**INTRODUCTION**

Bone marrow stromal cells (BMSCs) are multipotent progenitor cells that form osteoblasts and adipocytes in vivo, and have trilineage potential to form osteoblasts, adipocytes, and chondrocytes in vitro. Originally identified as cells forming fibroblast-like colonies (fibroblast colony-forming units [CFU-F]), recent studies have identified multiple markers to prospectively identify BMSCs, including CD146 in human tissues (Sacchetti et al., 2007), platelet-derived growth factor receptor (PDGFR) (Morikawa et al., 2009), Cxcl12 (Ding and Morrison, 2013; Greenbaum et al., 2013; Omatsu et al., 2010; Sugiyama et al., 2006), Scf (Ding et al., 2012), Nestin-GFP (Kunisaki et al., 2013; Mendez-Ferrer et al., 2010), Mx1-Cre (Park et al., 2012), Leptin receptor (Lepr)-Cre (Ding et al., 2012; Omatsu et al., 2014; Zhou et al., 2014), and Prx1-Cre (Greenbaum et al., 2013; Zhou et al., 2014) in mice. These markers identify a largely overlapping population of BMSCs. For instance, stromal populations that express high levels of Cxcl12 (Cxcl12-abundant reticular [CAR] cells) (Sugiyama et al., 2006) largely overlap with cells that can be identified by Prx1-Cre labeling (Greenbaum et al., 2013), Lepr-Cre labeling (Ding et al., 2012), and Nestin-GFP expression (Zhou et al., 2014). Genetic manipulations with these tools are beginning to reveal important insights into the role of bone marrow BMSCs in bone maintenance and hematopoiesis.

BMSCs are a component of the perivascular HSC niche along with endothelial cells, and express high levels of HSC growth factors (Kfoury and Scadden, 2015; Morrison and Scadden, 2014). HSCs identified by highly selective markers were found in close proximity to endothelial cells and the surrounding BMSCs (Acar et al., 2015; Chen et al., 2016; Kiel et al., 2005; Mendez-Ferrer et al., 2010; Sugiyama et al., 2006). Depletion of BMSCs in Cxcl12-diphtheria toxin receptor (DTR) or in Nestin-Cre; inducible-DTR mice reduced the numbers of HSCs in the bone marrow, affirming the importance of BMSCs in HSC maintenance (Mendez-Ferrer et al., 2010; Omatsu et al., 2010). Moreover, deletion of HSC growth factors produced by BMSCs, such as stem cell factor (SCF) or Cxcl12, depleted bone marrow HSCs, establishing that BMSCs support HSCs by providing these growth factors (Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013). In addition to producing HSC growth factors, the immature state of BMSCs is also important for HSC maintenance. Accumulation of marrow adipocyte is a hallmark of aging (Li et al., 2018), and this age-related expansion of adipocytes negatively affects HSCs (Ambrosi et al., 2017). Murine caudal vertebrae is enriched in adipocytes, making this location refractory for HSC expansion (Naveiras et al., 2009). Consistently, BMSC-specific deletion of Foxc1, a transcription factor highly expressed in BMSCs, results in the accumulation of adipocytes in the marrow and severely impairs hematopoiesis and HSC maintenance (Omatsu et al., 2014). On the other hand, stromal components committed to the adipocytic fate as defined by adiponectin expression play a positive role in hematopoietic recovery after myeloablation by producing SCF, possibly as an adaptive response to the damage (Zhou et al., 2017). Thus, not only the growth factor secretome but also the differentiation status of BMSCs is intimately linked to HSC maintenance. However, little is known regarding the mechanisms that regulate fate determination of BMSCs.
The polycomb group protein BMI1 is an epigenetic regulator involved in development and cell-fate determination (Schuettengruber et al., 2017). BMI1 is a component of the polycomb repressive complex 1 (PRC1) that catalyzes the repressive histone modification H2A ubiquitination (H2Aub) (de Naples et al., 2004; Wang et al., 2004a). The PRC1 was previously considered to depend on another polycomb complex, the PRC2, which catalyzes repressive histone H3 lysine 27 trimethylation (H3K27me3) to associate with chromatin (Wang et al., 2004b). However, recent studies have revealed a more complex interplay between PRC1 and PRC2, whereby PRC1-catalyzed H2Aub recruits and stabilizes PRC2 association with chromatin (Blackledge et al., 2015; Schuettengruber et al., 2017). PRC1 and PRC2 govern developmental programs in pluripotent and tissue stem cells, as exemplified by the finding that H2Aub and H3K27me3 decorates chromatin of gene loci that encodes developmental genes, preventing precocious expression of these genes in stem cells (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Lee et al., 2006). Consistently, Bmi1-deficient mice exhibit multiple developmental defects including skeletal transformation, neuronal and hematopoietic defects, growth retardation, and postnatal lethality (Alkema et al., 1995; Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; van der Lugt et al., 1994). Bmi1 regulates self-renewal of HSCs both cell intrinsically in HSCs and cell extrinsically through its maintenance of the microenvironment (Oguro et al., 2006; Park et al., 2003). Since Bmi1-deficient mice exhibit myriad developmental defects in many organs, it remains unclear in which cell type Bmi1 functions to support HSCs cell extrinsically, in addition to its cell-intrinsic role in HSC self-renewal.

Here we show that Bmi1 is required in BMSCs to maintain HSCs, by suppressing the adipogenic differentiation of BMSCs. We found that BMSCs expressed high levels of Bmi1, and its expression level was downregulated upon adipocytic differentiation and during aging. Conditional deletion of Bmi1 from BMSCs increased the numbers of adipocytes in the bone marrow, depleting HSCs and hematopoietic cells. Bmi1 was required to epigenetically repress multiple developmental programs in BMSCs, including a novel adipogenic differentiation program mediated by Pax3. Our findings establish that Bmi1 maintains the HSC niche by suppressing adipogenesis of BMSCs.

RESULTS

Bmi1 Is Expressed in Immature Bone Marrow BMSCs and Suppresses Senescence
To begin to study how Bmi1 in bone marrow stroma cells regulates HSCs cell extrinsically, we determined Bmi1 expression levels in BMSCs (CD140^+CD45^-Ter119^-), non-BMSC stroma cells (CD140^-CD45^-Ter119^-), and hematopoietic cells (CD45/Ter119^+) by quantitative PCR (qPCR). CD140^+CD45^-Ter119^- BMSCs expressed high levels of HSC niche factors Kifl (encoding SCF) and Cxc112 (Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013), BMSC markers Lepr and Pdgfra (Ding et al., 2012; Morikawa et al., 2009), and transcription factors Foxc1 and Grem1 (Omatsu et al., 2014; Worthley et al., 2015) (Figures 1A and 1B). As shown in Figure 1C, Bmi1 mRNA was also highly expressed in BMSCs compared with non-BMSC stroma cells and hematopoietic cells. To examine whether expression of Bmi1 in BMSCs changes upon lineage commitment, we isolated BMSCs from wild-type bone marrow and cultured them with or without differentiation stimuli for adipocytes or osteoblasts, and measured Bmi1 expression levels by qPCR. Bmi1 expression was increased upon osteogenic stimuli, while it was decreased in cells that were induced to undergo adipocytic differentiation (Figure 1D). These results suggest that Bmi1 is highly expressed in undifferentiated BMSCs and that reduction in Bmi1 expression accompanies commitment to the adipocytic fate.

To study the role of Bmi1 in BMSC function, we conditionally deleted Bmi1 using Prx1-Cre. Prx1-Cre has been shown to be expressed in CAR cells (Sugiyama et al., 2006), Lepr^+ stroma cells (Ding and Morrison, 2013; Greenbaum et al., 2013), Scf-expressing HSC niche cells (Ding et al., 2012), and Nestin^+ BMSCs (Mendez-Ferrer et al., 2010). Prx1-Cre;Bmi1^fl/fl mice were born normally and did not exhibit any changes in body weight, fat mass, or lean mass, although the amount of circulating adiponectin was lower than those in Bmi1^fl/fl mice (referred to as control) (Figure S1A). qPCR to detect Bmi1 in purified BMSCs from control Bmi1^fl/fl and Prx1-Cre;Bmi1^fl/fl mice showed that Bmi1 expression was significantly depleted in the BMSCs from Prx1-Cre;Bmi1^fl/fl mice, confirming efficient deletion of Bmi1 (Figure 1E). In young adult mice of 2–4 months of age, we did not detect any differences in the frequencies or total numbers of BMSCs in the long bones between Bmi1^fl/fl and Prx1-Cre;Bmi1^fl/fl mice (Figures 1F and S1B). Consistently, enumeration of BMSCs in a CFU-F assay showed that the numbers of CFU-F were not significantly affected by Bmi1 deletion (Figure 1G). We then serially passaged BMSCs in vitro to examine the proliferative capacity of Bmi1-deficient BMSCs. Whereas wild-type BMSCs expanded significantly over three passages, Bmi1-deficient BMSCs ceased proliferation upon passage 2 and failed to expand in later passages (Figure 1H). Since Bmi1 suppresses senescence by silencing the Ink4a/Arf locus (Jacobs et al., 1999), we examined whether Bmi1-deficient BMSCs undergo senescence. Bmi1-deficient BMSC cultures exhibited significantly more senescent-associated...
-gal (SAβ-gal) cells and had increased expression of Ink4a, a marker of senescent cells (Figures 1I–1J and S1C). These results establish that Bmi1 is highly expressed in BMSCs and suppresses senescence in vitro.

**Bmi1 Suppresses Adipogenic Differentiation of BMSCs**

The finding that Bmi1 expression is reduced in BMSCs upon commitment to the adipocytic fate prompted us to test whether Bmi1 deletion promotes adipocyte differentiation of BMSCs. We cultured BMSCs from Prx1-Cre;Bmi1fl/fl mice and stimulated BMSCs to undergo adipocytic, osteoblastic, or chondrocytic differentiation in vitro. While wild-type BMSCs underwent osteoblastic differentiation after 2 weeks of induction, osteoblastic differentiation of Prx1-Cre;Bmi1fl/fl BMSCs was delayed, and fewer alizarin red-positive osteoblasts were observed after 4 and 6 weeks of induction (Figures 2A and S2A). Chondrogenesis occurred in Bmi1-deficient BMSCs similar to wild-type BMSCs (Figure S2B). In contrast, the accelerated adipocytic differentiation of Prx1-Cre;Bmi1fl/fl

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**Figure 1. Bmi1 Is Highly Expressed in BMSCs and Suppresses Senescence**

(A) Schematic showing the gating strategy to isolate BMSCs (CD140a⁻CD45⁻Ter119⁻), non-BMSC stroma cells (CD140a⁺CD45⁻Ter119⁻), and hematopoietic cells (CD45/Ter119⁻).

(B and C) qPCR showing the expression of HSC niche factors and regulators (B) and Bmi1 (C) in BMSCs, other stroma cells, and hematopoietic cells (Hem) (n = 3).

(D) Bmi1 expression 3 weeks after osteogenic stimulation (left) and adipocytic induction (right) (n = 7–9).

(E) qPCR of Bmi1 expression in purified BMSCs and HSCs from Prx1-Cre;Bmi1fl/fl mice (n = 3–4).

(F) The frequencies (left) and total numbers (right) of BMSCs in Bmi1fl/fl and Prx1-Cre;Bmi1fl/fl mice (n = 6).

(G) The numbers of CFU-F per 1 × 10⁶ bone marrow cells following Bmi1 deletion (n = 6).

(H) Passaging of Bmi1fl/fl and Prx1-Cre;Bmi1fl/fl BMSCs (n = 5).

(I and J) The frequencies of SAβ-gal⁺ cells (n = 5) (I) and expression of Ink4a as determined by qPCR in Bmi1fl/fl and Prx1-Cre;Bmi1fl/fl BMSCs (normalized to 18S rRNA) (J) (n = 3).

In (B–E), expression values were normalized to β-actin. All data represent mean ± standard deviation (SD). *p < 0.05, **p < 0.01, and ***p < 0.001 by Student’s t test. ns, not significant. See also Figure S1.
BMSCs compared with wild-type BMSCs was observed after only 1 week of induction (Figures 2B and S2C). These changes in differentiation potential were confirmed by gene expression changes. Consistent with the accelerated adipocytic differentiation of Bmi1-deficient BMSCs, genes involved in adipogenesis such as Pparg, Fabp4, and Adipoq were increased in Bmi1-deficient BMSCs 3 weeks after induction compared with control BMSCs (Figure 2C). Conversely, genes involved in osteogenesis such as Sp7 (Osterix) and Runx2 were reduced in Bmi1-deficient BMSCs upon osteoblastic differentiation compared with control BMSCs (Figure 2D). In vitro bromodeoxyuridine (BrdU) incorporation assays revealed that Bmi1 deletion does not affect proliferation of BMSCs or adipocyte progenitors (Figure S2D). These results suggest that Bmi1 deletion primes BMSCs to undergo adipocytic differentiation.

We then determined whether Bmi1 deletion from BMSCs affects the bone marrow environment by regulating osteogenesis or adipogenesis in vivo. We first performed micro-computed tomography (μCT) scanning of intact femurs to examine the bone parameters. Bmi1 deletion from BMSCs did not affect the thicknesses, densities, or the bone volume to total volume ratios of cortical and trabecular bones or the numbers of trabecular bones (Figures 3A, 3B, and S3A–S3G), suggesting that Bmi1 is largely dispensable for bone formation and maintenance. Since Bmi1 expression was attenuated upon adipogenic stimuli (Figure 1D), we tested whether Bmi1 deletion affects adipogenesis in the bone marrow. To quantitatively analyze the adipocyte volume in the marrow, we performed μCT scanning of osmium tetroxide-stained femurs (Figures 3C and 3D). Two- to 6-month-old wild-type mice exhibited some adipocytes in the epiphyses, whereas adipocytes expanded significantly in the epiphyses and extended to the diaphyses of femurs in Prx1-Cre;Bmi1fl/fl mice (Figures 3C, 3D, and S3H). Cross-sections of the femurs stained with osmium tetroxide confirmed this finding (Figure 3E). In contrast to the expansion of adipocytes in the long bones, we did not detect significant changes in adipocyte volume in the lumbar vertebrae (Figure S3I), consistent with the selective expression of Prx1-Cre in the limbs (Logan et al., 2002). These results establish that Bmi1 is required to suppress accumulation of bone marrow adipocytes.

Accumulation of marrow adipocytes is a hallmark of aging (Li et al., 2018). The finding that the bone marrow of young adult Prx1-Cre;Bmi1fl/fl mice become filled with adipocytes prompted us to examine Bmi1 expression in BMSCs isolated from mice with different ages. CD140+CD45−Ter119−BMSCs were isolated from 3- to 5-month-old and 18- to 24-month-old wild-type mice and Bmi1 expression was quantified by qPCR. We found that older mice had lower Bmi1 expression in BMSCs compared with young mice (Figure 3F). These results illustrate the links between aging, adipocyte expansion, and loss of Bmi1 expression; aging is accompanied by the expansion of marrow adipocytes and loss of Bmi1 expression in BMSCs, while Bmi1 deletion from BMSCs causes precocious accumulation of marrow adipocytes.

Deletion of Bmi1 from BMSCs Reduces HSCs and Attenuates Hematopoiesis

To determine the effect of Bmi1 deletion on the hematopoietic microenvironment, we analyzed hematopoiesis in the bone marrow of Prx1-Cre;Bmi1fl/fl mice. At 2 months of age, we observed a slight reduction in bone marrow cellularity, possibly due to the expansion of bone marrow adipocytes following Bmi1 deletion. In contrast to this minor reduction at early time points, a more severe phenotype was observed at 6 months of age (Figures 4A–4H and...
Bone marrow cellularity significantly increased in Bmi1fl/fl mice from 2 to 6 months of age, but this increase was not observed in Prx1-Cre;Bmi1fl/fl mice, which had significantly reduced bone marrow cellularity at 6 months compared with age-matched control mice (Figure 4A). Hematopoietic progenitor cells also expanded from 2 to 6 months of age in Bmi1fl/fl mice but not in Prx1-Cre;Bmi1fl/fl mice. Overall, Bmi1 deletion resulted in significant depletion of HSCs, common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), and granulocyte macrophage progenitors (GMPs) (Figures 4B–4H and S4A–S4D). Fractionating HSCs into long- and short-term (LT and ST) HSCs using CD34 and FLT3 revealed similar depletion of HSCs (Figures 4C and S4C). As expected, Prx1-Cre did not affect the expression of Bmi1 in HSCs (Figure 1E). Multipotent progenitors (MPPs) and megakaryocyte erythroid progenitors (MEPs) also showed trends toward becoming depleted following Bmi1 deletion (Figures 4D and 4G). Total numbers of hematopoietic colony-forming cells in hind limbs were significantly reduced in 6-month-old Prx1-Cre;Bmi1fl/fl mice (Figure 4I). These results establish that deleting Bmi1 from BMSCs reduces the number of HSCs and other hematopoietic stem and progenitor cells (HSPCs) in the bone marrow, thereby impairing hematopoiesis in adult mice.

Previous reports have shown that adipocytes in the tail vertebrae promote quiescence and regenerative potential but reduce the numbers of HSCs (Naveiras et al., 2009). The expansion of adipocytes in Prx1-Cre;Bmi1fl/fl mice prompted us to examine the cell-cycle activity of HSCs in these mice by BrdU incorporation assays. Prx1-Cre;Bmi1fl/fl and control Bmi1fl/fl mice were treated with BrdU for 7 days and BrdU incorporation into HSCs was analyzed by flow cytometry. Interestingly, we found that BrdU incorporation specifically into HSCs was significantly reduced, whereas BrdU incorporation into other immature cells such as MPPs, HPC1, and HPC2 (Oguro et al., 2013) was not affected by deleting Bmi1 from BMSCs (Figures 5A and S4G). Importantly, deletion of Bmi1 from hematopoietic cells using Vav-Cre did not affect division of HSCs (Figure 5B), indicating that Bmi1 is required in the hematopoietic microenvironment, and not in HSCs themselves, to maintain HSC division. HSCs in Prx1-Cre;Bmi1fl/fl mice were capable of entering the cell cycle, since treating mice with cyclophosphamide and granulocyte colony stimulating factor increased BrdU+ HSCs in Prx1-Cre;Bmi1fl/fl mice comparable with Bmi1fl/fl mice (Figure S4H). To examine the function of the residual HSCs, we isolated 50 HSCs from Prx1-Cre;Bmi1fl/fl and control Bmi1fl/fl mice and performed a competitive HSC transplantation assay. We found that HSCs from Prx1-Cre;Bmi1fl/fl mice do not exhibit any defects in long-term multilineage...
reconstitution compared with control HSCs (Figure 5C).

We did not observe any differences in the lineage contribution of HSCs isolated from Prx1-Cre;Bmi1fl/fl or control Bmi1fl/fl mice (Figure 5D). Thus, deletion of Bmi1 from BMSCs causes quiescence and depletion of HSCs and reduces the numbers of many hematopoietic progenitor populations downstream of HSCs.

**Bmi1 Deletion Derepresses Developmental Programs in BMSCs**

Since PRC1 is known to repress developmental gene expression in embryonic stem cells and tissue stem cells, we next analyzed the gene expression profiles of Bmi1fl/+ and Prx1-Cre;Bmi1fl/fl BMSCs. Freshly isolated BMSCs from Bmi1fl/+ and Prx1-Cre;Bmi1fl/+ mice were subjected to
RNA sequencing (RNA-seq). Compared with whole bone marrow cells, many genes known to be expressed in BMSCs were highly enriched in our gene expression profiles, including Kitl, Cxcl12, Lepr, Pdgfra, Foxc1, and Grem1 (Figure 6A), attesting to the purity of the BMSCs we used for RNA-seq.

To broadly examine the gene expression changes that occur in BMSCs after Bmi1 deletion, we performed a gene set enrichment analysis (GSEA) between wild-type and Bmi1-deficient BMSCs using the MSigDB collection of gene sets. Consistent with the role of BMI1 in the PRC1 complex, we found positive enrichment of gene sets that represent PRC2 target genes and genes with histone H3K27me3 modification in Bmi1-deficient BMSCs (Figure 6D). These results suggest that polycomb target genes including posterior transcription regulators involved in developmental processes, such as organ morphogenesis, pattern specification, and anterior/posterior patterning, were all enriched in Bmi1-deficient BMSCs (Figure 6C). We also identified gene sets representing fat cell differentiation and adiponectin-activated pathways as enriched in Bmi1-deficient BMSCs. Consistent with the dysregulation of developmental processes after deleting Bmi1, several transcription regulators involved in developmental processes, including posterior Hox genes (Hoxc10-Hoxc13), several Paired box transcription factors (Pax3 and Pax9), Engrailed 2 (En2), Gli1, and Sal1 were derepressed in Bmi1-deficient BMSCs (Figure 6D). These results suggest that Bmi1 is required to suppress developmental gene programs in BMSCs, possibly through its role in the PRC1 complex and in collaboration with the PRC2.

To determine whether Bmi1-deficient BMSCs exhibit transcriptional changes indicative of precocious adipogenic differentiation, we compared the expression profiles of Bmi1-deficient BMSCs with those from a recent study that identified four fractions of bone marrow stroma cells with osteogenic and/or adipogenic potential;CD45−CD31−Sca-1−CD24+ stem cell-like cells (MSCs), CD45−CD31−Sca-1−PDGFRα osteochondrogenic progenitor cells (OPCs), CD45−CD31−Sca-1−CD24− adipogenic progenitor cells, and CD45−CD31−Sca-1−ZFP423+ pre-adipocytes (PreAd) (Ambrosi et al., 2017). We created gene sets that are upregulated or downregulated in Bmi1-deficient BMSCs. We then compared MSCs, OPCs, APCs, and PreAd in a pairwise GSEA (Subramanian et al., 2005; Viny et al., 2015) and determined the enrichment scores of each population in regard to our custom gene sets. These analyses revealed that pre-adipocytes are enriched in genes upregulated in Bmi1-deficient BMSCs (Figure 6E), whereas osteochondrogenic progenitor cells were enriched in genes downregulated in Bmi1-deficient BMSCs (Figure 6F). These results suggest that Bmi1-deficient BMSCs share gene expression similarity with pre-adipocytes, consistent with the accelerated adipogenic and delayed osteogenic differentiation of these cells. Together, these results indicate that Bmi1 deletion initiates a precocious adipocytic differentiation program in BMSCs.

We then examined the role of Bmi1 in maintaining the epigenome of BMSCs. BMSCs were isolated from Bmi1fl/fl and Prx1-Cre:Bmi1fl/fl mice (n = 3). BrdU incorporation into HSC, MPP, HPC1, and HPC2 of Bmi1fl/fl and Prx1-Cre:Bmi1fl/fl mice (n = 3). Long-term reconstitution assays with 50 HSCs isolated from Bmi1fl/fl or Prx1-Cre:Bmi1fl/fl mice (n = 3) (C). Lineage contribution from transplanted HSCs at 16 weeks after transplantation (n = 3) (D). All data represent mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student’s t test.

Figure 5. Deletion of Bmi1 from BMSCs Causes Quiescence of HSCs
(A) Incorporation of BrdU during a 7-day pulse into HSC, MPP, HPC1, and HPC2 of Bmi1fl/fl and Prx1-Cre:Bmi1fl/fl mice (n = 3). (B) BrdU incorporation into HSCs, MPPs, HPC1, and HPC2 in Bmi1fl/fl and Vav1-Cre:Bmi1fl/fl mice (n = 3). (C and D) Long-term reconstitution assays with 50 HSCs isolated from Bmi1fl/fl or Prx1-Cre:Bmi1fl/fl mice (n = 3) (C). Lineage contribution from transplanted HSCs at 16 weeks after transplantation (n = 3) (D). All data represent mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student’s t test.
H3K27me3. The Hoxc10-Hoxc13 cluster, particularly on the centromere-proximal side with the Hoxc13 gene, was covered by the repressive H2Aubi and H3K27me3 modification in wild-type BMSCs (Figure 7A). In Bmi1-deficient BMSCs, the H2Aubi modification was reduced in this region, consistent with the notion that the Bmi1-containing PRC1 catalyzes H2A ubiquitylation (de Napoles et al., 2004; Wang et al., 2004a) (Figure 7A). Interestingly, H3K27me3 catalyzed by PRC2 was also reduced in this region in Bmi1-deficient BMSCs, suggesting that the BMI1-containing PRC1 is required to maintain the PRC2-catalyzed H3K27me3 modification on chromatin. Furthermore, we found that the Cdkn2a locus, which encodes the Ink4a/Arf tumor-suppressor genes that are known to be repressed by PRC1, was covered by H2Aubi and H3K27me2 in a Bmi1-dependent manner, consistent with the derepression of Ink4a and Arf (Figure 7B). Interestingly, among the genes that encode transcription factors involved in developmental processes, we found significant changes in both H2Aubi and H3K27me3 in the promoter region of Pax3 in Bmi1-deficient BMSCs compared with controls (Figure 7C). Bmi1 deletion decreased enrichment of H2Aubi and H3K27me3 in the Pax3 promoter region. Together with the finding that Pax3 expression is derepressed in the absence of Bmi1 (Figure 6D), this finding raised the possibility that Pax3 is a direct target of BMI1 in BMSCs.
Derepressed Pax3 in the Absence of Bmi1 Promotes Adipogenic Differentiation of BMSCs

PAX3 is a transcriptional regulator that integrates cell extrinsic signals to cell-fate determination, regulating multiple tissue stem/progenitor cells including neural crest stem cells, muscle satellite cells, and melanoblasts (Blake and Ziman, 2014; Lang et al., 2005; Young and Wagers, 2010). Pax3 was highly expressed in BMSCs compared with hematopoietic cells and non-BMSCs and its expression level did not change with age (Figure S5A). To determine the role of Pax3 in BMSCs, we knocked down Pax3 expression using short hairpin RNA (shRNA) and tested whether the derepressed Pax3 regulates adipogenesis in the absence of Bmi1. As controls, we also knocked down the expression of Pparg, a master regulator of fat cell fate, and Ink4a/Arf, a prototypical target of the PRC1/2 in Bmi1<sup>fl/fl</sup> and Prx1-Cre;Bmi1<sup>fl/fl</sup> mice. CD140<sup>+</sup>CD45<sup>-</sup>/Ter119<sup>-</sup> BMSCs were isolated from Prx1-Cre;Bmi1<sup>fl/fl</sup> and control mice and infected with lentiviral shRNA constructs and stimulated to undergo adipogenic or osteoblastic differentiation. shRNA for these genes effectively knocked down the expression of Pax3, Pparg, and Ink4a/Arf in Bmi1-deficient BMSCs (Figures 7D, S5B, and S5C). Ink4a/Arf knockdown increased adipogenesis in both wild-type and Bmi1-deficient BMSCs (Figure S5D), suggesting that the derepressed Ink4a/Arf in Bmi1-deficient BMSCs counteracts...
rather than promotes adipogenesis. As expected, *Pparg* knockdown significantly attenuated the adipogenesis in both wild-type and *Bmi1*-deficient BMSCs (Figure S5E). Importantly, *Pax3* knockdown also significantly blunted the increased adipogenesis observed in *Bmi1*-deficient BMSCs (Figures 7E and S5F), indicating that the derepressed *Pax3* expression in *Bmi1*-deficient BMSCs is at least partly responsible for the enhanced adipogenesis. *Pax3* knockdown also increased osteogenesis in both wild-type and *Bmi1*-deficient BMSCs (Figures SF and S5G). Together, these results indicate that *Pax3*, which is repressed by *Bmi1* in immature BMSCs, promotes adipogenesis and suppresses osteogenesis in bone marrow BMSCs.

**DISCUSSION**

Recent studies have established the role of bone marrow BMSCs as a crucial component of the HSC niche, responsible for the production of multiple secreted or membrane-bound factors to support HSCs, and elucidated their contribution toward osteogenic and adipogenic cells in the marrow (Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013; Himburg et al., 2018; Omatsu et al., 2010; Park et al., 2012; Sugiyama et al., 2006; Worthley et al., 2015; Zhou et al., 2014). However, relatively little is known of how the cell fate of BMSCs is regulated and the consequences of dysregulated BMSC cell fate on HSC maintenance. Here we show that *Bmi1* is required in BMSCs to suppress adipogenic differentiation and thus maintain adult bone marrow HSCs. We found that *Bmi1* is required for the repression of *Pax3*, which we identified as a novel adipogenic regulator of BMSCs.

Several studies have shown that *Bmi1* expression is downregulated during aging in several tissues (Cordisco et al., 2010; Dhawan et al., 2009; Tschen et al., 2009). Consistent with these reports, we found that *Bmi1* expression is reduced in BMSCs isolated from older mice. Furthermore, we found that *Bmi1* expression was reduced upon exposing wild-type BMSCs to adipogenic stimuli, suggesting that reduced *Bmi1* expression facilitates adipogenic differentiation. These studies corroborate the expansion of adipocytes in the marrow with age, known as the age-related conversion of red to yellow marrow (Li et al., 2018). As lineage-tracing studies indicate that bone marrow BMSCs are the major source of marrow adipocytes (Ambrosi et al., 2017; Zhou et al., 2014), our results are consistent with the model that *Bmi1* expression is attenuated in bone marrow BMSCs with age, causing promiscuous differentiation of BMSCs to adipocytes.

Expansion of adipocytes after deleting *Bmi1* from BMSCs impaired HSC niche function, reducing proliferation and the number of HSCs, suggesting that *Bmi1*-deficient BMSCs or increased adipocytes create a quiescent HSC niche. Prior studies have also demonstrated that adipocytes negatively regulate the numbers of murine HSCs (Ambrosi et al., 2017; Naveiras et al., 2009). The maintenance of human HSCs in vitro is also improved by coculturing them with bone marrow adipocytes (Martiucci et al., 2018). Interestingly, although the numbers of HSCs were depleted in *Prx1-Cre;Bmi1*^fl/fl^ mice, residual HSCs were as functional as HSCs from control mice. As a consequence of HSC depletion, multiple downstream progenitor cells were also depleted and *Prx1-Cre;Bmi1*^fl/fl^ mice had lower white blood cell counts compared with wild-type mice. The effects of *Bmi1* deletion on peripheral blood cells were modest compared with the effects on bone marrow HSPCs, consistent with a study demonstrating that homeostatic blood production can be sustained with few HSPCs (Schoedel et al., 2016). The reduction in lymphocytes in the peripheral blood is consistent with aging; however, many changes that accompany HSC aging were not observed in *Prx1-Cre;Bmi1*^fl/fl^ mice. For example, myeloid bias of HSCs after transplantation, a defining feature of HSC aging (Geiger et al., 2013), was not observed in HSCs isolated from *Bmi1*-deficient mice. Thus, while reduction in *Bmi1* expression during aging may contribute to the age-related expansion of adipocytes in the HSC niche, it is insufficient to cause a major aging phenotype in HSCs. Additionally, whether *Bmi1* reduction contributes to age-associated bone loss or BMSC loss remains unclear. Future investigation should shed light on additional age-associated changes in the niche, such as in the endothelial cells, as well as molecules produced by BMSCs that induce quiescence and aging of HSCs.

We found that deletion of *Bmi1* from BMSCs reduces not only the H2Aub1 mark catalyzed by the PRC1 but also the H3K27me3 mark decorated by the PRC2. These findings are consistent with the recently proposed model describing that the PRC1-catalyzed H2Aub1 can stabilize PRC2 and the H3K27me3 modification on chromatin (Blackledge et al., 2015; Schuettengruber et al., 2017). Both H2Aub1 and H3K27me3 were reduced on known polycomb target genes, such as the *Hox* and the *Ink4a/Arf* genes, causing derepression of these genes. We also identified *Pax3*, a master regulator of neural crest development (Blake and Ziman, 2014), as a potential target of BMI1/PRC1 whose promoter region is modified by H2Aubi and H3K27me2 in a PRC1-dependent manner. *Pax3* has also been found to distinguish brown adipocytes from white adipocytes (Hafner et al., 2016; Mohsen-Kanson et al., 2014), but whether *Pax3* regulates adipogenesis of bone marrow BMSCs is unknown. Further work should delineate the role of *Bmi1* and *Pax3* in regulating the bone marrow niche for HSCs and age-related increase in adipogenesis.
EXPERIMENTAL PROCEDURES

Mice

C57BL/Ka-Thy-1.2 (CD45.1) mice (8–12 weeks of age) were used for HSC transplantation assays. Bmi1fl/fl mice were described previously (Mich et al., 2014). Prx1-Cre (JAX: 005584) and Vav1-Cre (JAX: 008610) mice were obtained from The Jackson Laboratory. Age- and sex-matched pairs were analyzed in each experiment otherwise specified in figure legends. Mice were housed in AAALAC-accredited, specific-pathogen-free animal care facilities at Baylor College of Medicine (BCM). All procedures were approved by BCM Institutional Animal Care and Use Committees.

Statistics

Statistics were determined with a paired or unpaired Student's t test or ANOVA with Prism software (GraphPad). Group data represent mean ± standard deviation in all results. No randomization or blinding was used in any experiments. Experimental mice were not excluded from analysis in any experiments. Sample sizes were selected on the basis of previous experience with the degree of variance in each assay.

CFU-F Assays

One million enzymatically digested bone marrow cells were cultured per well of a 6-well plate in α-minimal essential medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% human platelet lysate (STEMCELL Technologies, Vancouver, Canada). After 10–14 days, the cultures were washed with PBS and stained with 1% crystal violet (C0775, Millipore Sigma, Burlington, MA) in methanol. Colonies with more than 50 cells were counted as a colony-forming unit. SA-β-gal staining was done with the Senescence β-Galactosidase Staining Kit (#9860, Cell Signaling Technologies, Danvers, MA).

Body Composition and Adiponectin Level Measurements

Body composition of 6-month-old mice was assessed by magnetic resonance imaging and circulating adiponectin levels were measured by a Mouse Adiponectin ELISA kit (EZMADP-60K, Millipore) according to the manufacturer's instructions, both by the BCM Mouse Metabolic and Phenotyping Core.

BMSC Differentiation Assays

To induce osteogenic differentiation, we cultured BMSCs in α-MEM supplemented with 20% heat-inactivated FBS, 10 nM dexamethasone (D1759), 100 mM ascorbic acid (49752), and 10 mM β-glycerophosphate (G9422, all from Millipore Sigma). For evaluation of osteogenesis, cells were fixed in 4% paraformaldehyde (PFA), washed twice with PBS, and stained with 2% Alizarin red S solution for 15 min at 37°C. After washing with 60% isopropanol, cells were either imaged or oil red O extracted with 100% isopropanol and quantified by the absorption of 500 nm with a spectrophotometer. To induce chondrogenic differentiation, we cultured BMSCs in MSC chondrogenic differentiation medium (PFT-3003, Lonza, Allendale, NJ) supplemented with 10 ng/mL TGFβ3 (PT-4124, Lonza) according to the manufacturer's instructions. For evaluation of chondrogenesis, cell aggregates were cryosectioned, fixed in 4% PFA, stained with toluidine blue O solution (pH 4.5), and imaged.

μCT Analysis

Femurs were fixed in 4% PFA for 48 h at room temperature, washed in PBS, and stored in 70% ethanol before scanning. To quantify bone marrow adipocytes in the bone marrow, we fixed femurs in 10% formalin at 4°C overnight and washed them in running water. Bones were decalcified in 14% EDTA for approximately 14 days. Decalcified bones were stained in 1% osmium tetroxide with 2.5% potassium dichromate for 48 h at room temperature before washing and scanning. μCT scanning was performed with the following parameters: energy of 55 kV, a diameter of 16,384 μm, an intensity of 145 μA, and a maximum isometric voxel size of 16 μm using a Scanco Medical MicroCT-40 (Scanco Medical, Bruttisellen, Switzerland) and μCT V6.1 software. μCT scanning for osmium-stained samples was done with the voxel size of 8 μm.

Lentiviral shRNA Transduction

The pLKO shRNA plasmids were purchased from Sigma-Aldrich (St. Louis, MO). To generate lentiviral particles, we transiently transfected 293T cells with pLKO vectors with psPAX2 (12260) and pMD2.G (12259) from Addgene. Culture supernatant was collected after 48 h and used to transduce freshly isolated BMSCs for 48 h. After infection, medium was changed to α-MEM supplemented with 10% heat-inactivated FBS and 5% human platelet lysate.

HSC Transplantation

Fifty HSCs were sorted from mice and transplanted along with 200,000 competitor whole bone marrow cells from CD45.2 mice into lethally irradiated (two doses of 500 cGy) CD45.1 mice. Recipient mice were bled monthly and mononuclear cells were analyzed by flow cytometry.

Quantitative Real-Time PCR

BMSCs, stroma cells, and hematopoietic cells were sorted into TRIzol (Thermo Fisher Scientific, Waltham, MA) and RNA was isolated according to the manufacturer's instructions. cDNA was made with random primers and SuperScript IV VIO reverse transcriptase (Thermo Fisher Scientific). qPCR was performed using a Viia7 Real-Time PCR System (Thermo Fisher Scientific). Primers are listed in Table S2.

ACCESSION NUMBERS

The RNA-seq and ChIP-seq are available at GEO with accession number GEO: GSE121288.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.05.027.

AUTHOR CONTRIBUTIONS

T.H., A.K., and V.L. performed most experiments. B.D. performed and analyzed CT scanning results under the supervision of B.H.L. K.A.H analyzed high-throughput sequencing data. T.H. and D.N. designed the experiments, analyzed the results, and wrote the manuscript.

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