Lethal myelofibrosis induced by Bmi1-deficient hematopoietic cells unveils a tumor suppressor function of the polycomb group genes

Hideyuki Oguro,1,6 Jin Yuan,1,6 Satomi Tanaka,1,2 Satoru Miyagi,1,6 Makiko Mochizuki-Kashio,1,6 Hitoshi Ichikawa,3 Satoshi Yamazaki,4,7 Haruhiko Koseki,5 Hiromitsu Nakauchi,4,7 and Atsushi Iwama1,6

1Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
2Department of Hematology, Chiba University Hospital, Chiba 260-8670, Japan
3Division of Genetics, National Cancer Center Research Institute, Tokyo 104-0045, Japan
4Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
5Laboratory for Lymphocyte Development, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan
6Japan Science and Technology Agency (JST), Core Research for Evolutional Science and Technology and 7ERATO, Gobancho, Chiyoda-ku, Tokyo 102-0076, Japan

Polycomb-group (PcG) proteins form the multiprotein polycomb repressive complexes (PRC) 1 and 2, and function as transcriptional repressors through histone modifications. They maintain the proliferative capacity of hematopoietic stem and progenitor cells by repressing the transcription of tumor suppressor genes, namely Ink4a and Arf, and thus have been characterized as oncogenes. However, the identification of inactivating mutations in the PcG gene, EZH2, unveiled a tumor suppressor function in myeloid malignancies, including primary myelofibrosis (PMF). Here, we show that loss of another PcG gene, Bmi1, causes pathological hematopoiesis similar to PMF. In a mouse model, loss of Bmi1 in Ink4a−/−Arf−/− hematopoietic cells induced abnormal megakaryocytopoiesis accompanied by marked extramedullary hematopoiesis, which eventually resulted in lethal myelofibrosis. Absence of Bmi1 caused derepression of a cohort of genes, including Hmga2, which is an oncogene overexpressed in PMF. Chromatin immunoprecipitation assays revealed that Bmi1 directly represses the transcription of Hmga2. Overexpression of Hmga2 in hematopoietic stem cells induced a myeloproliferative state with enhanced megakaryocytopoiesis in mice, implicating Hmga2 in the development of pathological hematopoiesis in the absence of Bmi1. Our findings provide the first genetic evidence of a tumor suppressor function of Bmi1 and uncover the role of PcG proteins in restricting growth by silencing oncogenes.

Polycomb-group (PcG) proteins are transcriptional repressors that function in gene silencing by modulating chromatin structure. They form the chromatin-associated multiprotein complexes, polycomb repressive complex (PRC) 1 and PRC2 (Simon and Kingston, 2009). PcG proteins have been implicated in the maintenance of self-renewing stem cells (Pietersen and van Lohuizen, 2008; Konuma et al., 2010; Sauvageau and Sauvageau, 2010). Among PcG genes, Bmi1 plays a central role in the inheritance of somatic cell stemness, including hematopoietic stem cells (HSCs) and neural stem cells (Park et al., 2003; Iwama et al., 2004; Molofsky et al., 2003), and its forced expression augments their self-renewal capability (Iwama et al., 2004). One of the major targets of Bmi1 is the Ink4a/Arf tumor suppressor gene locus, and deletion of both Ink4a and Arf in Bmi1-deficient mice substantially restores the defective self-renewal capacity of HSCs (Oguro et al., 2006). PcG and trithorax-group proteins mark developmental regulator gene promoters with bivalent domains consisting of overlapping repressive and activating
histone modifications to keep them poised for activation in embryonic stem cells (Pietersen and van Lohuizen, 2008; Konuma et al., 2010). Likewise, we found that Bmi1 reinforces bivalent histone domains at key hematopoietic regulator gene promoters in multipotent hematopoietic stem and progenitor cells to maintain their multipotency (Oguro et al., 2010). Thus, Bmi1 functions in the maintenance of both the self-renewal capacity and multipotency of HSCs.

Bmi1 has also been implicated in the maintenance of the proliferative capacity of leukemic stem cells (LSCs). Co-expression of HoxA9 and Meis1, which can transform HSCs, induces leukemia from Bmi1-deficient fetal liver cells in primary recipient mice, but fails to sustain a leukemic state in the secondary recipients (Lessard and Sauvageau, 2003), suggesting that Bmi1 regulates the self-renewal of both HSCs and LSCs. In addition, we have recently reported that Bmi1 is essential for the faithful reprogramming of myeloid progenitors into LSCs, and that leukemic fusion genes require PcG proteins acting in concert to establish LSC-specific transcriptional profiles that confer full leukemogenic activity on LSCs (Yuan et al., 2011). Notably, a gain-of-function mutation of the PRC2 gene, EZH2, has recently been identified in a subset of lymphoma (Morin et al., 2010), highlighting the oncogenic properties of PRC2 genes. In contrast, inactivating mutations of EZH2 have also been identified in patients with myelodysplastic syndrome and myeloproliferative neoplasms (MPN), revealing that EZH2 also has a tumor suppressor function (Ernst et al., 2010; Nikoloski et al., 2010).

In this study, we found that Bmi1 antagonizes development of MPN in the absence of its major tumor-suppressive targets, Ink4a and Arf, and identified Hmga2, an oncogene, as one of the direct targets of Bmi1 involved in the development of MPN. Our findings suggest that PcG genes fine-tune the hematopoietic homeostasis by balancing the transcription of oncogenic and tumor suppressive target genes.

RESULTS AND DISCUSSION
Loss of Bmi1 augments repopulating capacity of BM cells in an Ink4a/Arf-null background
We previously reported that deletion of both Ink4a and Arf in Bmi1-deficient mice substantially restores the defective self-renewal capacity of HSCs (Oguro et al., 2006). In this study, we performed competitive repopulation assays using the same number of BM competitor cells as the test cells, and found that Bmi1–/– Ink4a-Arf–/– cells had higher repopulating activity in recipient mice than the wild-type and Ink4a-Arf–/– cells.

Figure 1. Loss of Bmi1 augments the repopulating capacity of BM cells in the absence of Ink4a/Arf.
(A) To perform a competitive repopulating assay, 10^6 pooled test BM cells from 4-wk-old mice (CD45.2+) of the indicated genotype were mixed with 10^6 competitor BM cells (CD45.1+) and injected into lethally irradiated recipient mice (CD45.1+). The percent chimerism of donor cells in the recipient PB is presented as the mean ± SD (n = 5).

(B) Relative numbers of donor-derived BM and splenic LSK cells, and BM CMPs, GMPs, and MEPs. Lethally irradiated wild-type recipient mice were infused with 2 × 10^6 BM cells of the indicated genotype and analyzed at 4 mo after transplantation. Data were normalized relative to wild type and are shown as the mean ± SD (BM LSK cells, n = 12; splenic LSK cells, n = 6; CMPs, GMPs, and MEPs, n = 5). * P < 0.05; ** P < 0.01; *** P < 0.001.

(C) Survival curve of the wild-type and Ink4a-Arf–/– cells. The data from four independent experiments were combined (wild type, n = 12; Ink4a-Arf–/–, n = 11; Bmi1–/– Ink4a-Arf–/–, n = 14). The significance of the difference in survival curves was calculated by log-rank test. * P = 0.0007. (D) PB analysis of white blood cells (WBC), platelets (PLT), and hemoglobin (HGB) of the wild-type recipient mice in C. Data are shown as the mean ± SD. * P < 0.05; ** P < 0.01; *** P < 0.001.
control BM cells (Fig. 1 A). To evaluate the repopulating capacity of Bmi1−/− Ink4a-Arf−/− BM cells precisely, we then transplanted wild-type, Ink4a-Arf−/−, and Bmi1−/− Ink4a-Arf−/− BM cells into lethally irradiated mice without competitor cells. At 4 mo after transplantation, the recipients repopulated with Bmi1−/− Ink4a-Arf−/− donor cells had significantly fewer lineage marker-negative (Lineage−) Sca-1+c-Kit+ (LSK) cells in BM than did the control recipients as we reported previously (Oguro et al., 2010; Fig. 1 B). However, they had twofold more megakaryocyte/erythroid progenitors (MEPs) than the controls (Fig. 1 B) and showed extramedullary hematopoiesis as evident from a significant increase in the number of LSK HSCs/multipotent progenitors (MPPs) in spleen (Fig. 1 B). All of the recipient mice repopulated with Ink4a-Arf−/− BM cells eventually developed sarcomas or lymphomas and died by 11 mo after transplant, as reported with the Ink4a-Arf−/− mice (Fig. 1 C; Serrano et al., 1996). Alternatively, the recipient mice repopulated with Bmi1−/− Ink4a-Arf−/− BM cells died much earlier than the Ink4a-Arf−/− controls (Fig. 1 C) and displayed a more progressive thrombocytopenia (Fig. 1 D).

**Bmi1-deficient hematopoietic cells induce lethal myelofibrosis**

The recipient mice repopulated by Bmi1−/− Ink4a-Arf−/− BM cells had marked hepatosplenomegaly (not depicted) and hypoplastic BM with severe fibrosis (Fig. 2, A and B) at their control BM cells (Fig. 1 A). To evaluate the repopulating capacity of Bmi1−/− Ink4a-Arf−/− BM cells precisely, we then transplanted wild-type, Ink4a-Arf−/−, and Bmi1−/− Ink4a-Arf−/− BM cells into lethally irradiated mice without competitor cells. At 4 mo after transplantation, the recipients repopulated with Bmi1−/− Ink4a-Arf−/− donor cells had significantly fewer lineage marker-negative (Lineage−) Sca-1+c-Kit+ (LSK) cells in BM than did the control recipients as we reported previously (Oguro et al., 2010; Fig. 1 B). However, they had twofold more megakaryocyte/erythroid progenitors (MEPs) than the controls (Fig. 1 B) and showed extramedullary hematopoiesis as evident from a significant increase in the number of LSK HSCs/multipotent progenitors (MPPs) in spleen (Fig. 1 B). All of the recipient mice repopulated with Ink4a-Arf−/− BM cells eventually developed sarcomas or lymphomas and died by 11 mo after transplant, as reported with the Ink4a-Arf−/− mice (Fig. 1 C; Serrano et al., 1996). Alternatively, the recipient mice repopulated with Bmi1−/− Ink4a-Arf−/− BM cells died much earlier than the Ink4a-Arf−/− controls (Fig. 1 C) and displayed a more progressive thrombocytopenia (Fig. 1 D).

**Figure 2.** Enhanced megakaryocytopoiesis and massive myelofibrosis induced by Bmi1−/− Ink4a-Arf−/− hematopoietic cells. (A) Hematoxylin and eosin (H&E) staining of BM, spleen, and liver sections. BM, spleen, and liver of representative wild-type recipient mice repopulated by 2 × 10⁶ BM cells of the indicated genotype were analyzed at 174 d after transplantation. Bars: 125 µm (BM); 500 µm (spleen and liver). (B) Silver staining of BM and spleen sections in A. Bars, 50 µm. (C) H&E staining of BM sections of representative recipient mice repopulated with the indicated mutant BM cells analyzed at 138 d after transplantation. Bars, 50 µm. (D) CD41 (green) and DAPI (blue) staining of spleen sections of representative recipient mice repopulated with indicated mutant BM cells analyzed at 138 d after transplantation. Bars, 125 µm.
terminal stage. The spleen structure was destroyed by extra-
mediullary hematopoiesis accompanied by massive fibrosis
(Fig. 2, A and B). Extramedullary hematopoiesis was also evi-
dent in the liver (Fig. 2 A). All these features resemble those
of human primary myelofibrosis (PMF). Notably, the recipi-
ent mice infused with Bmi1-/-/Ink4a-Arf-/- BM cells along
with competitor cells in Fig. 1 A also developed lethal
myelofibrosis in a similar fashion (not depicted).

PMF is the rarest and most severe chronic MPN (Tefferi
et al., 2007; Levine and Gilliland, 2008). Abnormal megakary-
cyctosis in the BM has been proposed as the main caus-
ative factor for myelofibrosis. Deregulated stem cell signaling,
resulting in part from mutated JAK2 and MPL, likely cause
abnormal megakaryocyctosis. Myelofibrosis is thought to be
the consequence of an excessive release/leakage of growth
factors within the BM by cells from pathological hematopo-
etic clones, especially by necrotic megakaryocytes. TGF-β1
is speculated to be one of the major causative growth factors
that activate mesenchymal cells (Martyre et al., 1994). Although
abnormal megakaryocyctosis was obscure in BM and spleen
because of severe fibrosis at the terminal stage of the disease,
the mice at earlier time points after transplantation had marked
megakaryocyctosis in both BM and spleen (Fig. 2, C and D).
These findings clearly implicate pathological megakaryocyctosis
in the development of lethal myelofibrosis, and indicate that
lethal myelofibrosis induced by Bmi1-/-/Ink4a-Arf-/- hema-
topoietic cells follows the natural course of human PMF.

Derepression of Hmga2 in Bmi1-deficient hematopoietic stem/progenitor cells
To identify the genes responsible for PMF-like disease in the
absence of Bmi1, we compared gene expression profiles of
LSK HSCs/MPPs and common myeloid progenitors (CMPs).
In total, 245 and 286 genes were derepressed by more than
twofold, specifically in Bmi1-/-/Ink4a-Arf-/- LSKs and
CMPs, respectively (Fig. 3 A). We then compared the list of
derepressed genes with a list of PMF-associated genes identi-
fied by gene expression profiling of CD34+ cells in human
PMF patients (Guglielmelli et al., 2007). Hmga2 appeared to
be commonly up-regulated in Bmi1-/-/Ink4a-Arf-/- CMPs
and PMF CD34+ cells. Hmga2 was found to be one of eight
genes that can distinguish PMF CD34+ cells from normal
CD34+ cells, and abnormal expression of Hmga2 was associ-
ated with the presence of JAK2V617F (Guglielmelli et al.,
2007). Moreover, overexpression of Hmga2 was reported
in 12 of 12 patients with myelofibrosis with myeloid meta-
plasia, among which two patients had a chromosomal trans-
location involving the Hmga2 gene at 12q (Andreux et al.,
2004). We therefore focused on Hmga2. Hmga2 expression
was up-regulated by 1.6- and 13.4-fold in Bmi1-/-/Ink4a-
Arn-/- LSKs and CMPs, respectively, in our microarray anal-
ysis (Fig. 3 A). We then quantified the Hmga2 expression in
each progenitor fraction by quantitative RT-PCR. Because the
BM environment of Bmi1-/- and Bmi1-/-/Ink4a-Arf-/- mice
is defective in supporting HSCs (Oguro et al., 2006), we pu-
rified progenitors from wild-type recipient BM reconstituted
with wild-type, Ink4a-Arf-/-, or Bmi1-/-/Ink4a-Arf-/- BM
cells to exclude any environmental effects. Hmga2 expression
was predominantly found in CMPs in wild-type mice, but in
the absence of Bmi1, it was derepressed in Flt3-LSK HSCs/
MPPs (2.4-fold compared with Ink4a-Arf-/- cells) and mark-
edly increased in myeloid-committed progenitors (CMPs,
14.1-fold; granulocyte/macrophage progenitors [GMPs], 268-
fold; MEPs, 11.5-fold), but was barely affected in Flt3-LSK
lymphoid-primed MPPs (LMPPs, 0.9-fold) or common lymph-
oid progenitors (CLPs, 0.74-fold; Fig. 3 B). To confirm that
this derepression is in fact mediated by loss of Bmi1, we per-
fomed RT-PCR analysis on Lineage-“c-Kit” Bmi1-/-
progenitors and found that Hmga2, as well as the canonical
targets Ink4a and Arf, are derepressed in a Bmi1-deficient set-
ting (Fig. 3 C). These results suggest that Bmi1 functions in
the silencing of Hmga2 in cell types ranging from HSCs to
myeloid progenitors.

Direct repression of Hmga2 transcription by Bmi1
The high-mobility group A (HMGA) nonhistone chromatin
proteins alter chromatin structure, and thereby regulate tran-
scription. HMGA proteins have been implicated in both be-
nign and malignant tumors through mechanisms that result in
HMGA overexpression. Chromosomal rearrangements in-
volving the region 12q13-15, in which the Hmga2 gene is
located, are one of the major mechanisms causing deregula-
tion of the Hmga2 gene, giving rise to a truncated transcript
lacking the C-terminal tail and/or 3’-UTR. Expression of
Hmga2 is negatively regulated by the let-7 family of mi-
croRNAs, which bind to the 3’-UTR of Hmga2 and re-
strict its expression. Thus, chromosomal rearrangements
within the Hmga2 locus cause overexpression of a full-
length or truncated Hmga2 with a preserved DNA-binding
capacity (Busco and Fedele, 2007; Young and Narita, 2007).
To examine whether Bmi1 directly represses transcription of
Hmga2, we next characterized the Hmga2 promoter by con-
ducting chromatin immunoprecipitation (ChIP) assays.
To obtain enough cells, we used BM Lineage-“c-Kit” progen-
itors depleted of cells committed to the lymphoid, myeloid,
and erythroid lineages. Binding of Bmi1 to the Hmga2 pro-
producer was detected, but not to the promoter of the control
gene, β-actin (Fig. 3 D), which was marked with H3K4me3,
an active histone mark (not depicted). PRC1 catalyzes the
monoubiquitination of histone H2A (H2Aub1) at lysine 119.
The Hmga2 promoter was marked with H2Aub1 in wild-type
BM Lineage-“c-Kit” progenitors (Fig. 3 D). The levels of
Bmi1 binding and H2Aub were significantly reduced in
Bmi1-/-/Ink4a-Arf-/- Lineage- progenitors compared with
the levels in wild-type cells (Fig. 3 E). These results indicate
that Bmi1 directly represses the expression of Hmga2 by mark-
ing its promoter with a repressive histone mark.

Hmga2 promotes expansion of progenitor cells and
enhances megakaryocytogenesis in vitro
The specific up-regulation of Hmga2 expression in PMF,
but not in other chronic MPNs, such as polycythemia vera
and essential thrombocytopenia, suggested a contribution of HMGA2 to the development and/or progression of PMF (Guglielmelli et al., 2007). To address this, we evaluated the proliferative and differentiation capacity of Hmga2-overexpressing CD34-LSK HSCs. In all experiments, transduction efficiency was ~90% as determined by flow cytometry using GFP as a marker (unpublished data). In liquid cultures supplemented with SCF, TPO, IL-3, IL-6/IL-6 receptor fusion protein (FP6), and EPO, Hmga2-overexpressing HSCs showed a growth advantage compared with the control (Fig. 4 A), and the Hmga2 culture contained significantly more LSK cells than the control at day 10 of culture

Figure 3. Derepression of Hmga2 in Bmi1<sup>−/−</sup> Ink4a-Arf<sup>−/−</sup> hematopoietic cells. (A) List of top 10 genes up-regulated in LSK cells and CMPs in the absence of Bmi1. IL-7Rα LSK cells purified from BM of 4-wk-old Ink4a-Arf<sup>−/−</sup> (DKO) and Bmi1<sup>−/−</sup> Ink4a-Arf<sup>−/−</sup> (TKO) mice and CMPs from recipients’ BM at 4 mo after infusion of DKO and TKO BM cells were subjected to microarray analyses and their profiles were compared. Highlighted genes were further characterized in this study. (B) Quantitative RT-PCR analysis of Hmga2 expression. mRNA levels in each progenitor fraction from the recipients’ BM re-populated by BM cells of the indicated genotype at 4 mo after transplantation were normalized to Hprt1 expression. Expression levels relative to those in the wild-type Flt3<sup>−/−</sup> LSK cells are shown as the mean ± SD for triplicate analyses. N.D. indicates not detected. **, P < 0.01. (D) ChIP analysis at the Hmga2 promoter in wild-type Lineage<sup>−</sup>c-Kit<sup>+</sup> cells. The Hmga2 locus indicating its genomic structure (based on the Ensemble data, transcript ID ENSMUST00000072777) is depicted in the top panel. Exons are demarcated by black boxes. The regions 1–6 amplified from the precipitated DNA by site-specific quantitative PCR are indicated. The binding of Bmi1 and the levels of H2Aub were determined by ChIP using control mouse IgG (mIgG), anti-Bmi1, and anti-H2Aub antibodies and site-specific real-time PCR. The data are shown as the mean ± SD for four independent experiments. The β-actin promoter (Actb) served as a negative control. *, P < 0.05. (E) ChIP analysis at the Hmga2 promoter in wild-type or Bmi1<sup>−/−</sup> Ink4a-Arf<sup>−/−</sup> Lineage<sup>−</sup> cells. The binding of Bmi1 and the levels of H2Aub were determined by ChIP using anti-Bmi1 or anti-H2Aub antibodies and site-specific real-time PCR as in D. The data are shown as the mean ± SD for triplicate PCRs from two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
We then cultured transduced HSCs for 9 d (10 d of culture in total) in the presence of SCF and TPO, which supports the proliferation of HSCs and progenitors rather than their differentiation (Ema et al., 2000). The total number of colony-forming cells (CFCs) was significantly increased in the Hmga2 culture (Fig. 4 E). Among CFCs, the number of high proliferative potential (HPP)-CFCs, which generate a colony with a diameter >1 mm, was increased with Hmga2 compared with the control. These results indicate that overexpression of Hmga2 promotes expansion of progenitor cells and also facilitates the proliferation and differentiation of megakaryocytic cells in vitro.

**Forced expression of Hmga2 promotes megakaryocytopoiesis and induces myeloproliferative hematopoiesis in vivo**

We next examined the effect of Hmga2 overexpression on hematopoiesis in vivo. We performed competitive repopulation assays using 30 CD34+ LSK HSCs transduced with Hmga2 (CD45.2) at day 3.5 of culture along with $2 \times 10^5$ fresh unfractionated BM cells (CD45.1) for radioprotection. Hmga2-overexpressing cells showed a greater, albeit not statistically significant, contribution to the myeloid lineage in peripheral blood (PB) compared with the control (Fig. 5 A). Recipients repopulated with Hmga2-overexpressing cells had significantly more LSK cells and myeloid progenitors in BM compared with the control (Fig. 5 C). Furthermore, they also had mild splenomegaly with extramedullary hematopoiesis as evident from a marginal increase in LSK HSCs/MPPs in the spleen and a significant increase in the PB (Fig. 5 B and C). The recipients repopulated with Hmga2-overexpressing cells appeared to have more megakaryocytes in the BM (not depicted) and platelets in the PB (Fig. 5 D). These findings indicate that derepression of Hmga2 could induce an MPN-like state with enhanced megakaryocytopoiesis and correspond well with findings recently obtained with transgenic mice carrying a $3'$-UTR–truncated Hmga2 cDNA (ΔHmga2 mice; Ikeda et al., 2011). ΔHmga2 mice developed MPN-like hematopoiesis with an increased number of megakaryocytes in the BM, although the number of platelets in the PB was not described. Together, these findings implicate Hmga2 in the PMF-like pathological hematopoiesis induced by Bmi1$^{-/-}$/Ink4a-Arf$^{-/-}$ BM cells.

Given that HMGA2 is highly derepressed in CD34+ stem and progenitor cells in PMF patients, PcG function could be compromised in PMF patients, partly by loss-of-function mutations of EZH2. Indeed, we confirmed that Ezh2 also regulates Hmga2 as Bmi1 does. Hmga2 was highly derepressed in Ezh2-deficient progenitor cells (unpublished data). ChIP assays demonstrated that the Hmga2 promoter is bound by Ezh2 and marked with H3K27me3 in wild-type BM Lineage$^{-c}$-Kit$^{+}$ progenitors (unpublished data). Interestingly, recipient mice repopulated with Ezh2-deficient BM cells showed an increase in the platelet count in PB similar to recipients repopulated with HSCs overexpressing Hmga2 (Mochizuki-Kashio et al., 2011). These data indicate that both Bmi1 and Ezh2 transcriptionally repress Hmga2. However, the...
Collectively, our findings indicate that Bmi1 antagonizes the development of MPN in the absence of its major tumor-suppressive targets, Ink4a and Arf, and we showed the tumor suppressive function of Bmi1 through epigenetic silencing of oncogenes. Although Bmi1-deficiency is self-limiting unless Ink4a and Arf are deleted first, INK4A and ARF are frequently inactivated by deletions or mutations, or transcriptionally repressed by DNA methylation at their promoters in human cancers. Therefore, the situations like the Ink4a/Arf-null background that we used in this study also happen in the initiation and progression of human cancers. Our findings suggest that in such situations, the tumor cells with impaired BMI1 function could outcompete cells with normal BMI1 function because the effects of derepressed oncogenes, such as HMGA2, appear to supersede the effects of derepressed tumor suppressor genes. Corresponding to our findings, PRC1 has been reported to exert tumor suppressor activity through epigenetic silencing of Notch and JAK-STAT signaling in Drosophila melanogaster eyes (Martinez et al., 2009; Classen et al., 2009). Furthermore, mice with hypomorphic mutations of Eed and Suz12 show enhanced hematopoiesis (Lessard et al., 1999; Majewski et al., 2008). All of these findings support the frequency of EZH2 mutations in PMF has been reported to be only 13% (Nikoloski et al., 2010). Thus, it would be intriguing to examine other mechanisms that compromise PcG function in PMF patients without EZH2 mutations.

Nevertheless, it should be noted that Hmga2 is not the only oncogenic target of Bmi1 responsible for the establishment of MPN-like disease, as fibrosis was not seen in recipient BM that was repopulated with Hmga2-overexpressing cells at 6 mo after transplantation (unpublished data). As evident from the significant increase in platelet counts, overexpression of Hmga2 does not compromise the terminal differentiation of megakaryocytes. It has been proposed that necrotic megakaryocytes in BM are the main causative factor for myelofibrosis. Although derepression of Hmga2 induces an MPN-like state with enhanced megakaryocytosis, it may require the derepression of other Bmi1 targets to eventually induce a fibrotic state after a preceding MPN-like state. Our microarray analysis also identified Hoxc4 and Foxa1 as being derepressed in Bmi1−/−Ink4a−Arf−/− cells (Fig. 3 A). We confirmed this derepression by RT-PCR (unpublished data), and Hoxc4 and Foxa1 also appeared to be direct targets of Bmi1 by ChIP assays (unpublished data). We tested the effects of forced expression of these genes in HSCs, but failed to induce an MPN-like disease in recipient mice (unpublished data).

Collectively, our findings indicate that Bmi1 antagonizes the development of MPN in the absence of its major tumor-suppressive targets, Ink4a and Arf, and we showed the tumor suppressive function of Bmi1 through epigenetic silencing of oncogenes. Although Bmi1-deficiency is self-limiting unless Ink4a and Arf are deleted first, INK4A and ARF are frequently inactivated by deletions or mutations, or transcriptionally repressed by DNA methylation at their promoters in human cancers. Therefore, the situations like the Ink4a/Arf-null background that we used in this study also happen in the initiation and progression of human cancers. Our findings suggest that in such situations, the tumor cells with impaired BMI1 function could outcompete cells with normal BMI1 function because the effects of derepressed oncogenes, such as HMGA2, appear to supersede the effects of derepressed tumor suppressor genes. Corresponding to our findings, PRC1 has been reported to exert tumor suppressor activity through epigenetic silencing of Notch and JAK-STAT signaling in Drosophila melanogaster eyes (Martinez et al., 2009; Classen et al., 2009). Furthermore, mice with hypomorphic mutations of Eed and Suz12 show enhanced hematopoiesis (Lessard et al., 1999; Majewski et al., 2008). All of these findings support the
tumor suppressor function of EZH2 observed in human myelodysplastic syndrome and MPN, and are suggestive of a broad range of target genes of the PcG proteins, including both oncogenes and tumor suppressor genes. Although tumor suppressor genes have been stressed as PcG targets, our findings shed light on the role of PcG proteins in the gene silencing of oncogenes. Thus, the PcG proteins fine-tune the growth of hematopoietic cells in both a positive and a negative manner to maintain hematopoietic homeostasis.

**MATERIALS AND METHODS**

**Mice.** Bmi1<sup>−/−</sup> mice (van der Lugt et al., 1994) and Ink4a-Arf<sup>−/−</sup> mice (Serrano et al., 1996) that had been backcrossed at least eight times onto a C57BL/6 (CD45.2) background were used. C57BL/6 (CD45.2) mice and C57BL/6 mice congenic for the Ly5 locus (CD45.1) were purchased from Japan SLC and Sankyo Laboratory Service, respectively. Littermates were used as controls in all experiments. All mice were bred and maintained in the Animal Research Facility of the Graduate School of Medicine, Chiba University in accordance with institutional guidelines. All experiments using mice received approval from the Chiba University Administrative Panel for Animal Care.

**Competitive repopulation assay.** BM cells (10<sup>6</sup>) from 4-wk-old CD45.2<sup>−/−</sup> mice were mixed with the same number of uninfected BM competitor cells (CD45.1) and transplanted into CD45.1<sup>−/−</sup> mice irradiated at a dose of 9.5 Gy. PB cells of the recipient mice were analyzed with a mixture of antibodies that included PE-Cy7-conjugated anti-CD45.1, Pacific blue-conjugated anti-CD45.2, PE-conjugated anti-Mac-1 and anti–Gr-1, APC-conjugated anti-B220, and APC-Cy7-conjugated anti-CD4 and anti-CD8<sup>α</sup> antibodies. Cells were analyzed on a FACSComp II (BD). Donor cell chimerism in the recipient PB cells was evaluated as percent donor chimerism calculated as (percent donor cells) × 100/(percent donor cells + percent recipient cells). PB cell counts were made using an automated cell counter (Celltec α; Nihon Kohden).

**Purification of mouse HSCs and progenitors.** Mouse HSCs (CD34<sup>−</sup> LSK cells) were purified from BM of 8-wk-old mice. Mononuclear cells were isolated on Ficoll-Paque PLUS (GE Healthcare). The cells were stained with an antibody cocktail consisting of biotinylated anti–Gr-1, Mac-1, IL-7Ra, B220, CD4, CD8<sup>α</sup>, and Ter119 monoclonal antibodies. Lineage<sup>−</sup> cells were depleted with goat anti–rat IgG microbeads (Miltenyi Biotec) through an LD column (Miltenyi Biotec). The cells were further stained with FITC-conjugated anti-CD34, PE-conjugated anti-Sca-1, and APC-conjugated anti–c-Kit antibodies. Biotinylated antibodies were detected with APC-Cy7-conjugated streptavidin. Analysis and sorting were performed on a FACS Aria II (BD). CMPs, GMPs, MEPs, and CLPs were analyzed as CD34<sup>−</sup> FceRI<sup>−/−</sup>-c-Kit<sup>−/−</sup>Sca-1<sup>−/−</sup> Lineage<sup>−</sup> IL-7Ra<sup>−</sup> cells, CD34<sup>−</sup> FceRI<sup>−/−</sup>-c-Kit<sup>−/−</sup>Sca-1<sup>−/−</sup> Lineage<sup>−</sup> IL-7Ra<sup>−</sup> cells, CD34<sup>−</sup> FceRI<sup>−/−</sup>-c-Kit<sup>−/−</sup>Sca-1<sup>−/−</sup> Lineage<sup>−</sup> IL-7Ra<sup>−</sup> cells, and CD34<sup>−</sup> FceRI<sup>−/−</sup>-c-Kit<sup>−/−</sup>Sca-1<sup>−/−</sup> Lineage<sup>−</sup>IL-7Ra<sup>−</sup> cells, respectively.

**Retroviral vectors expressing Hmga2 and virus production.** Full-length mouse Hmga2 cDNA in the pMY-ires-EGFP retroviral expression vector was kindly provided by K. Nakashima (Nara Institute of Science and Technology, Ikoma, Nara, Japan). A recombinant vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped high-titer retrovirus was generated by a 293gpq packaging cell line that had been engineered to express the VSV-G protein under the control of a tetracycline-inducible system. The virus in supernatants of 293gpq cells was concentrated by centrifugation at 6,000 rpm for 16 h. Viral titers were determined by infecting Jurkat cells (a human T cell line).

**Transduction of CD34<sup>−</sup> LSK HSCs.** CD34<sup>−</sup> LSK HSCs were transduced with the indicated retrovirus, as previously described, with minor modifications (Iwama et al., 2004). CD34<sup>−</sup> LSK HSCs were sorted into 96-well microtiter plates coated with the recombinant human fibronectin fragment CH-296 (RetroNectin; Takara Shuzo) at 100 cells per well, and then incubated in α-MEM supplemented with 1% FBS, 1% l-glutamine, penicillin, streptomycin solution (GPS; Sigma-Aldrich), 50 μM 2-mercaptoethanol (2-ME), 100 ng/ml mouse stem cell factor (SCF; PeproTech), and 100 ng/ml human thrombopoietin (PeproTech) for 24 h. Next, cells were transduced with the indicated retrovirus at a multiplicity of infection (MOI) of 1,500 in the presence of 1 μg/ml RetroNectin and 10 μg/ml protamine sulfate (Sigma-Aldrich) for 24 h. After transduction, cells were further incubated in the same medium for 9 d, subjected to in vitro colony assays or in S-Clone SF-O3 (Sanko Junyaku) supplemented with 0.2% BSA, 50 μM 2-ME, 1% GPS, 50 ng/ml SCF, and 50 ng/ml TPO for 2.5 d, and then subjected to competitive repopulation assays. The transduction efficiency was nearly 90%, as judged from GFP expression. Colony assays were performed using a methylcellulose medium (M3234; STEMCELL Technologies) supplemented with 20 ng/ml mouse SCF, 20 ng/ml mouse IL-3 (PeproTech), 50 ng/ml human TPO, and 3 U/ml human EPO (provided by Kyowa Hakko Kimia). Colony numbers were counted on day 10. CD34<sup>−</sup> LSK HSCs (CD45.2<sup>−/−</sup>) transduced with the indicated retrovirus were also transplanted intravenously into 8-wk-old CD45.1<sup>−/−</sup> mice irradiated at a dose of 9.5 Gy, together with 2 × 10<sup>5</sup> BM competitor cells from 8-wk-old CD45.1<sup>−/−</sup> mice.

**Microarray analysis.** IL-7Ra<sup>−/−</sup> LSK cells were purified from BM of 4-wk-old Ink4a-Arf<sup>−/−</sup> and Bmi1<sup>−/−</sup> Ink4a-Arf<sup>−/−</sup> mice and CMPs from recipient BM at 4 mo after infusion of Ink4a-Arf<sup>−/−</sup> or Bmi1<sup>−/−</sup> Ink4a-Arf<sup>−/−</sup> BM cells. Total RNA was isolated using TRIzol LS Reagent (Invitrogen), and its integrity was confirmed using LabChip RNA 6000 Nano chips and a 2100 Bioanalyzer (Agilent Technologies). Target cRNA was prepared from the total RNA equivalent to 10,000 cells (IL-7Ra LSK) or 8,000 cells (CMPs) with a two-cycle cDNA synthesis kit and 3′-amplification reagents for IVT labelling (Affymetrix), and then hybridized to a GeneChip Mouse Genome 430 2.0 oligonucleotide microarray (Affymetrix) according to the manufacturer’s instructions. The expression value of each gene was calculated and normalized using GeneChip Operating Software version 1.4 (Affymetrix). All data are MIAME compliant, and the raw data were deposited in Gene Expression Omnibus (accession no. GSE19796 and GSE31086).

**Quantitative reverse transcription (RT) PCR analysis.** Total RNA was isolated using TRIzol LS solution (Invitrogen) and reverse transcribed by the ThermoScript RT-PCR system (Invitrogen) with an oligo dT primer. Real-time quantitative PCR was performed with an ABI prism 7300 Thermal Cycler (Applied Biosystems) using FastStart Universal Probe Master (Roche). Hypoxanthine-guanosine phosphoribosyl transferase 1 (Hprt1) expression was used to calculate relative expression levels. The combination of primer sequences and probe numbers are as follows: Hmga2, probe #26, 5′-AAGGCAGCAAAAACAAGAGC-3′ and 5′-CCGGTTTTTCTCCAAATGGTTCT-3′; Hprt1, probe #95, 5′-TCTCTCTCAGACCGGCTT-3′, and 5′-CCTCTGTTCTATCATGCATATCAT-3′.

**ChIP assay.** Lineage<sup>−</sup> or Lineage<sup>−</sup> c-Kit<sup>−</sup> BM cells (2 × 10<sup>6</sup> cells/antibody) isolated by flow cytometry were cross-linked with 1% formaldehyde for 15 min at room temperature, and incubated for 5 min at 4°C after the addition of 0.125 M glycine. Cells were washed with PBS, lysed with cell lysis buffer (50 mM Tris- HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.5% SDS, and protease inhibitor cocktail [PIC; Roche]) on ice, and sonicated until the DNA fragments were 200–500 bp in mean size as measured by Bioanalyzer (Cosmo Bio). After centrifugation at 15,000 rpm for 10 min, sheared chroma- tin was diluted 10-fold in dilution buffer (50 mM Tris- HCl, pH 8.0, 150 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, and PIC), and pre- cleared by addition of Dynabeads Protein G (Invitrogen) for 1 h at 4°C. For anti-Bmi1 (clone 8A9; provided by N. Nozaki, MAB Institute, Yokosuka, Kanagawa, Japan), precleared chromatin was immunoprecipitated overnight at 4°C with antibody/Dynabeads Protein G mix. For anti–ubiquityl-Histone H2A (H2Aub; clone E6C5, 05–678; Millipore), precleared chromatin was immunoprecipitated overnight at 4°C with anti-H2Aub, followed by the
addition of anti-mouse IgG 

Brief Definitive Report


Mochizuki-Kashio, M., Y. Mishima, S. Miyagi, M. Negishi, A. Saraya, T. Konuma, J. Shinga, H. Koseki, et al. 2004. Enhanced self-renewal of hematopoietic stem cells in mice, respectively, N. Nozaki, K. Helin, T. Kitamura, and K. Nakashima for providing Bmi1+/- mice and Ink4a-Arf+/- mice, respectively, N. Ozaki, K. Helin, T. Kitamura, and N. Nakashima for the anti-Bmi1 antibody (BA9), the anti-Ezh2 antibody (AC22), the pMys-ires-EGFP vector, and the Hmga2 cDNA, respectively, and George Wendt for critical reading of the manuscript.

We thank M. van Lohuizen and R.A. DePinho for providing Bmi1+/- mice and Ink4a-Arf+/- mice, respectively, N. Ozaki, K. Helin, T. Kitamura, and N. Nakashima for the anti-Bmi1 antibody (BA9), the anti-Ezh2 antibody (AC22), the pMys-ires-EGFP vector, and the Hmga2 cDNA, respectively, and George Wendt for critical reading of the manuscript.

This work was supported in part by Grants-in-aid for Scientific Research (#21390268) and for the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment). MEXT, Japan, a Grant-in-aid for Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corporation (JST), and grants from the Takeda Science Foundation, Astellas Foundation for Research on Metabolic Disorders, and for the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment). MEXT, Japan, a Grant-in-aid for Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corporation (JST), and grants from the Takeda Science Foundation, Astellas Foundation for Research on Metabolic Disorders, and for the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment).

The authors have no conflicting financial interests.

Submitted: 12 August 2011
Accepted: 27 January 2012

REFERENCES


