SCF [8]. In addition, *lnk*$^{-/-}$ mice exhibit increased numbers of hematopoietic progenitors in the bone marrow, and the ability of hematopoietic progenitors to repopulate

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irradiated host animals was greatly enhanced by the absence of Lnk [10]. Independently, Velazquez et al. [11] have reported *lnk*-deficiency results in abnormal modulation of SCF and IL-3-mediated signaling pathways and augmented growth of bone marrow cells or splenocytes. SH2-B is originally identified as a protein associated with immunoreceptor tyrosine-based activation motifs (ITAMs) of FcεRI γ-chain by a modified two-hybrid (tribrid system) screening [6]. We have shown that SH2-B is a critical molecule for the maturation of reproduction organs that is at least in part mediated by insulin-like growth factor I (IGF-I) receptor signaling [12]. APS is identified as a potential substrate of c-Kit by two-hybrid system [7]. We also independently isolated the murine counterpart of APS as a protein homologous to Lnk and SH2-B [9]. APS is phosphorylated upon stimulation with various growth factors, including EPO-R, PDGF-R, insulin, nerve growth factor (NGF), and cross-linking B cell receptor (BCR) [9,13–16]. Recently, we generated $APS^{-/-}$ mice and found that B-1 cells in peritoneal cavity were increased, and humoral immune responses to type-2 antigen significantly enhanced in $APS^{-/-}$ mice [17].

Lnk-family adaptor proteins, Lnk, SH2-B, and APS, are all expressed in bone marrow-derived mast cells (BMMCs) [12]. In addition, various experiments using cell lines overexpressing those Lnk-family adaptor proteins suggested their possible functions in signaling mediated through c-Kit or FcεRI. We investigated and compared for the first time consequences of the deficiency either of Lnk, SH2-B or APS in mast cell functions using primary cultured cells. We established BMMCs from bone marrow progenitors of $lnk^{-/-}$, $SH2\text{-}B^{-/-}$, $APS^{-/-}$ mice, and their respective control wild-type mice. IL-3-dependent BMMCs were equally established even in the absence of Lnk, SH2-B or APS. SCF-dependent proliferation or adhesion was also not compromised and was comparable among $lnk^{-/-}$, $SH2\text{-}B^{-/-}$, and $APS^{-/-}$ BMMCs. Although FcεRI-mediated degranulation was not affected by the absence of Lnk or SH2-B, $APS^{-/-}$ BMMCs showed enhanced degranulation after cross-linking FcεRI. $APS^{-/-}$ BMMCs showed reduced filamentous actin (F-actin) assembly at steady state and was resistant to inhibitors disrupting F-actin microfilaments in FcεRI-mediated degranulation responses. These results suggest that APS plays a role in negative regulation of mast cell degranulation by controlling actin dynamics.

Materials and methods

Cells and culture. Bone marrow cells were obtained from 8- to 10-week-old $lnk^{-/-}$ [8], $SH2\text{-}B^{-/-}$ [12], $APS^{-/-}$ mice [17], and their respective wild-type littermates, and cultured in RPMI1640 supplemented with 5 ng/ml murine IL-3 (PeproTech), 8% fetal calf serum (FCS), nonessential amino acids (Gibco-BRL), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM of 2-mercaptoethanol. Cells were split and supplied with fresh medium every 4 or 5 days. After 4 weeks of cultivation, greater than 95% of cells were c-Kit and FcεRI positive as assessed by flow cytometry.

Flow cytometry and cytochemistry. For the detection of FcεRI, BMMCs were incubated in a supernatant of IGEL a2 (15.3) hybridoma containing mouse anti-DNP IgE monoclonal antibody (mAb) and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE mAb (LO-ME-2, Oxford Biomarketing, UK). For the detection of c-Kit, cells were stained with phycoerythrin (PE)-conjugated anti-CD117 mAb (2B8, Pharmingen). For measurements of F-actin content, cells were fixed in 3.7% formaldehyde for 6 h at 4 °C permeabilized with 0.2% Triton X-100 in PBS for 30 min and then stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 h. Stained cells were then analyzed by flow cytometry using a FACSCalibur (Becton–Dickinson).

Unstimulated or stimulated BMMCs were resuspended in PBS and deposited onto microscope slides using a Cytospin 3 (Shandon Scientific, Cheshire, England). After staining with May–Gruenwald's and Giemsa's solutions (MERCK), cellular morphology was assessed by a light microscope.

Proliferation and survival assays. BMMCs (5×10^4) were cultured in 0.2 ml of flesh medium containing various concentrations of SCF (PeproTech) in a 96-well multi-well plate for 72 h. Cells were pulsed with [^{3}H]thymidine (0.2 μCi/well) in the last 12 h of culture and harvested and incorporated [^{3}H]thymidine was measured in triplicate determination using a MATRIX 96 Direct Beta Counter (Packard, Meriden, CT). Cells were cultured in media alone or in the presence of various concentrations of anti-DNP IgE mAb (SPE-7, Sigma). Percentage of viable cells was determined by trypan blue exclusion.

Adhesion assay. Adhesion assays to fibronectin were performed as previously described [18]. In brief, 5×10^4 BMMCs labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein (BCECF: Molecular Probes, Eugene, OR) were incubated in triplicate in a 96-well polystyrene plate (Lynbro-Titertek, Aurora, OH) coated with fibronectin (Sigma) in the presence of various concentrations of SCF or 10 ng/ml PMA at 37 °C for 30 min. Unbound cells were removed by washing the plates with binding medium RPMI 1640 containing 10 mM Hepes (pH 7.4), and 0.03% BSA four times. Adhered cells were quantified by measuring fluorescence of input and bound cells using a Fluorescence Concentration Analyzer (IDEXX Laboratories, Westbrook, ME).

Degranulation assay. BMMCs were sensitized with anti-DNP IgE at 37 °C for 18 h, washed, and resuspended in Tyrode's buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA). Cells (5×10^5 in 0.2 ml) were then stimulated with various concentrations of DNP-BSA or 10 ng/ml PMA plus 400 ng/ml ionomycin at 37 °C for 1 h. Enzymatic activities of β-hexosaminidase in supernatants and cells solubilized in 0.5% Triton X-100 Tyrode's buffer were measured using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminidase (Sigma) as substrates. Degranulation was calculated as the percentage of β-hexosaminidase released from cells in the total amount of the enzyme in the supernatants and cell pellets as described before [18]. For the experiment using latrunculin, sensitized BMMCs were pretreated with various concentrations of latrunculin for 15 min at 37 °C before assays. Histamine released into culture supernatants after degranulation was measured using ELISA kit (Immunotech, Marseille, France) according to manufacturer's recommendation.

Calcium measurements. Sensitized BMMCs were incubated with 6 μM Fura PE3/AM (TEFLABS, Austin, TX) in PBS containing 20 mM Hepes (pH 7.4), 5 mM glucose, 0.025% BSA, and 1 mM $CaCl_2$ (HBS) at 37 °C for 60 min. Cells were washed and resuspended in HBS (1×10^5 cells/0.1 ml) in a stirring cuvette. Fluorescence was monitored continuously with a fluorescence spectrophotometer (CAF-110: JASCO, Osaka, Japan) at an emission wavelength of 500 nm and two different excitation wavelengths (340 and 380 nm).

Immunoblotting. Cell lysates from stimulated BMMCs were subjected to immunoprecipitation and Western blot analysis as previously

described [9]. The proteins were resolved by SDS–8% PAGE and transferred to PVDF membranes (Immobilon, Millipore). After blocking with 5% BSA, membranes were probed with anti-phosphotyrosine mAb (4G10, Upstate Biotechnology) and incubated with HRP-conjugated secondary antibodies. Blots were washed in 0.05% Tween 20/Tris-buffered saline, pH 7.6, and proteins were detected by chemiluminescence (Perkin–Elmer Life Sciences).

Results

Establishment of BMMCs lacking either Lnk, SH2-B or APS

Lnk, SH2-B, and APS were all expressed in normal BMMCs [12]. To investigate possible functions of those adaptor proteins in mast cells, we established IL-3-dependent BMMCs from bone marrow progenitors of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, and *APS*$^{-/-}$ mice and their responses were compared with those of BMMCs established from respective control wild-type littermates. IL-3-dependent growth of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ or *APS*$^{-/-}$ bone marrow progenitor cells was almost comparable to that of respective control progenitor cells (Fig. 1A). Established *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ or *APS*$^{-/-}$ BMMCs were not distinguishable from the wild-type BMMCs in terms of surface expression of FcεRI and c-Kit (Fig. 1B). Mast cell differentiation and proliferation induced by IL-3 was not affected at all even in the absence of Lnk, SH2-B or APS.

Functions of lnk$^{-/-}$, *SH2-B*$^{-/-}$ *or APS*$^{-/-}$ *BMMCs*

First, we examined proliferative responses of established BMMCs to SCF and found no difference among *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$, and respective control BMMCs (Fig. 2A). Adhesion to fibronectin induced by SCF or PMA was also not affected in the absence of Lnk, SH2-B or APS (Fig. 2B). We then examined degranulation of those BMMCs induced by cross-linking FcεRI by measuring β-hexosaminidase and histamine released after stimulation. Degranulation from *lnk*$^{-/-}$ or *SH2-B*$^{-/-}$ BMMCs was almost comparable to that from control wild-type BMMCs (Fig. 2C). In contrast, *APS*$^{-/-}$ BMMCs showed enhanced degranulation responses upon cross-linking FcεRI (Fig. 2C). Degranulation from *APS*$^{-/-}$ BMMCs, determined by β-hexosaminidase releasability, was 130–140% of that from control cells at each stimulation condition, and the enhancement was statistically significant at the concentrations of DNP-BSA over 0.5 μg/ml (Table 1). Histamine released after cross-linking FcεRI was also augmented in *APS*$^{-/-}$ BMMCs (data not shown).

FcεRI-mediated cellular responses in APS$^{-/-}$ *BMMCs*

To clarify the possible molecular mechanisms leading to the enhanced degranulation in the absence of APS,

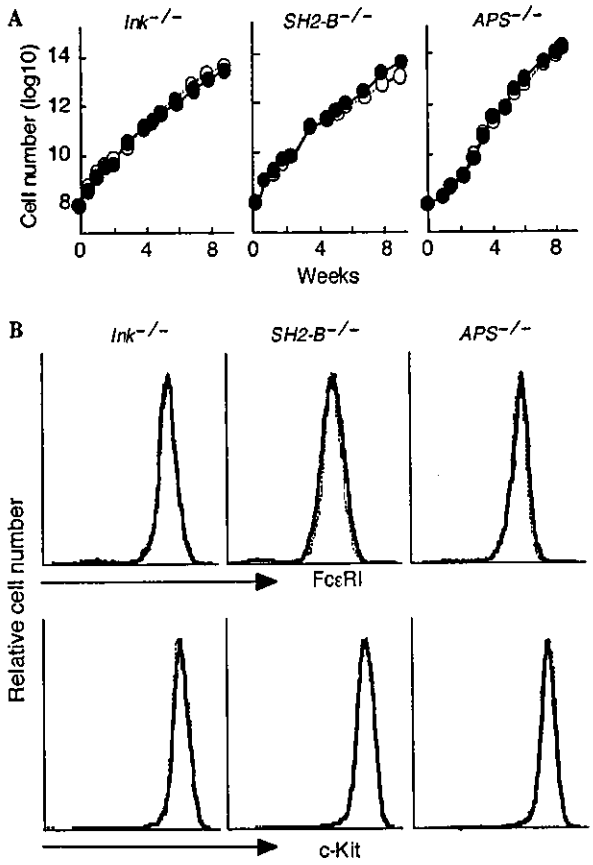

Fig. 1. (A) Cumulative cell numbers of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$ (closed circles), and respective wild-type control (open circles) BMMCs. Differentiation of BMMCs from progenitors and their cell growth induced by IL-3 was comparable in the absence of either Lnk, SH2-B or APS. Representative results obtained from multiple independent pairs of BMMCs are shown. (B) Surface expressions of FcεRI (upper panels) or c-Kit (lower panels) on *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$ (bold lines), and respective wild-type control (dotted lines) BMMCs. After IgE sensitization, BMMCs were stained with anti-c-Kit or anti-IgE antibodies and analyzed by flow cytometry. Representative results of multiple independent experiments are shown.

we tried to evaluate various cellular events induced by cross-linking FcεRI. We first cytochemically evaluated the proportion of degranulated BMMCs after stimulation. Percentage of degranulated cells increased in a dose-dependent manner as the concentration of antigens increased. Importantly, the ratio of degranulated BMMCs in each stimulation condition was comparable between *APS*$^{-/-}$ and wild-type BMMCs (Fig. 3A). The enhanced degranulation from *APS*$^{-/-}$ BMMCs was thus due to augmented degranulation from each mast cell but not to increased proportion of cells that underwent degranulation. We then analyzed calcium influx induced by cross-linking FcεRI, however, we did not observe significant difference in initial peak and following sustained increase of intracellular free calcium between *APS*$^{-/-}$ and control BMMCs (Fig. 3B). Cell survival