Complementary and Independent Function for *Hoxb4* and *Bmi1* in HSC Activity

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Determinants regulating short- and long-term repopulating hematopoietic stem cell (STR-HSC and LTR-HSC) self-renewal remain largely uncharacterized. To gain further insights into HSC self-renewal, we investigated possible genetic interactions between two well-recognized regulators of this process: *Bmi1* and *Hoxb4*. Using complementation and overexpression strategies in mouse HSCs, we document that *Bmi1* is not required for the in vivo expansion of fetal HSCs but is essential for the long-term maintenance of adult HSCs. Importantly, we show that *Hoxb4* overexpression induces an expansion of *Bmi1*^{−/−} STR-HSCs leading to a rescue of their repopulation defect. In contrast to *Hoxb4*, we also show that *Bmi1* fails to induce HSC expansion ex vivo. Consistent with these results, we report high levels of Angptl3 and Cbx7 in *Hoxb4*- and *Bmi1*-transduced cells, respectively. Together, these results support the emerging concept that fate and sustainability of this fate are two critical components of self-renewal in adult stem cells such as HSCs.

Self-renewal implies the coordination between cell division and fate such that for at least one progeny, cell differentiation, proliferative senescence, and apoptosis are excluded. In contrast to mouse embryonic stem cells that undergo symmetrical self-renewal divisions in response to leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) signaling, most adult stem cells have the capacity to undergo two distinct types of self-renewal divisions: symmetrical and asymmetrical (Shen et al. 2002). In the hematopoietic system, HSCs undergo at least two rounds of mobilization that largely overlap with changes in proliferative status: Indeed, most HSCs are in cycle and symmetrically self-renew (thus expand) at a specific time in the mouse fetal liver (FL) and they then switch to a more quiescent program later in the bone marrow environment where the population is maintained for the entire life of the animal (no expansion, but maintenance of the population). Whether this maintenance phase is established by a balance between symmetrical self-renewal and stem cell loss, by asymmetric self-renewal division, or a combination of these processes remains unclear. In adult mice, HSC symmetrical self-renewal divisions can be experimentally triggered (reactivation of an embryonic program?) under specific conditions such as during the early phase following HSC transplantation (Pawliuk et al. 1996; Antonchuk et al. 2001), and this can be dramatically enhanced upon the engineered overexpression of the *Hoxb4* transcription factor or other homeobox genes (Sauvageau et al. 1995; Thorsteinsdottir et al. 1999, 2002; Antonchuk et al. 2001, 2002; Krosi et al. 2003; Cellot et al. 2007; Ohta et al. 2007).

Recent studies have documented that at least two different stem cell populations with more or less equal dif-

ferentiation potential can be prospectively isolated. These include the long-term repopulating hematopoietic stem cells (LTR-HSCs) that supply hematopoiesis for the life span of the animal and the short-term repopulating HSCs (STR-HSCs) that contribute to repopulation for up to 3 months after transplantation (Morrison et al. 1997; Christensen and Weissman 2001; Wognum et al. 2003). Until recently, however, this property of short-term versus long-term repopulation was limited to phenotypically defined populations, but new studies have validated, at the single-cell level, the concept that the HSC compartment is highly heterogeneous and includes STR-HSCs and LTR-HSCs (Dykstra et al. 2007; N.N. Iscove, pers. comm.). In these studies, single STR-HSCs could provide multilineage reconstitution for up to 3–4 months post-transplantation, whereas LTR-HSCs contributed to life-long repopulation. This level of blood cell production, estimated at above 10¹² progeny per STR-HSC, argues that these cells can extensively self-renew, thus introducing the notion (of N.N. Iscove) that it is the ability to sustain self-renewal rather than self-renewal itself that distinguishes LTR-HSCs from STR-HSCs.

Together, these data suggest the existence of two mandatory components that define self-renewal (SR) in LTR-HSCs: one that coordinates fate with cell division (referred to as “execution of SR”) and one that ensures the sustainability of this execution through cell divisions (referred to as “sustainability of SR”). Several observations suggest that although *Hoxb4* overexpression enhances the execution of SR in defined conditions, the Polycomb group (PcG) gene *Bmi1* may be critical for regulating the sustainability of this process. Indeed, by phenotypic analysis, *Bmi1*^{−/−} HSCs are present in normal

numbers in FL, but they progressively decrease from embryonic to adult life (Park et al. 2003; Iwama et al. 2004). In addition, although *Bmi1* homozygous null HSCs fail to reconstitute myeloablated recipients for periods extending for more than a few weeks, this defect is complemented by the experimental reintroduction of *Bmi1* in these cells, thus indicating the independence on *Bmi1* for the generation of FL LTR-HSCs. Moreover, a single *Hoxa9* + *Meis1* leukemia-initiating cell that lacks *Bmi1* expression expands sufficiently to induce lethality in mice, but this expansion is nevertheless limited in time (Lessard and Sauvageau 2003; Park et al. 2003; Iwama et al. 2004). Finally, *Bmi1* is not critical for stem cell homing and does not appear to regulate apoptosis (Park et al. 2003; Iwama et al. 2004). With this in mind, we now report a series of experiments suggesting that *Bmi1* ensures the sustainability of SR in LTR-HSCs and, in contrast to *Hoxb4*, fails to enhance the execution of SR under comparable experimental conditions.

***Bmi1* IS REQUIRED FOR ACTIVITY OF ADULT HSC**

Bmi1 homozygous null fetal liver cells (FLCs) were infected using retroviral vectors (Fig. 1a) and manipulated to assess *Bmi1* function on progenitor and stem cell activities (Fig. 1b). Gene transfer efficiencies for all experiments were between 14% and 100% (typically between 60% and 100%). As previously reported, progenitor activity is markedly reduced in *Bmi1*^{-/-} FLCs kept in short-term culture (Fig. 1c). These cells also lack in vivo reconstitution ability (Fig. 1d) (Lessard and Sauvageau 2003). Retroviral transduction of *Bmi1* in *Bmi1*^{-/-} FLCs rescues both their progenitor and in vivo reconstitution activities (Fig. 1c,d). Moreover, complemented *Bmi1*^{-/-} cells (referred to hereafter as *Bmi1*^{-/-}*Bmi1* cells) have the capacity to differentiate into myeloid (Gr-1⁺ and CD11b⁺) and lymphoid (CD45R⁺ and CD4⁺) lineages in vivo (Fig. 1e), and they can provide hematopoietic reconstitution when transferred into secondary recipients (data not shown). Because hematopoietic reconstitution is observed when wild-type HSCs are transplanted into *Bmi1*^{-/-} mice (Park et al. 2003; Iwama et al. 2004), these results support a cell-autonomous dysfunction for *Bmi1* homozygous null HSC.

To further investigate the requirement for *Bmi1* in adult HSC activity, RNA interference (RNAi) studies were conducted using an approach that we previously validated (Chagraoui et al. 2006). *shBmi1*-transduced progenitor cells were significantly compromised in their ex vivo response to hematopoietic growth factors when compared to control cells (Fig. 1f). Interestingly, multipotent progenitors (i.e., CFU-GEMM) were most sensitive to the acute reduction in *Bmi1* levels (Fig. 1g). Consistent with these data, *shBmi1*-transduced cells were impaired in their in vivo reconstitution activity with only one mouse reconstituted in both the myeloid and the lymphoid compartments 16 weeks post-stem-cell transfer (SCT) (Fig. 1h, right panel, solid dot).

Together with previous reports confirming that *Bmi1*^{-/-} HSCs are not impaired in their survival and homing potential (Park et al. 2003; Iwama et al. 2004), these results indi-

cate that *Bmi1* is not required for HSC specification but is essential for their long-term maintenance in vivo.

***Bmi1*^{-/-} FL HSCS UNDERGO SR DIVISIONS**

Taking into consideration that mouse HSC populations rapidly expand between embryonic days 10 (E10) and 14.5 (E14.5) (Rebel et al. 1996), experiments were designed to test whether *Bmi1* is required for HSC expansion (i.e., symmetrical self-renewal divisions). In these assays, freshly isolated FLCs were infected with a *Bmi1* retrovirus before their transplantation at limiting dilution into lethally irradiated hosts, and stem cell frequencies were evaluated using the CRU (competitive repopulating unit) assay (Szilvassy et al. 1990). Results from these experiments indicated that HSC frequencies (Fig. 1i,j) and absolute numbers (data not shown) are similar in *Bmi1*^{-/-} or *Bmi1*^{+/+} fetal livers, suggesting that although essential for HSC maintenance, *Bmi1* is not required for embryonic HSC expansion and therefore fetal-liver-derived HSCs can symmetrically self-renew in the absence of this gene.

***Bmi1* OVEREXPRESSION DOES NOT INDUCE HSC EXPANSION**

We next investigated whether *Bmi1* overexpression might trigger HSC expansion and regulate *Hoxb4* expression levels. For these experiments, bone marrow cells (BMCs) were transduced using control, *Hoxb4*, *Bmi1*, or both *Hoxb4* + *Bmi1* retroviruses (Figs. 1a and 2a). Western blot and quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR) analyses of transduced cells showed that levels of *Hoxb4* are not affected in *Bmi1*-transduced cells and similarly that *Bmi1* is not regulated by *Hoxb4* (Fig. 2b and data not shown).

When compared to controls, *Hoxb4*-transduced KLS (c-Kit⁺Lineage⁻Sca-1⁺) cells showed a modest expansion during a 7-day culture period (Fig. 2c). In contrast, expansion of *Bmi1*-transduced KLS cells was significantly higher (Fig. 2c). Accordingly, cell cycle distribution studies indicated a greater proportion of KLS cells in S phase in cultures initiated with *Bmi1*-transduced cells than in green fluorescent protein (GFP) control cultures (data not shown). In agreement with previous findings, both *Hoxb4* and *Bmi1* significantly enhanced the generation of myeloid progenitors in culture compared to controls (Fig. 2d) (Sauvageau et al. 1995; Iwama et al. 2004).

The effect of *Bmi1* overexpression on in vivo hematopoietic cell reconstitution and ex vivo HSC expansion was evaluated using the CRU assay. In contrast to results obtained within the same experiment with *Hoxb4*, *Bmi1*-overexpressing LTR-HSCs did not expand ex vivo and reconstituted primary recipients similarly to control cells (Fig. 2e,f). These experiments were also conducted with cells derived from E14.5 FL and likewise, *Bmi1* overexpression had no effect on LTR-HSC expansion (data not shown). Importantly, control and *Bmi1*-transduced HSCs showed similar expansion and differentiation properties, thus indicating that *Bmi1* overexpression is not detrimental for stem cell activity (Fig. 2f,g). Interestingly, *Bmi1* over-

Figure 1. *Bmi1* is required for hematopoietic stem cell function. (a) Retroviral constructs used in this study. (Sequences are available upon request to the author.) (b) Experimental outline. *Bmi1*^{+/+}, *Bmi1*^{+/-}, or *Bmi1*^{-/-} E14.5 fetal liver cells (FLCs) were transduced with a control, *Bmi1*, or *ShBmi1* retroviral vector. Stem cell transplantation (SCT) and colony-forming cell (CFC) assays were performed as described previously (Chagraoui et al. 2006). (c) CFC assays were performed 7 days postinfection ($n = 60$ colonies analyzed per condition) (mean $\pm$ s.d.). (d) Transduced *Bmi1*^{-/-} FLCs (3.3×10^6 cells) were transplanted ($n = 3$ mice per condition), and a proportion of donor-derived (Ly5.2⁺) peripheral blood cells was assessed 12 weeks later (mean $\pm$ s.d.). (e) Fluorescence-activated cell sorting (FACS) profiles of hematopoietic tissues from a representative *Bmi1*^{-/-}*Bmi1* chimeric mouse to determine myeloid (Gr-1, CD11b) and lymphoid (CD45R and CD4) reconstitution by donor cells ($n = 3$ mice were analyzed). (f) Expansion of CFCs during a series of 7-day ex vivo cultures (compared CFC numbers were determined at D0) (mean $\pm$ s.d.). (g) Relative distribution of CFC subtypes following a 7-day ex vivo culture. (h) Donor-derived reconstitution of recipients of FLCs transduced with control versus *shBmi1* virus, as indicated in a. Reconstitution was performed with a cell dose of 10^7 (highest dilution) to 40,000 (lowest dilution) cells per mouse. $n = 10$ mice per dilution. Each dot represents a mouse (solid vs. open dots: presence vs. absence of donor-derived lympho-myeloid reconstitution). Similar results were confirmed with bone marrow cells (BMCs) (data not shown). (i) Transduced FLCs were transplanted ($n = 10$ mice per condition) at limiting dilution (from 10^6 to 20,000 cells) and peripheral blood cell reconstitution was assessed as in h. (j) HSC frequencies were evaluated using reconstitution results presented in i (error bars indicate a 95% confidence interval). Results (i and j) were confirmed in two independent experiments. (BFU-E) Burst-forming-unit erythroid; (BM) bone marrow; (CFU-GEMM) colony-forming-unit granulocyte/erythroid/monocyte/megakaryocyte; (L) large (CFU-G/M/GM colonies with >50 cells); (S) small (CFU-G/M/GM colonies with <50 cells); (Sp) spleen; (Th) thymus.

Figure 2. *Bmi1* overexpression is not associated with ex vivo stem cell expansion. (a) Experimental outline. 5-Fluorouracil-treated bone marrow cells (5-FU BMCs) were transduced with control, *Hoxb4*, *Bmi1*, or *Hoxb4 + Bmi1* retroviral vectors. CFC ($n = 50$ – 100 colonies analyzed per condition) and stem cell transplantation ($n = 10$ mice per condition) assays were performed on days 0, 7, and 21. (b) Western blot analysis of BMCs transduced with control, *Hoxb4*, *Bmi1*, or *Hoxb4 + Bmi1* retroviral vectors and immunoblotted with *Hoxb4*, *Bmi1*, or ribosomal protein S6 (loading) antibodies. (c) Expansion of c-Kit⁺ Lineage⁺ Sca-1⁺ (KLS) cells during a 10-day culture period ($n = 3$ experiments) (mean ± S.D.). (d) CFC expansion during 7 (black) or 21 days (gray) of ex vivo cultures (compared to D0 control CFC numbers) (mean ± S.D.). (e) Transduced BMCs were transplanted at limiting dilution (from 10⁶ to 500 cells), and reconstitution was assessed 16 weeks later. (Upper, middle, lower panels) Experiments performed with cells isolated at days 0, 7, and 21 after initiation of the cultures, respectively (see also a). Cell dose for the experiments is represented in the upper panel: 10⁶ to 500 cells. (Middle, lower panels) All cultures were seeded with 10⁶ cells and recipients were transplanted with a range of 200,000 to 20 (D7) or 2,000 to 20 (D21) cells. Results for D0 and D7 were confirmed in three independent experiments and also with FLCs (data not shown). (f) Seven-day (black) and 21-day (gray) ex vivo HSC expansions (in comparison to D0 control HSC numbers) were evaluated using reconstitution of recipients shown in *e* (≥95% confidence interval). (g) FACS profiles of hematopoietic tissues from selected recipients of GFP (control) versus *Bmi1*-transduced cells from *f*. Abbreviations are the same as those in Fig. 1.

expression conferred a small proliferative advantage to repopulating cells (i.e., higher reconstitution level per stem cell transplanted) (Fig. 2e, compare middle [*Bmi1*] and first [control] panels).

***Bmi1* IMPAIRS HOXB4-INDUCED HSC EXPANSION**

As shown in the previous sections, *Bmi1* overexpression enhances proliferation of phenotypically primitive KLS hematopoietic cells, whereas *Hoxb4* mostly triggers HSC self-renewal divisions. On the basis of these results, we inferred that these two genes may display potent genetic interaction leading to enhanced HSC expansion (e.g., enhanced proliferation [*Bmi1*] of self-renewing [*Hoxb4*] HSCs). To verify this hypothesis, control, *Hoxb4*, and *Hoxb4* + *Bmi1* BMCs were kept in culture for up to 21 days and progenitor/stem cell contents were evaluated at various time points (Fig. 2a). As shown in Figure 2d, *Hoxb4* progenitor activity was not enhanced by the engineered cooverexpression of *Bmi1* after 7 or 21 days of culture. Importantly, expanded BMCs generated a large proportion of clones that expressed both *Hoxb4* and *Bmi1*, documenting the absence of negative interaction between these two genes in progenitors (data not shown).

In vivo reconstitution experiments performed with these same ex vivo expanded cells revealed that *Hoxb4*-induced HSC expansion (e.g., 400-fold at 21 days; Fig. 2f) is abrogated by the cooverexpression of *Bmi1* (Fig. 2f). Importantly, the loss of HSC activity in cultures initiated with doubly transduced cells is not due to nonspecific toxicity of *Bmi1* on HSC, because *Hoxb4* + *Bmi1* doubly transduced HSC are as competitive as *Hoxb4* cells in vivo when transplanted immediately after gene transfer (Fig. 2e, upper panel). Thus, ex vivo, where the HSC-expanding activity of *Hoxb4* is most prominent (Antonchuk et al. 2002), *Bmi1* dominantly interferes with *Hoxb4* activity while enhancing proliferation. As demonstrated using Annexin V staining, this effect was not consequential to a modulation of apoptosis, which was minimal in the KLS subfraction of all cultures analyzed (data not shown).

***Bmi1*^{-/-} STR-HSC FUNCTION IS RESCUED BY HOXB4**

In an attempt to further investigate the genetic interaction between *Hoxb4* and *Bmi1*, experiments were performed to assess the possibility that *Hoxb4* might complement the *Bmi1*^{-/-} progenitor and stem cell defects (Fig. 3a). As shown in Figure 3b and c, *Bmi1*^{-/-} progenitor numbers, differentiation potential, and proliferative activity are fully rescued by the engineered overexpression of *Hoxb4*.

In vivo experiments further revealed that *Hoxb4* also complements the short-term hematopoietic reconstitution defects of *Bmi1*^{-/-} cells to levels similar to those obtained for *Bmi1*^{-/-}*Bmi1* cells (Fig. 3d, upper panel). Confirmation that reconstitution was derived from *Hoxb4*-transduced *Bmi1*^{-/-} cells was obtained by PCR-based analyses of peripheral blood samples isolated from several indepen-

dent mice (Fig. 3d, middle [250-bp band] and lower panels) and from protein analysis of a representative specimen (Fig. 3e). Importantly, *Bmi1*^{-/-}*Hoxb4* cells were competent in myeloid (Gr-1⁺), B lymphoid (CD45R⁺), and T lymphoid (CD8a⁺) differentiation in vivo (Fig. 3f).

Interestingly, the reconstitution potential of *Bmi1*^{-/-}*Hoxb4* cells progressively decreased, such that by 12 weeks posttransplantation, it became almost undetectable (Fig. 3g). The rare donor-derived cells that persisted at 12 weeks in recipients of *Bmi1*^{-/-}*Hoxb4* grafts were derived from myeloid (CD11b⁺) and lymphoid (CD45R⁺ and CD4⁺CD8a⁺) lineages (Fig. 3h). As might be expected from the results presented above, *Bmi1*^{-/-} cells transduced with both *Hoxb4* and *Bmi1* persistently reconstituted all mice analyzed at 12 weeks post-SCT (Fig. 3g), and evaluation of the stem cell content in these grafts reveals that HSCs had expanded 20–35 times more than control HSCs (i.e., *Bmi1*^{-/-}*Bmi1*) (data not shown). Together, these results suggest that *Bmi1*^{-/-} FL-derived HSCs were complemented by *Hoxb4* overexpression for reconstitution lasting up to 8–12 weeks. However, *Bmi1* is required to further sustain long-term stem cell activity (i.e., >12 weeks).

HOXB4 TRIGGERS EXPANSION OF *Bmi1*^{-/-} HSCS

A series of additional experiments were conducted to further understand the nature of *Hoxb4*-induced rescue of *Bmi1*^{-/-} repopulating cells. We specifically designed these experiments to determine whether *Hoxb4* influenced the expansion (self-renewal) or the proliferative output of these *Bmi1*^{-/-} cells (Fig. 4a). These experiments first showed that *Hoxb4* overexpression induced a significant expansion of Lin⁻CD48⁻CD150⁺ populations in cultures initiated with *Bmi1*^{-/-} FLCs to levels similar to those determined in cultures initiated with wild-type cells (Fig. 4b and data not shown). Furthermore, and consistent with results reported by Iwama et al. (2004), Lin⁻CD48⁻CD150⁻ wild-type and *Bmi1*^{-/-} cells, lacking long-term reconstitution potential (Kiel et al. 2005; Kim et al. 2006), were poorly responsive to *Hoxb4* overexpression (Fig. 4c).

The ex vivo expansion of *Hoxb4*-*Bmi1*^{-/-} repopulating cells was assessed at an early time point (i.e., 4 weeks) using the CRU assay as described originally (Szilvassy et al. 1990). Results from these experiments showed that *Hoxb4* overexpression similarly triggered the net expansion of short-term repopulating (STR-HSC) cells from both genotypes (*Bmi1*^{-/-} and *Bmi1*^{+/+}) to values representing 2.5- to 3.9-fold, whereas untransduced STR-HSCs decreased by approximately 10-fold in these cultures (Fig. 4d). Thus, independent of *Bmi1* genotype, cultures initiated with *Hoxb4*-transduced cells contained between 25 and 39 times more STR-HSCs than the control (Fig. 4d). Importantly, these cells maintained lymphoid and myeloid differentiation capacity but lacked the potential for long-term reconstitution (i.e., >12 weeks).

Confirmation of the lympho-myeloid potency of individual STR-HSCs was obtained for at least two clones where identical proviral integration profiles were identified in sorted myeloid (Gr-1⁺) and lymphoid (CD45R⁺)

Figure 3. *Hoxb4* overexpression rescues *Bmi1*^{-/-} STR-HSCs. (a) Experimental outline. *Bmi1*^{-/-} and *Bmi1*^{+/-} FLCs were transduced with control, *Bmi1*, *Hoxb4*, or *Hoxb4* + *Bmi1* retroviral vectors. CFC and SCT assays were performed at the indicated time points. (b,c) Transduced *Bmi1*^{-/-} FLCs were kept in culture for 7 days and plated in methylcellulose-containing medium for 7 days for CFC evaluation as described by Chagraoui et al. (2006). Pictures were taken at day 7 after initiation of the methylcellulose cultures (n = 60 colonies analyzed per condition). (b, Lower panel) Genomic DNA was isolated from individual colonies picked from methylcellulose cultures initiated with *Hoxb4*-transduced *Bmi1*^{-/-} (or *Bmi1*^{-/-}*Hoxb4*) cells and subjected to PCR analysis to confirm that *Hoxb4*-rescued clones were of *Bmi1*^{-/-} genotype (indicated by the ~250-bp fragment). (d, Upper panel) Transduced *Bmi1*^{-/-} and *Bmi1*^{+/-} FLCs were transplanted (3.3 $\times$ 10⁶ cells per mice, n = 3), as indicated in a, and donor-derived reconstitution was evaluated 4 weeks later (mean $\pm$ S.D.). (d, Middle panel) Same analysis as detailed in b (lower panel) except that DNA was extracted from peripheral blood isolated from recipients 178–186 at 4 weeks posttransplantation. (d, Lower panel) Detection of *Hoxb4* provirus by PCR in the same DNA. (e) Western blot analysis of protein extracts from sorted (Ly5.2⁺) BMCs isolated from recipients of wild-type control or *Bmi1*^{-/-}*Hoxb4* cells. Membranes were immunoblotted with antibodies that specifically recognize *Hoxb4* or Tubulin β (loading). (f) Representative FACS profiles of hematopoietic tissues from a selected recipient of *Bmi1*^{-/-}*Hoxb4*-transduced cells. Analysis was performed using cells isolated at 4 weeks posttransplantation (n = 3 mice analyzed). (g) Time course showing donor-derived reconstitution (mean $\pm$ S.D.) of several recipients of *Bmi1* heterozygous or homozygous null FLCs transduced with the indicated retroviruses. (Asterisks) Results shown at 4 weeks are derived from d. (h) The presence of *Hoxb4*-integrated provirus in the DNA of BMCs isolated from *Bmi1*^{-/-}*Hoxb4* chimeric mice at 12 weeks posttransplantation. BMCs were sorted for myeloid (CD11b⁺), B-lymphoid (CD45R⁺), and T-lymphoid (CD4⁺CD8a⁺) populations, and amplification of the *Hoxb4* provirus was performed by PCR (n = 3 mice analyzed, one representative shown). Total BMCs were used as a negative control. Results were confirmed in three independent experiments.

Figure 4. *Hoxb4* induces expansion of STR-HSCs in the absence of *Bmi1*. (a) Experimental outline. *Bmi1*^{+/+} and *Bmi1*^{-/-} FLCs were transduced with control; *Bmi1* or *Hoxb4* retroviral vectors and cells were analyzed as indicated. (b,c) Proportion of Lin⁻CD48⁻CD150⁺ and of Lin⁻CD48⁻CD150⁺ cells in cultures initiated with the indicated cell populations. FACS analyses were performed 10 days after the cultures were initiated (mean $\pm$ S.D.). (d) Determination of STR-HSC net expansion during a 10-day culture as described in a–c. STR-HSC determination was performed using the CRU assay with a minimum of ten recipients per dilution. Assessment of donor-derived reconstitution was performed at 4 weeks posttransplantation, hence the definition of STR-HSC in these experiments (error bar indicates a 95% confidence interval). (e) Southern blot analysis of genomic DNA isolated from *Hoxb4*-transduced *Bmi1*^{-/-} BMCs and sorted based on the expression of Gr-1⁺ (myeloid) or CD45R⁺ (B-lymphoid) surface markers. Note that cells were collected at 4 weeks and 9 weeks posttransplantation for mouse 896 and mice 897–900, respectively. The DNA was digested with EcoRI, which cuts once in the integrated proviruses, and membranes were probed with a radiolabeled *neomycin* resistance gene fragment. Each fragment documents a unique insertion site for the *Hoxb4* provirus in the sorted populations. (f) Q-RT-PCR analyses of *Hoxb4* (left upper panel), *Bmi1* (left lower panel), *Angptl3* (right upper panel), and *Cbx7* (right lower panel) expression in sorted Lin⁻CD48⁻CD150⁺ *Bmi1*^{+/+} and *Bmi1*^{-/-} cells engineered to overexpress GFP (control) or *Hoxb4*-GFP or *Bmi1*-GFP. Results are presented as levels relative to those derived from GFP-transduced *Bmi1*^{+/+} cells. Quality of RNA was evaluated using *Gapdh* primer sets. Primer sequences available upon request.

populations isolated from selected recipients 9 weeks post-SCT (Fig. 4e, mice 897 and 900). Further analysis of proviral integrations in bone-marrow-derived cells from these recipients revealed that at least six *Hoxb4*-transduced clones contributed to repopulation (see minimal clone numbers indicated at the bottom of Fig. 4e).

Most importantly, recipients 899 and 900 were repopulated by identical retrovirally marked clones, a typical signature for SR that occurred during the 10-day culture period before transplantation (Fig. 4e). The *Bmi1*^{-/-} cell type (HSC vs. progenitor) that underwent SR as a result of *Hoxb4* overexpression is undefined. However, three arguments suggest that this cell type was an HSC. First, *Hoxb4* overexpression fails to recruit non-HSCs (progenitors) into this compartment (Park et al. 2003; Iwama et al. 2004). Second, the results presented in Figure 1i and j indicate that LTR-HSCs are present in normal numbers in *Bmi1*^{-/-} fetal livers. Third, complementation experiments summarized in Figure 1j indicate that *Bmi1*^{-/-} LTR-HSCs can expand normally in vivo during embryonic development.

DIFFERENT PATHWAYS ARE REGULATED BY *Bmi1* AND *Hoxb4*

The different impact of *Hoxb4* and *Bmi1* on HSC activity suggests that they affect distinct gene subsets. To gain insight into this possibility, we performed Q-RT-PCR studies on sorted Lin⁻CD48⁻CD150⁺ cells engineered to overexpress *Hoxb4* or *Bmi1* and compared the expression of approximately 110 candidate gene targets in these cells. These genes included *Hox* members and cofactors, Polycomb group genes, and other stem cell regulators such as *Etv6* (Wang et al. 1998), *Gfi1* (Hock et al. 2004; Zeng et al. 2004), and *Lnk* (Ema et al. 2005). The expression of most of these genes was unaffected by *Hoxb4* or *Bmi1* (Table 1). However, we found that *Angptl3* (but not *Angptl2*) expression was strongly induced by *Hoxb4* in HSC-enriched cells and that this regulation was observed

in wild-type and *Bmi1*^{-/-} cells (Fig. 4f). Conversely, the Polycomb group gene *Cbx7* is down-regulated by *Bmi1* and not by *Hoxb4* (Fig. 4f).

Bmi1 INTEGRATES MULTIPLE PATHWAYS IN THE REGULATION OF HSC ACTIVITY

Hoxb4-induced complementation of *Bmi1*^{-/-} repopulating cells is reminiscent of that recently described by our group for the loss of function of *E4F1* (Chagraoui et al. 2006) and by other groups for *arf/ink4a* and *p53* (Jacobs et al. 1999; Bruggeman et al. 2005; Molofsky et al. 2006; Oguro et al. 2006; Akala et al. 2008). In fact, besides the recent results with *p53* (Akala et al. 2008), it remains challenging to identify a single gene whose gain or loss of function can fully rescue *Bmi1*^{-/-} LTR-HSC activity. The recent identification of the *arf/ink4a* locus as a direct target for *Bmi1*, together with its direct interaction with *E4F1*, may suggest that both pathways are simultaneously regulated in self-renewing HSCs. Furthermore, the essential role for *Bmi1* as a cofactor to Ring1/2 ubiquitin E3 ligase in possibly promoting monoubiquitination of H2A (K119) suggests that other regulators may be involved (Fig. 5a).

CONCLUSIONS

Because HSC expansion occurs strictly as a result of symmetrical self-renewal divisions, we conclude that *Bmi1* is not essential for this function (at least before E14.5 in vivo and likely as a result of *Hoxb4* overexpression in vitro). This provides evidence to propose that execution and sustainability of SR are independently regulated in HSCs and that although the first appears to be regulated by *Hox* genes and their targets, the former is under the control of *Bmi1* (Fig. 5b). On the basis of this, it is tempting to speculate that in the absence of *Bmi1*, LTR-HSCs become functionally equivalent to STR-HSCs.

Table 1. Genes not regulated by *Hoxb4* and *Bmi1* overexpression in Lin⁻CD48⁻CD150⁺ cells

<i>Acin1</i>	<i>Egr1</i>	<i>Hoxa9</i>	<i>Nfat5</i>	<i>Rnf2</i>
<i>Angptl2</i>	<i>Epc2</i>	<i>Hoxb1</i>	<i>Notch1</i>	<i>Runx1</i>
<i>Asxl1</i>	<i>Etv6</i>	<i>Hoxb13</i>	<i>Numb</i>	<i>Runx2</i>
<i>Asxl2</i>	<i>Exh1</i>	<i>Hoxb2</i>	<i>Pard3</i>	<i>Scmh1</i>
<i>Atf4</i>	<i>Ezh2</i>	<i>Hoxb3</i>	<i>Pard6a</i>	<i>Scml2</i>
<i>Baz1b</i>	<i>Foxo3a</i>	<i>Hoxb5</i>	<i>Pard6b</i>	<i>Sf1</i>
<i>Bmp2</i>	<i>Gata2</i>	<i>Hoxb6</i>	<i>Pcgf2 (Mel18)</i>	<i>Sfp1 (Pu.1)</i>
<i>Bmp4</i>	<i>Gfi1</i>	<i>Hoxb7</i>	<i>Pcgf6</i>	<i>Sh2b3 (Lnk)</i>
<i>Bmp7</i>	<i>Gli1</i>	<i>Hoxb8</i>	<i>Phc1 (Rae-28)</i>	<i>Smarcc1</i>
<i>Bteb1</i>	<i>Gli2</i>	<i>Hoxb9</i>	<i>Phc2</i>	<i>Stat3</i>
<i>Casp8ap2</i>	<i>Gli3</i>	<i>Irx5</i>	<i>Phc3</i>	<i>Stat5a</i>
<i>Cbfb</i>	<i>H3f3b</i>	<i>L3mbtl1</i>	<i>Phf1</i>	<i>Suz12</i>
<i>Cbx2</i>	<i>Hoxa1</i>	<i>L3mbtl2</i>	<i>Pknox1 (Prep1)</i>	<i>Tal1</i>
<i>Cbx4</i>	<i>Hoxa10</i>	<i>L3mbtl3</i>	<i>Pknox2 (Prep2)</i>	<i>Tcf4</i>
<i>Cbx6</i>	<i>Hoxa11</i>	<i>Lef1</i>	<i>Prdm16</i>	<i>Tcf2a (E2a)</i>
<i>Cbx8</i>	<i>Hoxa13</i>	<i>Lmo2</i>	<i>Prox1</i>	<i>Tgif</i>
<i>Cebpa</i>	<i>Hoxa2</i>	<i>Mef2c</i>	<i>Pten</i>	<i>Trim2</i>
<i>Crebbp</i>	<i>Hoxa3</i>	<i>Meis1</i>	<i>Rap1a</i>	<i>Trim28</i>
<i>Crem</i>	<i>Hoxa4</i>	<i>Mrg1 (Meis2)</i>	<i>Rarb</i>	<i>Xbp1</i>
<i>Dnmt3b</i>	<i>Hoxa5</i>	<i>Mrg2 (Meis3)</i>	<i>Rela</i>	
<i>Eed</i>	<i>Hoxa6</i>	<i>Myb</i>	<i>Rest</i>	
<i>Egfr</i>	<i>Hoxa7</i>	<i>Ncl</i>	<i>ring1</i>

Figure 5. Complementary and independent function for *Hoxb4* and *Bmi1* in HSC activity.

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