Conditional c-*myb* knockout in adult hematopoietic stem cells leads to loss of self-renewal due to impaired proliferation and accelerated differentiation

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Hematopoietic stem cells (HSCs) have a unique capacity to undergo self-renewal and multi-lineage differentiation to provide a lifetime supply of mature blood cells. By using conditional knockout technology, we disrupted the c-*myb* proto-oncogene specifically in adult bone marrow (BM) to demonstrate that this transcription factor is a regulator of self-renewal and multi-lineage differentiation of adult HSCs. Targeted disruption of the c-*myb* gene resulted in a critical depletion of the HSC pool. In addition, BM hematopoiesis in adult mice was impaired, resulting in profound reductions of various hematopoietic lineages including neutrophilic, monocytic, B lymphoid, erythroid, and, unexpectedly, megakaryocytic cells. Serial BM transplantation into lethally irradiated recipient mice indicated an essential role for c-*myb* in the self-renewal process. Furthermore, in vitro functional assays demonstrated that deletion of the c-*myb* gene leads to a slightly reduced proliferative capacity and an aberrant and accelerated differentiation of HSCs. In addition to long-term HSCs, functional studies also show that c-*myb* plays a critical role in short-term HSCs and multi-potential progenitors. Collectively, our data indicate a critical role for c-*myb* in adult BM hematopoiesis and in self-renewal and multi-lineage differentiation of adult HSCs.

hematopoiesis | proto-oncogene | development | bone marrow

Hematopoiesis is the development of mature blood cells through the ordered regulation of gene expression. This tightly controlled process originates with the pluripotent long-term (LT) hematopoietic stem cells (HSCs) that have lifelong capacity to undergo multi-lineage differentiation to produce the entire gamut of terminally differentiated blood cells. Crucial to this entire process is the self-renewal capacity of HSCs, which endow them with the ability for self-maintenance and expansion. In addition, LT-HSCs give rise to short-term (ST) HSCs, which have limited self-renewal capacity and can commit further to generate multi-potential progenitors (MPPs). MPPs have completely lost the capacity for self-renewal. However, similar to LT-HSCs and ST-HSCs, MPPs have the potential to undergo multi-lineage differentiation (1).

The proto-oncogene c-*myb* is the founding member of the *myb* gene family, which also includes A-*myb* and B-*myb*. c-*myb* was first identified as the cellular counterpart of the transforming v-*myb* gene carried by the AMV and E26 retroviruses, both of which induce leukemias in chickens (2). The importance of the c-Myb transcription factor can be discerned by the fact that homozygous null mice died at embryonic d 15 during development as a result of a failure to transition from fetal to adult erythropoiesis (3). Hence, most of what is known about the gene comes from cell lines, which implicate a role for c-*myb* in cell proliferation, survival, and/or differentiation (4).

To circumvent the embryonic lethality due to c-*myb* nullizygosity, several mutant mouse models were generated to examine the role of c-*myb* in adult lymphoid development (5–10) and adult bone marrow (BM) hematopoiesis and HSCs, including a knockdown allele, wherein c-Myb expression was reduced to approximately 10% of control, and a mutation in the trans-activation domain M303V, which hindered binding of c-*myb* to p300 (11, 12). However, both of these models were found to have limitations. For instance, both the knockdown and M303V mutations do not specifically target adult HSCs; and therefore, the phenotypes reported could be carried over from fetal HSCs. Furthermore, the fate of adult HSCs when c-*myb* is disrupted remains unknown. By using conditional knockout technology to target the disruption of the c-*myb* gene specifically in adult BM tissue, we provide a model that is in striking contrast to the knockdown and M303V models.

To determine whether c-*myb* has a role in adult hematopoiesis and HSCs, we crossed our c-*myb* floxed mice ($myb^{f/f}$) (9) with the inducible MxCre mice (13), whereby induction of gene deletion can be achieved upon administration of IFN or synthetic double-stranded RNA polyinosinic-polycytidylic acid (pIpC) to mice. Here, we demonstrated that conditional disruption of the c-*myb* gene specifically in adult HSCs leads to a depleted HSC pool, an abolishment of self-renewal, a slightly reduced proliferative capacity, and a complete loss of colony growth and multi-lineage differentiation on methylcellulose. In addition, we showed that c-*myb* is required for the development of diverse BM lineages in the adult mice, including granulocytic, erythroid, monocytic, B lymphoid, and megakaryocytic lineages.

Our results from the conditional targeting c-*myb* mice are very different from those of the knockdown and the M303V models. Total BM cells from the knockdown and M303V mutant mice were able to successfully repopulate lethally irradiated recipient hosts (11, 12), indicating that both mutations did not affect self-renewal. In contrast, total BM cells from the deleted c-*myb* floxed mice could not repopulate lethally irradiated recipient mice. In fact, the M303V mutant animals had 10-fold more HSCs than the control mice (11, 12). In the knockdown mice, the absolute number of $LKS^{+}Flt3^{-}$ cells, representing the LT-HSCs, was unchanged compared with the control mice (11, 12). In stark contrast, LT-HSCs in our model were dramatically diminished when the c-*myb* gene is disrupted. In addition, both the M303V and the knockdown animals had HSCs with increased proliferative capacity (11, 12). In contrast, disruption of the c-*myb* gene in our model leads to a slightly decreased proliferation of HSCs. Moreover, whereas HSCs from the knockdown mice could form colonies equally as well as those of controls, the colonies were skewed toward monocytic and megakaryocytic lineages (11, 12). Conversely, HSCs from the M303V mice could form approximately 25% of control colonies; however, all of the colonies formed were of the megakaryocytic lineage (11, 12). In contrast, HSCs from our conditional disrupted c-*myb* mice could not form colonies and undergo multi-lineage differentiation on methylcellulose. Hence, in this report, we show that c-*myb* is a

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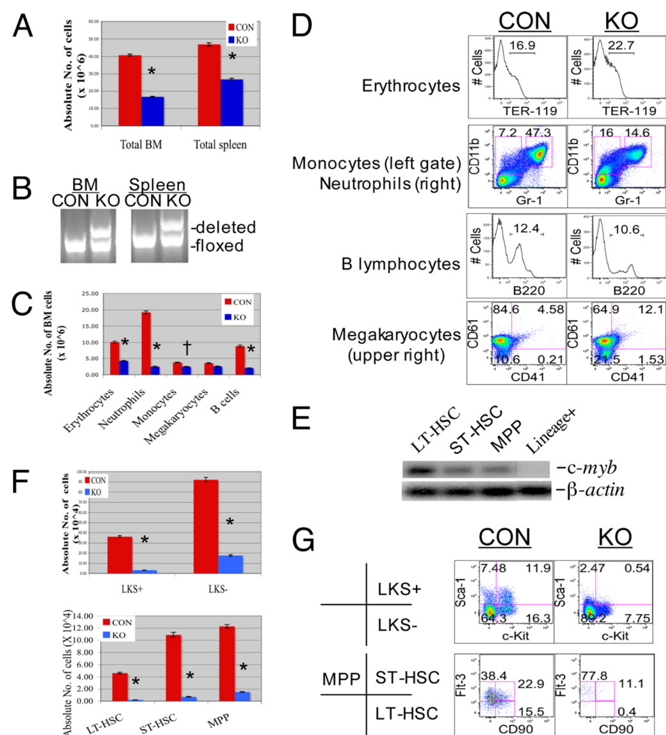

Fig. 1. c-*myb* is required for adult BM hematopoiesis and maintenance of HSCs. (*A*) Cellularity of BM and spleen of pIpC-induced *myb*$^{f/f}$/MxCre mutant (*KO*) and litter-mate control *myb*$^{f/f}$ (*CON*) mice. (*B*) PCR analysis of genomic DNA from BM and spleen of KO and CON mice. (*C*) Absolute number of cells in various BM lineages is depicted on a bar graph. (*D*) Representative 2-color flow cytometric or histogram analysis shows the percentages of various BM populations. (*E*) Expression of c-*myb* in hematopoietic stem cells as determined by semiquantitative RT-PCR. (*F*) Absolute number of cells in the hematopoietic progenitor (Lin$^-$c-Kit$^+$Sca-1$^-$, LKS$^-$) and stem cell (LKS$^+$) compartments as well as LT-HSC, ST-HSC, and MPP cells. (*G*) Representative 2-color flow cytometry showing the percentages of LKS$^-$, LKS$^+$, LT-HSC, ST-HSC, and MPP cells. (*, $P < 0.001$; †, $P < 0.05$. Numbers are presented as mean ± SEM; $n = 25$ mice.)

master regulator of adult BM hematopoiesis and is critical for self-renewal and multi-lineage differentiation of adult HSCs.

Results

Disruption of c-*myb* Gene Impairs Adult Hematopoiesis in the BM. The BM cellularity of the *myb*$^{f/f}$/MxCre (KO) mice was significantly reduced to 41% of litter-mate controls after pIpC administration to induce disruption of the c-*myb* gene (Fig. 1*A*). There were no notable abnormalities among *myb*$^{+/+}$, *myb*$^{f/+}$, *myb*$^{f/f}$, *myb*$^{+/+}$/MxCre, and *myb*$^{f/+}$/MxCre mice in the hematopoietic compartment. DNA analysis of total BM cells indicated that the c-*myb* floxed alleles were partially deleted (Fig. 1*B*). In addition, we observed a tight correlation between the levels of decreased BM cellularity and deletion efficiency of the c-*myb* floxed allele. The cellular number and percentages of neutrophils and B lymphoid cells in the pIpC-induced KO mice were also concomitantly reduced compared with the control mice, as determined by flow cytometric analysis (Fig. 1 *C* and *D*). Although the percentages of erythroid, monocytic, and megakaryocytic cells were increased in the pIpC-induced KO mice, the absolute number of these cells indicated a significant decrease by 57% for erythroid cells and 34% for monocytes and a nonsignificant reduction of 28% for megakaryocytes (Fig. 1 *C* and *D*). Together these data indicate that c-*myb* is required for BM hematopoiesis of the adult mice.

Deletion of the c-*myb* Gene Results in a Dramatic Reduction of HSCs. Consistent with published reports, c-*myb* is expressed in LT-HSCs and ST-HSCs (Fig. 1*E*). In addition, c-*myb* is also expressed in MPPs (Fig. 1*E*). The percentages of cells in the myeloid progenitor compartment (Lin$^-$c-Kit$^+$Sca-1$^-$, LKS$^-$) and the stem cell compartment (Lin$^-$c-Kit$^+$Sca-1$^+$, LKS$^+$) of the pIpC-induced KO mice were dramatically decreased by 53% and 96%, respectively, compared with those of controls (Fig. 1 *F* and *G*). Further fractionation of the LKS$^+$ by the Thy1 and CD135/Flt3 surface markers indicated that the numbers of LT-HSCs (LKS$^+$Thy^{lo}Flt3$^-$), ST-HSCs (LKS$^+$Thy1^{lo}Flt3$^+$), and MPPs (LKS$^+$Thy1$^-$Flt3$^+$) were also greatly reduced by 96%, 94%, and 88%, respectively, in the pIpC-induced KO mice compared with controls (Fig. 1 *F* and *G*). Likewise, fractionation of the LKS$^+$ into LT-HSCs, ST-HSCs, and MPPs by CD34 and Flt3 antigens produced the same dramatic decreases in cell numbers. Furthermore, staining for LT-HSCs and MPPs with the surface markers CD150/CD48/CD244 (14) along with lineage markers and c-Kit resulted in decreases of 94% for LT-HSCs and 87% for MPPs [supporting information (SI) Fig. S1]. These results demonstrate that c-*myb* is required for the maintenance of adult HSCs.

HSCs Have an Intrinsic Requirement for c-*myb* for Their Maintenance. As c-*myb* has been reported to be expressed in BM stromal cells (15), we performed syngeneic competitive BM transplantation (BMT) as previously described (16) to determine the nature of the requirement of c-*myb* in HSCs. Total blood cells obtained via retro-orbital bleeding before pIpC administration indicated that the chimeric mice were reconstituted closely to the desired ratio of 2 to 1, with 2 parts CD45.2 experimental (*myb*$^{f/f}$ or KO) cells to 1 part CD45.1 competitive cells (Fig. S2).

After pIpC administration, the ratios of CD45.2 to CD45.1 BM cells for all lineages examined in the pIpC-induced KO mice were inverted compared with those of the control chimeric mice: more CD45.1+ competitor cells than CD45.2+ pIpC-induced KO cells were present (Fig. 2 *A* and *B*). The CD45.2+ BM cells of erythroid, neutrophilic, monocytic, megakaryocytic, B lymphoid, and T lymphoid cells were dramatically decreased by 43% to 83% in the pIpC-induced chimeric KO mice compared with controls (Fig. 2*B*). Surprisingly, the decrease in BM CD45.2+ megakaryocytes of the pIpC-treated KO chimeric mice was significant compared with the nonsignificant decrease in the pIpC-induced KO mice (Figs. 1*C* and 2 *A* and *B*). In addition, the pIpC-induced chimeric KO CD45.2+ myeloid progenitor and stem cell compartments were severely diminished to 15% and 17%, respectively, of those of control CD45.2+ cells (Fig. 2 *D* and *E*). Concurrently, CD45.2+ LT-HSCs, ST-HSCs and MPPs in the pIpC-induced chimeric KO mice were all severely depleted to less than 20% of those in the control mice (Fig. 2 *D* and *E*). When *myb*$^{f/+}$/MxCre cells were used as donor cells to produce the mixed chimeras, heterozygous cells in blood, BM, and spleen behaved identically to *myb*$^{f/f}$ control cells before and after pIpC induction. These competitive BMT experiments indicated that HSCs have an intrinsic requirement for c-*myb* for their maintenance.

c-*myb* Is Required for Self-Renewal of Adult HSCs. To test rigorously whether c-*myb* is required for self-renewal of adult HSCs, serial transplantation was performed. Total BM cells from 3 primary transplants were isolated and pooled from each of the 2 experimental groups (Fig. 3*A*). Cells (1.5×10^6) were transplanted into the lethally irradiated secondary recipient mice. The secondary transplants were examined at 10 weeks after reconstitution. The inverted ratio of CD45.2 to CD45.1 cells of various BM lineages as well as HSCs in the secondary KO chimeras were maintained from the primary pIpC-induced KO chimeric mice (Fig. 3*D*). These results were consistent with DNA analysis from BM of the secondary KO transplants, which indicated a nearly complete loss of the mutant alleles (Fig. 3*C*). Likewise, the reconstituted ratio of CD45.2 to CD45.1 cells in the secondary control chimeras was similar to that of the primary control transplants (Figs. 2 and 3). The data gathered from the second-

Fig. 2. Hematopoietic stem cells have an intrinsic requirement for c-*myb* to maintain their populations. Percentages of experimental CD45.2 (blue bar) and competitive CD45.1 (yellow bar) lineage (*A*) and stem cells (*D*) in the BM of induced *myb*$^{f/f}$/MxCre chimeras (*KO*) and controls. Representative histogram and 2-color flow cytometric analysis showing the percentages of CD45.2 and CD45.1 cells in various BM lineages (*B*) and stem cells (*E*) of the first transplanted mice after pIpC administration. (*C*) DNA analysis of total BM cells from mixed chimeric mice after pIpC administration. For controls and conditional KO for all lineages except whole blood-derived erythroid cells, $n = 10$ and $n = 11$, respectively. For whole blood erythroid cells, $n = 3$ for both groups. Data are expressed as mean ± SEM. Error bars are shown for CD45.2 cells only. (*, $P < 0.001$; †, $P < 0.05$.)

ary transplants demonstrated that c-*myb* is required for self-renewal of adult HSCs.

Disruption of c-*myb* Leads to Impaired Proliferation and Accelerated Differentiation of HSCs. To understand the functions of c-*myb* in HSC development, we analyzed the proliferative, survival, and differentiation capacities of purified LT-HSCs, ST-HSCs, and MPPs via BrdU incorporation, caspase staining, and lineage marker expression, respectively. Because initial studies on sorted LKS$^+$ and LKS$^+$CD34- and LKS$^+$Thy^{lo} HSCs showed no noticeable changes in BrdU incorporation or poly-caspase staining between IFN-induced purified KO and control cells at 24 h following IFN treatment but revealed alterations at 48 h, we performed all our functional studies on purified LT-HSCs, ST-HSCs, and MPPs at the 48 h time point.

At 48 h following IFN treatment, there were no differences in poly-caspase staining among IFN-induced KO LT-HSCs, ST-HSCs, and MPPs compared with controls (Fig. S3). However, despite the incomplete deletion of the c-*myb* floxed allele (Fig. S4*A*), BrdU incorporation was statistically significantly reduced, although modestly, in IFN-treated KO LT-HSCs, ST-HSCs, and MPPs (Fig. 4*C*). These studies indicate that c-*myb* is required for the proliferative capacity of HSCs.

Interestingly, the number of CD11b+ and CD41+ IFN-induced KO purified LT-HSCs were increased by twice as much as controls, indicating that disruption of c-*myb* accelerates differentiation (Fig. 4*A*). Surprisingly, there exists an approximately 20% atypical population in the IFN-induced KO LT-HSCs that expressed both CD11b and CD41 antigens on their cell surface, as opposed to less than 1.3% in the controls (Fig. 4*A* and Fig. S4), signifying that loss of c-*myb* also leads to aberrant development. In contrast, there were either no or subtle changes in the percentages of IFN-induced KO LT-HSCs that expressed Gr-1 or both Gr-1 and CD11b compared with controls (Fig. S4). Furthermore, forward scatter/side scatter morphological gating, which specifies size and granularity of the cells, was increased in the IFN-induced KO LT-HSCs, which is consistent with the increased expression of lineage markers and indicates accelerated differentiation (Fig. S3). In addition, surface expression of c-Kit was down-regulated in IFN-induced KO LT-HSCs but not in the controls (Fig. 4*D*). These results demonstrated that disruption of c-*myb* gene accelerates differentiation of LT-HSCs.

Similar to the IFN-induced KO LT-HSCs, IFN-induced KO ST-HSCs and MPPs had no differences in poly-caspase staining, slightly decreased BrdU incorporation and c-Kit expression, and caused increases in CD11b$^+$, CD41$^+$ and CD11b$^+$CD41$^+$ populations and greater forward/side scatter compared with controls (Fig. 4 *B–D* and Fig. S3 and Fig. S4). In contrast to IFN-induced KO LT-HSCs, IFN-induced KO ST-HSCs and MPPs displayed an approximate fourfold increase in the CD11b$^+$Gr-1$^+$ population and a distinct four- to fivefold decrease in the CD11b$^-$Gr-1$^+$ cells compared with controls (Fig. S4). Results obtained using LT-HSCs, ST-HSCs, and MPPs, which were sorted by surface expression of CD34 and Flt3, showed the same impaired proliferation and altered differentiation patterns as described earlier. Furthermore, IFN-induced purified KO LKS+ cells exhibited the same defective phenotypes as IFN-induced KO LT-HSCs, ST-HSCs, and MPPs. Therefore, c-*myb* regulates the proliferation and differentiation programs of LT-HSCs, ST-HSCs, and MPPs.

Disruption of c-*myb* Leads to Increased Presence of Aberrant and Differentiated HSCs in Mice. To determine whether the results seen in vitro with sorted HSCs were artifacts or represented a naturally occurring phenomenon of the disrupted c-*myb* gene, we

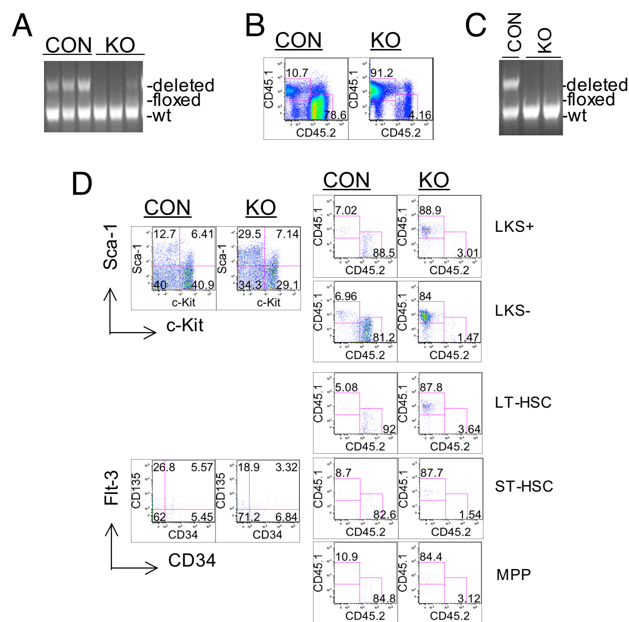

Fig. 3. c-*myb* is required for self-renewal of hematopoietic stem cells. (*A*) BM DNA from pIpC-treated first transplants ($myb^{f/+}$/MxCre or $myb^{f/f}$/MxCre chimeras) that were pooled to use as donor cells to reconstitute lethally irradiated mice to generate second transplants. (*B*) Percentages of CD45.2 and competitor CD45.1 cells in total BM of second transplanted recipient mice. (*C*) PCR analysis of genomic DNA from BM of second transplants. (*D*) Representative 2-color flow cytometric analysis showing the percentages of CD45.2 and CD45.1 cells in LKS$^-$, LKS$^+$, LT-HSC, ST-HSC, and MPP cells of second transplants. (n = 4 in each group.)

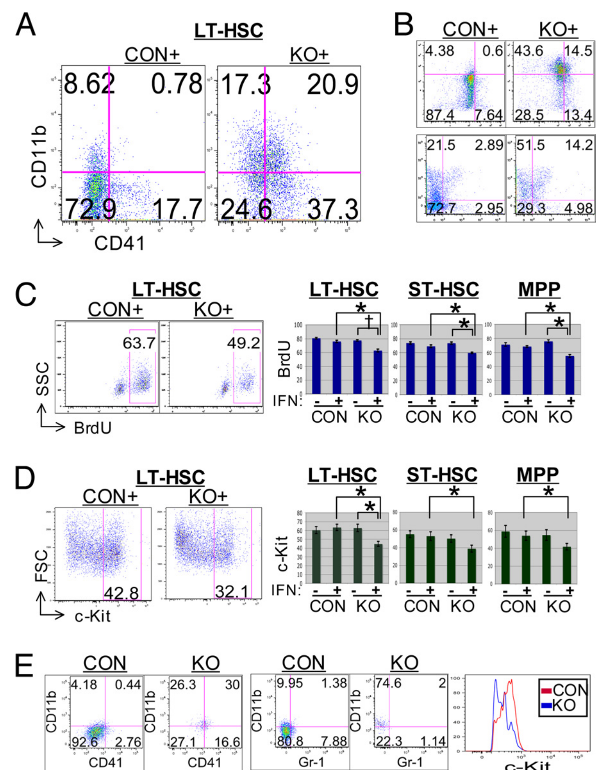

Fig. 4. Disruption of c-*myb* in hematopoietic stem cells leads to impaired proliferation and accelerated differentiation. Following 48 h IFN treatment to induce disruption of c-*myb*, the purified LT-HSCs, ST-HSCs, and MPPs were labeled with BrdU (*C*) and then stained for surface expression of lineage markers and c-Kit (*D*). Shown are the representative 2-color flow cytometric analysis of CD11b (y-axis) and CD41 surface antigens for LT-HSCs (*A*) (n = 3), ST-HSCs (*B*, *Top*) (n = 3), and MPPs (*B*, *Bottom*) (n = 4). Representative 2-color flow cytometric analysis of BrdU incorporation (*C*) and c-Kit expression (*D*) for LT-HSCs (n = 6) are shown. (*E*) Cells in the lin-c-Kit+Sca-1+ (LKS+) compartment of pIpC-induced KO mice were analyzed for surface expression of CD11b, CD41, Gr-1, and c-Kit. n indicates the number of experiments. Data are expressed as mean ± SEM. (*, $P < 0.05$; †, $P < 0.01$.) IFN (KO+) or pIpC (KO)-treated $myb^{f/f}$/MxCre.

re-examined the BM of the pIpC-induced KO mice. The LKS$^+$ compartment of the pIpC-induced KO mice exhibited decreased levels of c-Kit expression and increased levels of CD11b$^+$CD41$^+$ cells compared with control cells (Fig. 4*E*). These results from the pIpC-induced KO mice were consistent with the in vitro experiments using sorted HSCs and confirmed that c-*myb* regulates the differentiation of HSCs.

Disruption of c-*myb* Results in Loss of Growth on Methylcellulose and Multi-Lineage Differentiation. It has recently been shown that knockdown c-*myb* LT-HSCs, ST-HSCs, and MPPs could form colonies on cytokine-containing methylcellulose for assessing multi-lineage differentiation (11). Hematopoietic colonies in IFN-induced, purified KO LT-HSCs and MPPs were significantly reduced compared with those of nontreated $myb^{f/f}$/MxCre and $myb^{f/f}$ controls (Fig. 5*A*). In fact, colony formation using IFN-induced purified KO LKS$^+$ cells was profoundly diminished by greater than 80% (Fig. 5*A*). Analysis of genomic DNA, extracted from the remaining colonies that did grow on the plates of any of the aforementioned sorted cells, revealed presence of only the c-*myb* floxed allele (Fig. 5*B*), indicating that cells with a disrupted c-*myb* gene will not grow and form multi-lineage colonies. These results demonstrated that c-*myb* is critical for HSC growth and multi-lineage differentiation.

Disruption of c-*myb* Results in Altered Gene Expression in HSCs. To determine the molecular mechanism by which c-*myb* functions in HSCs, we first examined changes in the abundance of 5 genes (*gfi-1*, *cxcr4*, *cebpa*, *flt3*, *bcl2*) that were shown by microarray analysis of purified Lin$^-$c-Kit$^+$ BM cells to be altered when c-*myb* expression was lost (Table S1). These down-regulated genes were confirmed by semiquantitative RT-PCR and Northern blot analysis (Fig. S5). In addition, microarray analysis of purified LKS$^+$ cells also demonstrated reduced expression of these genes.

We therefore investigated the expression levels of the aforementioned genes, as well as *c-myc*, in purified LT-HSCs and ST-HSCs. *Cxcr4*, *c-myc*, and *bcl2* were decreased in IFN-induced KO LT-HSCs compared with those from controls (Fig. 5*C*). In IFN-induced KO ST-HSCs, *cxcr4*, *gfi-1*, c-*myc*, *bcl2*, and *flt3* were reduced compared with those from controls (Fig. 5*D*). These studies indicate that these genes play a role in c-*myb*-regulated development of LT-HSCs and ST-HSCs.

Discussion

Our study, which used the inducible disruption of c-*myb* floxed allele in adult mice, supports a critical role for c-*myb* in adult BM hematopoiesis and HSCs. Disruption of the c-*myb* gene leads to profound reductions in various BM hematopoietic lineages, including neutrophilic, monocytic, B lymphoid, and erythroid cells. In addition, megakaryocytes were significantly reduced in the BM of pIpC-induced c-*myb* KO chimeric mice compared with controls, which contradicts the earlier notion that c-*myb* is not required for megakaryopoiesis. Therefore, c-*myb* is a master regulator of BM hematopoiesis. Furthermore, our findings indicate a role for c-*myb* in LT-HSCs, ST-HSCs, and MPPs. Specifically, c-*myb* is critical for self-renewal and multi-lineage differentiation of HSCs.

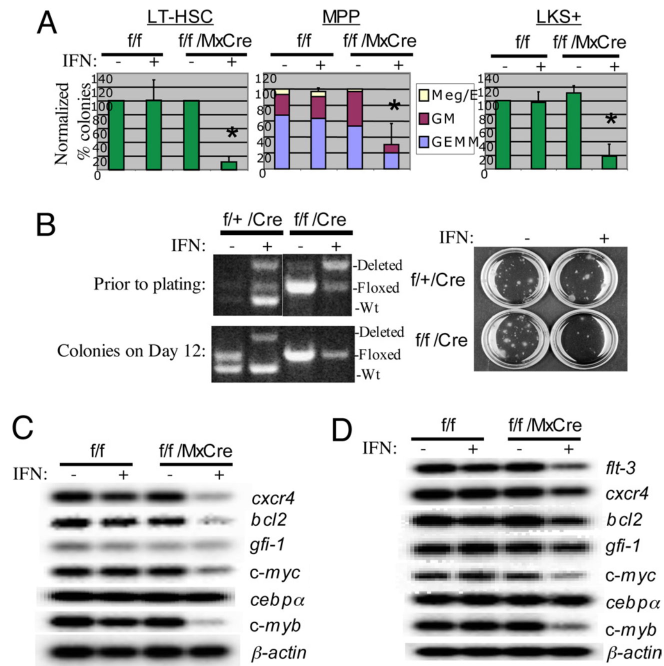

Fig. 5. Disruption of the c-*myb* gene results in impaired HSC growth and multi-lineage differentiation on methylcellulose and altered gene expression. (*A*) Hematopoietic colony assays performed using purified LT-HSCs, MPPs, and LKS+ cells. The number of colonies from the 3 groups was normalized to the untreated control, which was set to 100%. Data are expressed as mean ± SD, $n = 3$ experiments. (*B*) PCR analysis of genomic DNA from 18 h IFN-treated LKS+ cells before plating on methylcellulose containing dishes (*Top*) and cells from colonies growing on dishes after 12 d (*Bottom*). Semiquantitative RT-PCR analysis of gene expression of LT-HSCs (*C*) and ST-HSCs (*D*) after 18 h of IFN treatment. Results reflect ≥3 sorted experiments. *GEMM*, granulocytic, erythroid (*E*), monocytic, and megakaryocytic (*Meg*) colonies; *GM*, granulocytic and monocytic colonies. (*, $P < 0.001$.)

Our knowledge of the roles that c-*myb* plays in adult HSCs has increased recently with the generation of 2 mutant mouse models, one with a knockdown c-*myb* allele and another with the M303V c-Myb mutation (11, 12). However, both these models have limitations. The M303V mutant reflected the interaction of c-*myb* with only p300. It is known that c-*myb* binds to a vast number of proteins with multifaceted functions (17). As for the knockdown model, it showed the effects of persistently reduced steady-state c-*myb* levels during hematopoiesis. Furthermore, the altered phenotype in adult knockdown LT-HSCs could be carried over from the fetal liver, because the same defective hematopoietic lineage profile was observed in knockdown fetal liver (11, 18). In addition, HSCs from adult knockdown mice expressed CD11b antigen (11, 18), a feature that is characteristic of normal fetal HSCs (19). Nevertheless, these mutations also do not tell us about the fate of adult HSCs when the c-*myb* gene is disrupted. Data from embryonic stem (ES) cell chimeras are conflicting. The chimeric c-*myb*-null ES cell *rag*-deficient mice indicate that c-*myb* is not required for HSCs because early T cell precursors were found in the chimeras (20). In contrast, no mature cells along the lymphoid or myeloid lineages were found in the c-*myb*-null ES cell chimeric mice generated by Sumner et al. (21).

Our results indicated that c-*myb* is indeed required for adult HSCs. The percentages and absolute numbers of LKS$^+$ and LT-HSCs were severely depleted in contrast to those observed in the knockdown and M303V models. This signifies that some expression of c-*myb* is required for the maintenance of HSCs. As gene knockout indicates the earliest requirement of the gene, the reductions seen in ST-HSCs and MPPs could be a result of reduced numbers of LT-HSCs. However, our colony assays and in vitro functional studies demonstrated that c-*myb* is also critical for ST-HSCs and MPPs.

The reasons for the reduction in LT-HSCs are not clear in that it may be caused by apoptosis, a proliferative defect, or loss resulting from differentiation. The lack of poly-caspase staining in our in vitro functional assay does not rule out the possibility that these cells are undergoing apoptosis (Fig. S3). It is possible that cell death pathways were activated after the 48-h IFN treatment. In support of this, analysis of DNA isolated from cells remaining on the IFN-induced KO methylcellulose plates after 7 to 12 d demonstrated only the presence of the floxed allele (Fig. 5*B*), indicating that a cell survival defect may be an underlying mechanism for the reduced LT-HSC pool. Nevertheless, the significantly reduced percentages of BM cells in the live cell gate of the pIpC-induced KO mice compared with the control mice (Fig. S6) indicate a general role for c-*myb* in cell survival of hematopoietic cells, which is consistent with many published reports (4). Our in vitro functional assay indicated that decreased proliferative capacity and loss caused by aberrant and accelerated differentiation contribute to the diminished HSC pool. In support of this notion, the LKS$^+$ cells from the pIpC-induced KO mice showed an increased expression of CD11b and CD41 antigens and a reduced level of c-Kit expression (Fig. 4*E*). Hence, the modest reduction in proliferation and the aberrant and accelerated differentiation contributed to the reduction in the HSC pool when the c-*myb* gene is disrupted.

In the knockdown mutant, colony growth is intact but multi-lineage differentiation is skewed toward a megakaryocytic phenotype. In contrast, disruption of the c-*myb* gene results in the absence of colony formation and therefore absence of multi-lineage differentiation. This indicates that a minimal level of c-*myb* expression is required to sustain growth whereas a greater abundance is critical for normal multi-lineage differentiation, at least for the granulocytic, erythroid, monocytic, and megakaryocytic lineages. This is also supported by the severe reductions in various BM lineages including neutrophilic, erythroid, monocytic, and B lymphoid cells. Although megakaryocytes were dramatically increased in the knockdown and M303V mutants, there was a slight but nonsignificant decrease in BM megakaryocytes of pIpC-induced KO mice compared with control animals (Fig. 1 *C* and *D*). However, in the induced KO chimeric mice, there was a significant reduction in the number of megakaryocytes in the BM (Fig. 2 *A* and *B*), indicating that disruption of c-*myb* also affects adult megakaryopoiesis. Our results demonstrating that c-*myb* is required for adult megakaryopoiesis are consistent with the findings of Sumner et al. (21), which showed an absence of megakaryocytic CFUs and a reduction in the absolute number of megakaryocytes in the c-*myb*-null fetal liver.

Total BM cells derived from the knockdown and M303V mutant mice were able to preserve their self-renewal capacities. However, disruption of c-*myb* gene which does not produce a 19- to 20-kDa protein fragment (see *SI Materials and Methods*), leads to a loss of repopulating capacity, suggesting that some expression of c-*myb* is critical for self-renewal of HSCs. At the molecular level, several genes could explain the loss of self-renewal when c-*myb* gene is disrupted. Down-regulation of *gfi-1* and *cxcr4* could negatively affect self-renewal capacity whereas the reduction of c-*myc* could block multi-lineage differentiation (22–24). In addition, a decrease in *bcl-2* expression could hinder cell survival and the maintenance of HSCs (25). Furthermore, down-regulation of the c-Kit surface antigen could contribute to the impairment of self-renewal and multi-lineage differentiation (26, 27). Thus,

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our study unequivocally demonstrates a role for c-*myb* in self-renewal and multi-lineage differentiation of adult HSCs.

Materials and Methods

Mice and Genotyping. The construction of a conditional c-*myb* floxed mouse was previously reported (9). The c-*myb* mutant mice were backcrossed for at least 6 to 10 generations to C57BL/6 mice. The mutant mice were genotyped by using a 3-primer PCR amplification method: mybG2e 5′-ATT CCA GTG GTT CTT GAT AGC ATT ATC-3′; mybG11e, 5′-GCC GCT AAG CCA CAA TGG AAG GGC-3′; mybG19e, 5′-CCT TGA CTC TGA GTA AGA AAG TAA AC-3′.

In Vivo and in Vitro Deletion of the c-*myb* floxed Allele. For in vivo disruption of the c-*myb* floxed allele, the $myb^{f/f}$/MxCre and control mice were given 250 μL of 2-mg/mL pIpC (P-1530; Sigma) by i.p. injection every other day for a total of 7 to 9 injections and analyzed 1 or 2 d after the last injection. pIpC was dissolved in sterile PBS solution by heating at 56 °C for 30 min and then stored in frozen aliquot at −20 °C. For injections, defrosted pIpC solution was heated at 56 °C for 8 min and allowed to cool at room temperature.

For in vitro deletion, 2×10^4 units of IFN-α (R&D Systems) per mL SCF/IL3/IL6 cytokine-containing DMEM as described by Pear et al. (28) and listed in the legend for Fig. S4. For cell culture, regardless of the purified cell number, a minimum of 200 μL medium was used with a maximum concentration of 1×10^6 cells/mL.

Mixed BM Chimeras. For the competitive BMT experiments, 1×10^6 unfractionated $myb^{f/f}$/MxCre or control BM cells and 0.5×10^6 unfractionated competitor B6-CD45.1 BM cells were injected intravenously into the tail veins of lethally irradiated C57BL6/J mice (1,100 rad). FACS analysis was used to monitor for reconstitution at 10 weeks to 4 months post-transplantation via retro-orbital bleeding, and shortly afterward mice were given pIpC injections. For the second transplant, 1.5×10^6 unfractionated BM cells from the pIpC-induced first transplant mice were used as donor cells. Mice were analyzed at 10 weeks post-transplantation.

Colony Assay on Methylcellulose. See *SI Materials and Methods* for a detailed description of the colony assay on methylcellulose.

Flow Cytometry, Sorting, and Antibodies. See *SI Materials and Methods* for a detailed description of flow cytometry, sorting, and antibodies.

In Vitro Functional Assay. At the indicated time following IFNα treatment, purified cells were pulsed with BrdU (BD Biosciences) in fresh cytokine medium for 2 h at 37 °C in a tissue culture incubator under humidified conditions with 5% CO_2. Then the indicated fluorochrome inhibitor of caspases for detecting poly-caspase activity (V35117 FLICA kit; Invitrogen) was then added, and cells were returned to the incubator for an additional 1 h. After the incubation, purified cells were washed, stained with surface antibodies, and then fixed and permeabilized for anti-BrdU staining as specified by the manufacturer (BD Biosciences).

Semiquantitative RT-PCR Determination of mRNA Levels. See Table S2 and *SI Materials and Methods* for a detailed discussion of semiquantitative RT-PCR determination of mRNA levels.

Statistical Analysis. Data are expressed as mean ± SEM. Comparisons were analyzed by using Student 2-tailed paired or unpaired (with equal variance) *t* tests. Differences were considered significant at $P < 0.05$.

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