Rb is dispensable for self-renewal and multilineage differentiation of adult hematopoietic stem cells

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Stem cells have been identified as essential for maintaining multiple organ systems, including the hematopoietic system. The distinct cell fates of self-renewal and differentiation of hematopoietic stem cells (HSCs) depend on cell division. Recently, several negative regulators of the cell cycle, such as the cyclin-dependent kinase inhibitors $p21^{Cip1}$, $p27^{Kip1}$, and $p16^{INK4a}/p19^{ARF}$, have been demonstrated to have a role in regulating HSC fate decisions, suggesting that regulation of the G_1–S phase transition can contribute to HSC self-renewal. Because the retinoblastoma protein, Rb, plays a central role in the regulation of the G_1–S phase cell cycle, we sought to determine whether it has an intrinsic role in the regulation of HSC fate. Surprisingly, we found that HSC function was essentially normal in the absence of Rb. $Rb^{\Delta/\Delta}$ HSCs contributed normally to both myeloid and lymphoid lineages in both primary and secondary recipients, and no evidence of transformation was observed. Additionally, we observed a mild myeloid expansion and decrease in mature B cells within the $Rb^{\Delta/\Delta}$ bone marrow but a similar contribution to phenotypic HSC populations compared with nondeleted bone marrow. The Rb family members p107 and p130 were not deregulated in cells in which Rb had been deleted, as determined by quantitative RT-PCR on the highly enriched stem and primitive progenitor cell $lin^-c\text{-}Kit^+Sca\text{-}1^+$ population. These studies demonstrate that Rb is not intrinsically required for self-renewal and multilineage differentiation of adult HSCs.

retinoblastoma | conditional mutation | bone marrow transplantation

Hematopoietic stem cells (HSCs) produce all blood lineages throughout the lifetime of an organism through a tightly regulated balance of differentiative divisions, generating functional mature cells, and self-renewal divisions, resulting in additional HSCs. Although factors influencing the balance between self-renewal and differentiation of an HSC division are largely unknown, entry into the cell division cycle is thought to be an essential part of this process.

Recently, a number of studies have reported intrinsic roles for negative regulators of the cell division cycle in determining the fate of HSCs (1–5). These studies have predominantly focused on inhibitors of G_1–S phase cell cycle progression, in particular the CIP/KIP and INK4 family of cyclin-dependent kinase inhibitors, and they have revealed that the loss of these genes can alter HSC self-renewal and differentiation. The action of the INK4 and CIP/KIP families of cell cycle inhibitors on Cdk4/6- or Cdk2-containing complexes, respectively, is thought to lead ultimately to inhibition of phosphorylation of the retinoblastoma protein, Rb, and the related p107 and p130 proteins.

Loss of $p21^{Cip1}$ led to an increase in HSC cycling under homeostatic conditions but an increased sensitivity to stress-induced HSC exhaustion (2). Deletion of $p27^{Kip1}$ resulted in an increased frequency of serially transplantable HSCs in addition to an expanded progenitor compartment (1, 3). In contrast to loss of $p18^{INK4c}$, loss of both $p16^{INK4a}$ and $p19^{INK4d}$ had little effect on HSC function, with only a subtle increase in serial repopulation reported (5). Loss of $p18^{INK4c}$, however, resulted in enhanced repopulating capacity and frequency of HSCs (4). Recently, studies investigating the roles of both Bmi-1 and ATM in HSCs implicated cell cycle regulation through Rb as critical to the phenotypes observed in these respective mutants (6, 7). It is important to note that all of these studies have used HSCs from animals with germ-line gene deletion. Collectively, these studies focus attention on the role that Rb may play in determining the fate of HSCs.

Understanding the role of Rb in the regulation of stem cell self-renewal and differentiation may also allow for insight into tumorigenesis. The "Rb pathway" is universally inactivated in human cancer (8). It is thought that during transformation the tumor-initiating cell, or cancer stem cell as it has become termed, develops many properties that are shared by nonneoplastic stem cells, such as the capacity to self-renew (9–12). Understanding the role(s) Rb plays in the normal regulation of self-renewal may provide important insights into its role in transformation.

To date, no studies have directly assessed the role that Rb may play in the regulation of stem cell biology. Because of the lethality of germ-line Rb mutants, we have used conditional somatic mutagenesis to determine directly whether Rb has an intrinsic role in the regulation of self-renewal and differentiation of adult HSCs (13–17). For this purpose, whole bone marrow was transplanted into wild-type congenic recipients. When hematopoiesis was established, Rb was deleted, and the effects on hematopoiesis were monitored. We report that deletion of Rb did not result in any change in the contribution of HSCs to hematopoiesis in the peripheral blood of either primary or secondary transplant recipients and that, with the exception of a mild anemia, multilineage differentiation is largely unaffected by the loss of Rb. These results demonstrate that Rb is dispensable for normal self-renewal and differentiation of HSCs.

Results

Rb Is Dispensable for Multilineage Hematopoiesis. To determine directly whether Rb has an intrinsic role in the regulation of self-renewal and differentiation of adult HSCs, we have used a conditional somatic deletion strategy similar to that described in refs. 15 and 18. We used mice containing a conditional allele of Rb that has been demonstrated to result in an Rb-null after recombination (16, 19). $Rb^{fl/fl}$ homozygous mice were bred to the IFN-inducible *Mx-Cre* transgene that elicits highly efficient gene deletion throughout the hematopoietic system, including the HSCs (14, 15, 20).

Whole bone marrow from either $Mx\text{-}Cre^-Rb^{fl/fl}$ (control) or $Mx\text{-}Cre^+Rb^{fl/fl}$ mice was pooled and transplanted at varying ratios (1:1, 2:1, and 10:1) with wild-type competitor whole bone marrow, and the recipients were allowed to recover for 5 weeks after transplantation. After establishing the levels of donor reconstitution in the peripheral blood at 5 weeks posttransplan-

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Abbreviations: fl, floxed; HSC, hematopoietic stem cell; pIpC, poly(I)·poly(C); Rb, retinoblastoma.

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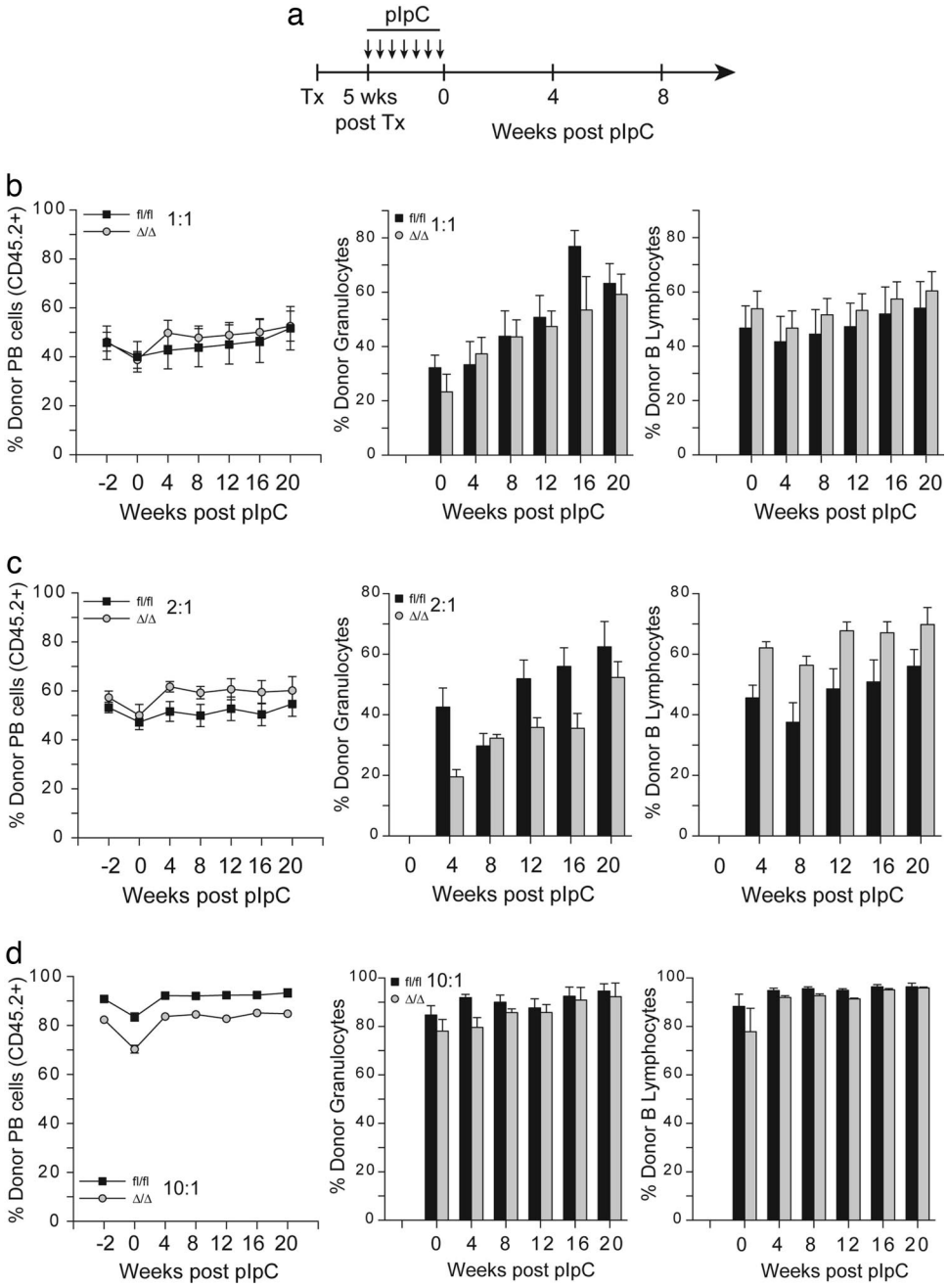

Fig. 1. Rb is dispensable for normal contribution of stem cells to hematopoiesis. (*a*) Schematic diagram of experimental design. (*b*) *Mx-Cre*$^{-}$*pRb*$^{fl/fl}$ (black squares, control) and *Mx-Cre*$^{+}$*pRb*$^{fl/fl}$ (gray circles, becomes *Rb*$^{\Delta/\Delta}$ after administration of pIpC) whole bone marrow was transplanted in a 1:1 ratio with WT competitor bone marrow. Recipients were treated as outlined in *a*. Data represent the average percentage peripheral blood chimerism for 20 weeks after the administration of pIpC (last day of pIpC is time 0). Data are pooled from two independent experiments and are expressed as means ± SEM; $n = 4$ or 5 recipients per group in each experiment. Contributions to myeloid (Gr-1^{+}/Mac-1^{+}) and B lymphoid (B-220^{+}) lineages are from cells of the indicated genotype from the 1:1 cell dose transplant. (*c*) *Mx-Cre*$^{-}$*pRb*$^{fl/fl}$ and *Mx-Cre*$^{+}$*pRb*$^{fl/fl}$ (Rb$^{\Delta/\Delta}$ after administration of pIpC) whole bone marrow was transplanted in a 2:1 ratio with WT competitor bone marrow. Contributions to myeloid and lymphoid lineage are from cells of the indicated genotype. Data are expressed as means ± SEM; $n =$ 5 recipients per group. (*d*) *Mx-Cre*$^{-}$*pRb*$^{fl/fl}$ and *Mx-Cre*$^{+}$*pRb*$^{fl/fl}$ (Rb$^{\Delta/\Delta}$ after administration of pIpC) whole bone marrow was transplanted in a 10:1 ratio with WT competitor bone marrow. Shown is the contribution to myeloid and lymphoid lineage from cells of the indicated genotype. Data are expressed as means ± SEM; $n = 5$ recipients per group.

tation, all groups of recipients were treated with poly(I)·poly(C) (pIpC), transiently inducing expression of Cre recombinase from the *Mx-Cre* transgene, causing Rb gene deletion in cells bearing the *Mx-Cre* transgene (for scheme, see Fig. 1*a*). After pIpC administration, the peripheral blood of recipients was monitored to determine the effects of loss of Rb on the self-renewal and multilineage differentiation of HSCs. This transplantation strategy allowed for a direct assessment of the intrinsic requirement for Rb in the HSC and hematopoietic system independent of potential requirements for Rb in the bone marrow microenvironment (21, 22).

Remarkably, conditional deletion of Rb did not alter the contribution of HSCs to hematopoiesis for 5 months of analysis after gene deletion in primary recipients (Fig. 1 *b–d*). The

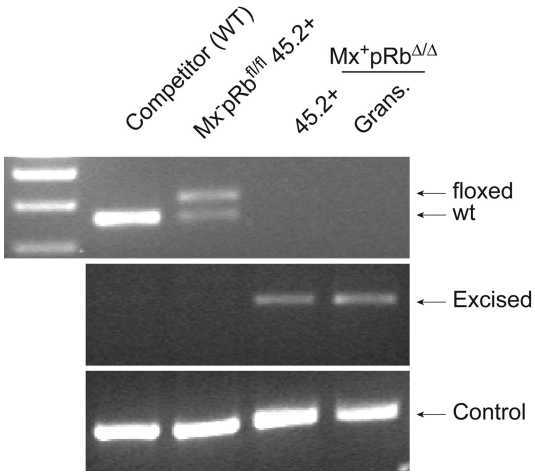

Fig. 2. Rb is efficiently deleted by using the *Mx-Cre* transgene. Genomic PCR analysis of sorted WT competitor cells (CD45.1$^+$/CD45.2$^+$), CD45.2$^+$ *Mx-Cre*$^-$*pRb*$^{fl/fl}$ cells (control), and *Rb*$^{\Delta/\Delta}$ CD45.2$^+$ and Gr-1$^+$ cells from primary transplant recipients 7 months after pIpC. PCR was performed on genomic DNA. Note the complete excision of the floxed allele from both *Rb*$^{\Delta/\Delta}$ CD45.2$^+$ cells and neutrophils (Gr-1$^+$).

contribution of HSCs in which Rb was deleted (Δ/Δ) was stable compared with the contribution of these same cells before deletion of Rb (-2 week analysis time point; Fig. 1 *b–d*) at all cell doses tested during the course of analysis. With the exception of the 10:1 cell dose, where the level of initial engraftment was lower for *Mx-Cre*$^+$*Rb*$^{fl/fl}$ than for the *Mx-Cre*$^-$*Rb*$^{fl/fl}$ recipients (*Mx*$^+$, 82.34 $\pm$ 1.17; *Mx*$^-$, 90.78 $\pm$ 0.55; $n = 5$ per group, $P = 0.006$; Fig. 1*d*), *Rb*$^{fl/fl}$ HSCs and *Rb*$^{\Delta/\Delta}$ cells exhibit comparable levels of contribution to hematopoiesis (Fig. 1 *b–d*). Loss of Rb did not alter the contribution of HSCs to either the lymphoid [B lymphoid, B220$^+$, Fig. 1 *b–d*; T lymphoid, CD4$^+$/CD8$^+$-derived cells at 20 weeks post-pIpC 1:1 transplant: 33.7 $\pm$ 7.6% *Rb*$^{fl/fl}$-derived, 37.7 $\pm$ 7.7% *Rb*$^{\Delta/\Delta}$-derived; $P = 0.72$; 2:1 and 10:1 transplant (data not shown)] or myeloid (Gr-1$^+$/CD11b$^+$, Fig. 1 *b–d*) lineages. We did observe mild anemia in recipients of *Rb*$^{\Delta/\Delta}$ cells in all transplant groups, suggesting an erythroid cell intrinsic requirement for Rb. However, this anemia was subtle and stable during the course of analysis [hemoglobin levels 24 weeks postdeletion 1:1 cell dose: fl/fl, 13.61 $\pm$ 0.43 g/dl; Δ/Δ, 11.55 $\pm$ 0.25 g/dl ($P < 0.01$); 10:1 cell dose: fl/fl, 13.68 $\pm$ 0.22 g/dl; Δ/Δ, 11.58 $\pm$ 0.43 g/dl ($P < 0.01$)]. No evidence of transformation had been observed up to 10 months after Rb deletion in primary recipients.

Rb Is Efficiently Deleted in Primary Recipients by Using the *Mx-Cre* Transgene. To confirm that we had deleted Rb from the HSC, cells were isolated from the bone marrow of primary recipients 7 months postdeletion, and genomic PCR was performed. As shown in Fig. 2*a*, excision was complete. We did not detect the nonrecombined allele in *Rb*$^{\Delta/\Delta}$ cells, and we observed only the genomic fragment generated after deletion of Rb in either unfractionated donor bone marrow (CD45.2$^+$ donor cells) or donor-derived myeloid cells (CD45.2$^+$Gr-1$^+$; Fig. 2*a*), indicating that excision was highly efficient in the HSC. These data, therefore, demonstrate that hematopoiesis is essentially normal in the absence of Rb.

Alterations in B Cell Development Within the Bone Marrow After Deletion of Rb. In addition to analysis of the peripheral blood, we undertook analysis of bone marrow hematopoiesis after deletion of Rb in primary recipients in the 2:1 transplant group. The analysis revealed that for the most part, contribution to bone

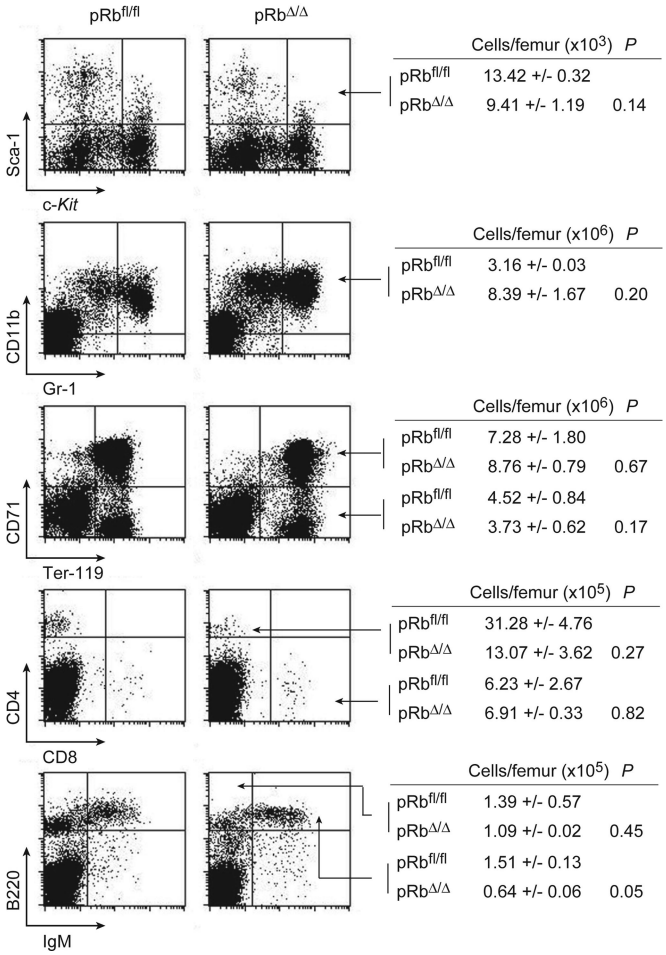

Fig. 3. Alterations in B cell development within the bone marrow after deletion of Rb. Shown are representative FACS profiles of bone marrow populations within the bone marrow of control (*Rb*$^{fl/fl}$) and *Rb*$^{\Delta/\Delta}$ 2:1 cell dose recipients. Populations represent stem and primitive progenitor cells (lineage$^-$c-Kit$^+$Sca-1$^+$), neutrophils (CD11b$^+$/Gr-1$^+$), proerythroblasts (CD71$^+$/Ter119$^+$), and mature erythroid cells (CD71$^-$/Ter119$^+$), CD4$^+$ and CD8$^+$ single-positive mature T lymphoid cells, immature B lymphoid (IgM$^-$/B220$^+$) cells, and mature B lymphoid (IgM$^+$/B220$^+$) cells. Data are expressed as cells per femur (means $\pm$ SEM).

marrow hematopoiesis was comparable between *Rb*$^{fl/fl}$ and *Rb*$^{\Delta/\Delta}$ cells. There was a significant reduction in the numbers of mature B cells (B220$^+$/IgM$^+$) per femur, but the numbers of immature B cells (B220$^+$/IgM$^-$) cells per femur were not significantly altered (Fig. 3), and the contribution to peripheral blood B lymphopoiesis was also not altered (Fig. 3). Surprisingly, despite the subtle peripheral blood anemia, no significant differences were observed in the erythroid-specific populations (Fig. 3). A previous report describing hematopoiesis in chimeric animals derived from *Rb*$^{-/-}$ ES cells showed that over time, bone marrow hematopoiesis became dominated by neutrophils (23). In contrast, we did not observe this dominance of bone marrow hematopoiesis in our transplant recipients, but we did observe an increase in neutrophil numbers in the bone marrow. This increase in neutrophils did not significantly impact on the contribution of other lineages within the bone marrow, but it resulted in an increased cellularity of the marrow (*Rb*$^{fl/fl}$, 16.7 $\pm$ 1.1 $\times$ 10^6 cells per femur; *Rb*$^{\Delta/\Delta}$, 28.4 $\pm$ 3.1 $\times$ 10^6 cells per femur). Spleen size was not significantly elevated in these recipients (*Rb*$^{fl/fl}$, 149.3 $\pm$ 38.5 mg; *Rb*$^{\Delta/\Delta}$, 103.0 $\pm$ 8 mg).

Rb Is Not Required for HSC Self-Renewal. To test rigorously whether Rb plays a role in HSC self-renewal, serial transplantation was

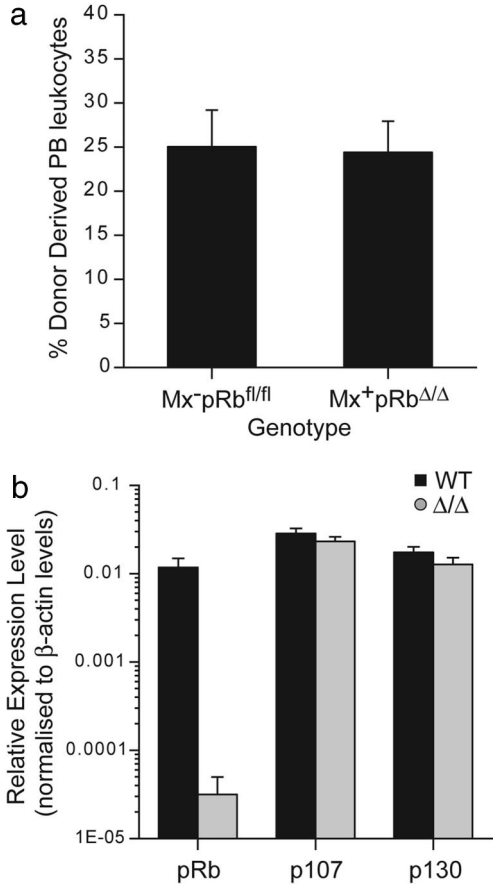

Fig. 4. Rb is not required for HSC self-renewal. (*a*) Secondary analysis of cells from the 1:1 primary transplant. Data represent the average percentage peripheral blood chimerism 9 weeks after transplantation, expressed as the mean ± SEM; $n = 5$ recipients per group. (*b*) Analysis of expression of Rb, p107, and p130 in isolated lin$^-$c-Kit$^+$Sca-1$^+$ cells as analyzed by quantitative RT-PCR from secondary transplant recipients. $n = 4$ independent cDNA samples; data are expressed as means ± SEM.

performed. Bone marrow from all recipients of the 1:1 cell dose transplants was isolated and pooled within each group, and 3×10^6 cells were transplanted into lethally irradiated recipients. Recipients were analyzed at 9 weeks posttransplantation, and no difference was observed in the peripheral blood chimerism (Fig. 4*a*). Subtle differences in hemoglobin levels were observed in secondary recipients, possibly reflecting the reduced level of chimerism observed in these secondary recipients compared with the primary recipients (fl/fl, 14.9 ± 0.13 g/dl; Δ/Δ, 14.5 ± 0.25g/dl; $P = 0.16$). These data demonstrate that Rb is not required for the normal self-renewal of primary and secondarily transplantable HSCs. Together, these data reveal that deletion of Rb is dispensable for both HSC self-renewal and multilineage hematopoiesis.

p130 and p107 Are Not Deregulated in Rb-Deficient HSCs. One possible explanation for the lack of phenotype in Rb-deficient HSCs is that there is compensatory expression of the other pocket protein family members p107 and p130. It was reported in ref. 16 that expression of p107 is significantly up-regulated after germ-line mutation of Rb. To determine whether this up-regulation occurred after acute mutation of Rb in HSCs, the stem cell-enriched lineage-negative, c-Kit-positive, Sca-1-positive (LKS$^+$) population was isolated from the bone marrow of secondary recipient animals by FACS as described in refs. 3 and 24. $Rb^{\Delta/\Delta}$ or $Rb^{fl/fl}$ cells were separated from the competitor bone marrow and endogenous cells based on the congenic CD45 allele ($Rb^{\Delta/\Delta}$ or $Rb^{fl/fl}$ cells are CD45.2$^+$/CD45.1$^-$; competitor cells are CD45.2$^+$/CD45.1$^+$; endogenous cells are CD45.2$^-$/CD45.1$^+$). cDNA was prepared from the isolated cells, and quantitative real-time PCR was performed to determine whether there were any differences in the expression levels of either p107 or p130. Consistent with previous reports, we were able to detect expression of all three transcripts in the stem cell-enriched LKS$^+$ population (25), demonstrating that these transcripts are normally expressed in the stem and primitive progenitor cell populations (Fig. 4*b*). As expected, the level of the Rb transcript was significantly reduced in $Rb^{\Delta/\Delta}$LKS$^+$ cells; however, no alterations in the expression levels of either p107 or p130 transcripts could be detected (Fig. 4*b*). These results demonstrate that loss of Rb from HSCs does not result in alterations in the expression other pocket proteins and that Rb is not required for normal HSC function.

Discussion

The aim of these studies was to determine whether Rb serves an intrinsic role in the regulation of HSC self-renewal and differentiation. The data presented here demonstrate that hematopoiesis is essentially normal in the absence of Rb and that HSC self-renewal is not compromised as a result of Rb deletion. We have utilized a simple transplantation and excision strategy that allows for an assessment of an intrinsic role for Rb in HSC homeostasis. Previous studies have suggested that Rb plays both intrinsic and extrinsic roles in erythroid development and stress erythropoiesis (23, 26, 27). We observed a subtle but stable anemia after somatic deletion of Rb in adult HSCs but normal numbers of phenotypic erythroid cells and progenitors in the bone marrow. We did not observe the progressive myeloid expansion and eventual dominance that was reported after chimera analysis, possibly reflecting inherent differences between germ-line and somatic cell deletion of Rb (23), but we did observe an increase in neutrophil content and cellularity in the bone marrow that was not reflected in the periphery. Alternatively, this increase may be the result of a nonhematopoietic cell role for Rb in the regulation of hematopoiesis, although a myeloid expansion is also observed in p107$^{-/-}$ animals on a BALB/c strain background, suggesting an intrinsic myeloid role for pocket proteins (28). In contrast to that reported for germline deletion of Rb, we did not observe up-regulation of p107 after somatic deletion of Rb (16). Taken together, our findings indicate that Rb does not play an essential intrinsic role in the normal self-renewal of HSCs and is in large part dispensable for hematopoiesis.

The role of Rb in HSC biology has not been clearly defined. Previous studies investigating the roles of the cyclin-dependent kinase inhibitors p21^{Cip1}, p27^{Kip1}, p16^{INK4a}/p19^{ARF}, and p18^{INK4c} have suggested that disruption of normal cell division control may influence the fate of the HSCs (1–4). However, with the exception of p18^{INK4c}, the HSC phenotype of these mutants is relatively subtle, with a 2-fold increase in HSC frequency after the loss of p27^{Kip1} (3). The loss of p21^{Cip1} reveals a significant biological difference in HSC function only after quaternary transplantation (2), with the loss of both p16^{INK4a} and p19^{ARF} resulting in only a modest increase in serial transplant potential (5). Although p18^{INK4c} does result in an increased frequency of HSCs and increased serial transplantation potential, it also results in a lymphoproliferative disorder and predisposes the animals to leukemogenesis (4, 29, 30). Collectively, these results would suggest that cell cycle regulation results in relatively subtle changes in HSC function; consistent with this hypothesis, the loss of Rb does not result in a significant HSC phenotype.

Chien *et al.* (31) have recently reported that transplantation of wild-type bone marrow into a p27$^{Kip1-/-}$ environment/niche

resulted in an expansion of lymphoid cells to a level comparable with that observed in the $p27^{Kip1-/-}$ mutant itself. They demonstrate that the lymphoid expansion in the $p27^{Kip1-/-}$ animals is not intrinsic to the hematopoietic cells, raising the possibility that the expanded HSC population reported in ref. 3 is actually the result of a microenvironment defect or of non-cell cycle regulatory roles of $p27^{Kip1}$ (31, 32). This result is particularly interesting because we observed subtle changes in hematopoiesis after the specific deletion of Rb in the adult HSC, raising the possibility that a significant proportion of the role attributed to cell cycle regulation in HSC self-renewal and differentiation may be mediated by the microenvironment or niche. The nonhematopoietic intrinsic effects observed in the $p27^{Kip1-/-}$ mutant reveal the need for an assessment of the nonintrinsic role of cell cycle regulation in the modulation of HSC biology.

In addition, Rb has been implicated in the phenotype observed in $ATM^{-/-}$ HSCs (7). In the case of the ATM mutant, there was a progressive decline in HSC function that was attributed to the increased cell damage by reactive oxygen species, accompanied by anemia. This phenotype could be ameliorated by overexpression of the viral oncoprotein E7 that is thought to inactivate the Rb. However, the loss of Rb does not phenocopy the loss of ATM. Although we do see a subtle anemia, we did not observe a progressive decline in contribution of the HSCs to hematopoiesis, and $Rb^{\Delta/\Delta}$ HSCs were secondarily transplantable. The results derived from analysis of somatic deletion of Rb would suggest that what role Rb does play in the $ATM^{-/-}$ HSC is minimal; however, loss of Rb in an ATM−/− HSC may not be equivalent to its loss from a wild-type HSC. Although unable to discriminate between intrinsic and extrinsic effects, it has also been recently reported that the Rb homolog plays a role in the maintenance of stem cells in *Arabidopsis* (33).

Our results would suggest that, at least in HSCs, Rb is dispensable. Further analysis of the roles that p107 and p130 play in HSC biology, both in isolation and in combination with Rb, will be important to address whether the pocket proteins do play an important role in regulating stem cell function.

Methods

Experimental Animals. $pRb^{fl/fl}$ mutant mice were generously provided by T. Jacks (Massachusetts Institute of Technology, Cambridge) (16). *Mx-Cre* transgenic mice are described in refs. 15, 18, and 34. All control mice were from within the colony, and all mice were on the same C57BL/6J background (two generations backcrossed onto C57BL/6J from C57BL/6J×129/SV hybrid). Congenic B6.SJL-Ptprc^{a}Pep3^b/BoyJ (Ptprca or CD45.1) mice were purchased from Taconic Farms. For HSC analysis, cells derived from the first-generation cross of C57BL/6J and Ptprca mice were used as the source of competitor bone marrow cells (CD45.1/CD45.2 heterozygous). All experiments were performed with the approval of the respective Animal Ethics Committees (Dana–Farber Cancer Institute and Children's Hospital Boston).

Bone Marrow Transplantation Analysis. Female Ptprca (CD45.1) mice were irradiated with a total of 10.5 Gy (1 Gy = 1 J/kg) (5 Gy and 5.5 Gy 3 h apart) on the day of transplantation. Whole bone marrow was isolated and pooled from $Mx\text{-}Cre^{-}pRb^{fl/fl}$ and $Mx\text{-}Cre^{+}pRb^{fl/fl}$ animals (referred to as test cells). Whole bone marrow competitor cells (2×10^5 or 5×10^5) from wild-type × Ptprca F_1 mice ($CD45.1^+CD45.2^+$ by surface phenotype) were coinjected with increasing numbers of test cells [2×10^5 (1:1), 1×10^6 (2:1), and 2×10^6 (10:1)]. Four or five recipients per cell dose per genotype in each experiment were transplanted with the 1:1 cell dose, performed in duplicate. Recipients were allowed to recover for 5 weeks posttransplant, and then pRb was deleted by i.p. injection of pIpC (Sigma) as described in refs. 15 and 18 (seven doses of 25 μg/g, with the last day of injection designated day 0). Recipients were analyzed before pIpC administration and monthly after completion of pIpC.

For secondary bone marrow transplantation, bone marrow from all individual recipients in the 1:1 cell group was pooled, and then 3×10^6 whole bone marrow cells were injected into cohorts of six lethally irradiated Ptprca recipients.

Analysis of Transplant Recipients. Peripheral blood from each individual recipient was obtained from the retroorbital plexus at the indicated time points posttransplant, and it was analyzed as described in refs. 3 and 35. Peripheral blood hemoglobin levels were measured on an AcT10 analyzer (Beckman Coulter).

For confirmation of deletion of Rb, cells were isolated with a FACSAria flow cytometer (Becton Dickinson). DNA was extracted, and PCR was performed by using standard methods (3, 16). Quantitative real-time PCR was performed after isolation of the LKS^+ fraction ($n = 4$ $Rb^{\Delta/\Delta}$ and $Rb^{fl/fl}$ recipients) on a FACSAria flow cytometer as described in refs. 3 and 36. RNA was isolated with the Micro RNA kit (Qiagen), and cDNA was prepared with SuperScript III by using standard procedures (Invitrogen). Quantitative RT-PCR was performed on an iCycler (Bio-Rad), and values were normalized to β-actin levels.

Statistical Analyses. Statistical analyses were performed with paired and unpaired Student *t* tests. The standard error of the mean was used to determine the deviation from the mean.

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