Rb Regulates Interactions between Hematopoietic Stem Cells and Their Bone Marrow Microenvironment

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SUMMARY

Hematopoiesis is maintained by stem cells (HSCs) that undergo fate decisions by integrating intrinsic and extrinsic signals, with the latter derived from the bone marrow (BM) microenvironment. Cell-cycle regulation can modulate stem cell fate, but it is unknown whether this represents an intrinsic or extrinsic effector of fate decisions. We have investigated the role of the retinoblastoma protein (RB), a central regulator of the cell cycle, in hematopoiesis. Widespread inactivation of RB in the murine hematopoietic system resulted in profound myeloproliferation. HSCs were lost from the BM due to mobilization to extramedullary sites and differentiation. This phenotype was not intrinsic to HSCs, but, rather, was the consequence of an RB-dependent interaction between myeloid-derived cells and the microenvironment. These findings demonstrate that myeloproliferation may result from perturbed interactions between hematopoietic cells and the niche. Therefore, RB extrinsically regulates HSCs by maintaining the capacity of the BM to support normal hematopoiesis and HSCs.

INTRODUCTION

Under homeostatic conditions, the adult hematopoietic system is maintained by a small number of stem cells (HSCs) that reside in the bone marrow in a specialized microenvironment, termed the niche (Adams and Scadden, 2006; Schofield, 1978). It is within the niche that HSCs undertake fate decisions, including differentiative divisions to generate progenitor cells and self-renewal divisions necessary to sustain HSCs throughout life. Both intrinsic and extrinsic cues are integrated within the niche to maintain effective control over HSCs, ensuring contribution to hematopoiesis without aberrant proliferation (Fuchs et al., 2004; Moore and Lemischka, 2006). Whereas the majority of HSCs are in a slowly dividing state, termed relative quiescence, with a cell-division cycle in the mouse in the range of 2–4 weeks, progenitor cells exhibit rapid cycling (Bradford et al., 1997; Passegue et al., 2005). HSCs can also be stimulated to rapidly enter the cell cycle and contribute to hematopoiesis (Li and Johnson, 1994). In part, the dramatic contrast in cell-cycle status between stem and progenitor cells has led to the hypothesis that cell-cycle regulation plays a fundamentally important role in stem cell fate determination.

Decisions to enter the cell cycle are regulated by the G1-S phase restriction point (Sherr and Roberts, 2004). The sequential phosphorylation and subsequent inactivation of the retinoblastoma protein (RB) is an important part of this transition (Weinberg, 1995). RB is phosphorylated by cyclin-cyclin-dependent kinase (Cdk) complexes. Several negative regulators of Cdk activity have been studied in the context of HSC biology. Loss of the Cdk2 inhibitors p21Cip1 and p27Kip1 revealed a divergent role in HSC regulation, with loss of p21Cip1 resulting in a subtle increase in sensitivity to stress-induced exhaustion apparent in vivo after quaternary transplant (Cheng et al., 2000). Loss of p27Kip1 resulted in a 2-fold increase in the number of long-term repopulating HSCs in addition to an enlarged progenitor compartment (Walkley et al., 2005). Loss of both Cdk4/6 inhibitors p16INK4a and p19ARF revealed a small increase in serial transplant potential (Stepanova and Sorrentino, 2005), with a similar phenotype observed in p16INK4a single mutant HSCs (Janzen et al., 2006). Loss of p18INK4c resulted in increased HSC repopulation and frequency (Yuan et al., 2004).

Collectively, these studies suggest that negative cell-cycle regulators that impact directly on RB-family protein function may influence HSC fate. It is indeterminate if
these phenotypes reflect intrinsic or extrinsic effects on HSCs and hematopoiesis, as all studies to date have utilized nonconditional mutant alleles that are not hematopoietic restricted in their effects. The analysis of HSCs from germ-line deficient animals does not allow for the clear delineation of intrinsic and extrinsic contribution to the observed HSC phenotype. Such studies have largely not accounted for effects on HSC genesis or potentially defective niche support that affect HSCs prior to transplantation analysis. While serial transplant studies are suggestive of an intrinsic role for Cdkis in HSC biology, they do not exclude a role for the environment from which these cells were removed, necessitating analysis utilizing hematopoietic restricted deletion. Indeed, a recent study demonstrated that the p27<sup>Kip1</sup><sup>/−</sup> microenvironment mediates lymphoid expansion observed in the p27<sup>Kip1</sup><sup>/−</sup> animals, possibly indicating that the HSC expansion observed in p27<sup>Kip1</sup><sup>/−</sup> bone marrow is extrinsic in nature (Chien et al., 2006; Walkley et al., 2005). This result suggests that cell-cycle regulators may play a role in regulating the competence of the hematopoietic niche in addition to intrinsic roles in HSC fate determination.

Recent studies have begun to characterize the adult bone marrow niche (Schofield, 1978). Osteoblasts appear to comprise an important component of the HSC niche, as modulation of osteoblast number and function influences hematopoiesis and HSC fate via extrinsic mechanisms (Calvi et al., 2003; Vasiijiev et al., 2004; Zhang et al., 2003). Additionally, numerous extrinsic factors modulate HSC function. These factors include retinoic acid, extracellular calcium, osteopontin, angiopoietins, and Notch ligands (Adams et al., 2006; Ari et al., 2004; Purton et al., 2000; Stier et al., 2005; Varum-Finney et al., 1998; Zhang et al., 2006a). Extrinsic regulation of homeostatic HSC numbers can be dominant to intrinsic cues in vivo. For example, HSCs engineered to overexpress HoxB4 expand in vivo only to the level of normal HSCs despite markedly enhanced in vivo self-renewal and proliferative capacity (Krosi et al., 2003). Additionally, systemic factors contained in the peripheral blood of young animals may reactivate self-renewal-associated pathways in progenitors of older animals, suggesting an important role for extrinsic signaling in stem cell regulation (Conboy et al., 2005). While these studies have begun to define the bone marrow niche, little is currently known regarding molecular regulators of the niche and their role in influencing HSC fate decisions. Regulatory interactions between the hematopoietic cells and the nonhematopoietic-derived microenvironment are largely unknown. Moreover, the regulators of these potential interactions and how they affect hematopoiesis and HSC function are unexplored.

Here we have utilized a conditional deletion strategy to investigate the role of the RB in the regulation of adult HSC fate. We found that widespread inactivation of RB resulted in the development of a myeloproliferative disease, characterized by extramedullary hematopoiesis and mobilization of primitive cells into the periphery. HSCs were lost from the BM as a result of increased differentiation and mobilization from the BM. The phenotype is not HSC intrinsic, as it was not recapitulated upon inactivation of RB in HSCs maintained in a wild-type environment (Walkley and Orkin, 2006). Strikingly, however, concomitant deletion of RB from myeloid-derived cells and the microenvironment generated the myeloproliferative disorder, thereby demonstrating that RB is an essential regulator of the interaction between myeloid-derived cells and the BM microenvironment. Thus, RB extrinsically controls HSCs by maintaining the competence of the BM to support normal HSCs and hematopoiesis.

**RESULTS**

**Rb Deletion Leads to Myeloproliferation**

RB was inactivated in hematopoietic cells, including HSCs, using the interferon-inducible Mx-Cre transgene and pRb<sup>ΔΔ</sup> animals (Kuhn et al., 1995; Sage et al., 2003; Walkley and Orkin, 2006). We performed PCR on both genomic DNA and cDNA from whole BM samples to confirm Rb deletion (BM, Figures 1A and 1B). Rb was quantitatively and stably deleted from hematopoietic cells, and expression of the related p130 and p107 was not altered as a result of Rb loss. Thus, with this conditional system, we achieve specific loss of Rb without compensatory gain of expression of other genes coding for pocket proteins.

Analysis of the peripheral blood of control (Mx-Cre<sup>−</sup>pRb<sup>ΔΔ</sup> plpC injected) and Rb<sup>−/−</sup> animals following plpC treatment revealed that Rb<sup>−/−</sup> animals developed a mild but stable anemia immediately following Rb deletion (C.R.W and S.H.O, unpublished data) and by 6 weeks developed thrombocytosis (Figure 1C). While Rb<sup>−/−</sup> animals developed pan-leukocytosis (Figure 1D) that was accompanied by elevated levels of circulating progenitor cells, as determined by in vitro colony-forming capacity (CFU-GEMM and CFU-G) and phenotypic staining (lin<sup>−</sup>c-Kit<sup>−</sup>Sca-1<sup>−</sup>, LKS<sup>−</sup>; Figures 1E and 1F; Okada et al., 1992). Although leukocytosis was apparent by 4 weeks post-Rb deletion, increased circulating progenitors could be detected as early as 2 weeks after plpC (LKS+ increased 3.7-fold, p ≤ 0.01, n = 7 per genotype; CFU-GEMM increased 2.4-fold, p ≤ 0.01, CFU-M/GM increased 3.9-fold, p ≤ 0.01, n = 6 per genotype). Surprisingly, the levels of circulating progenitors were comparable to those achieved during pharmacologically induced mobilization of stem and progenitors in the C57Bl/6 strain background (Ghiaur et al., 2006). However, this was a chronic, rather than an acute, response in the Rb<sup>−/−</sup> mutant.

BM cellularity was not initially altered; however, at 12 weeks post-plpC it was increased by 40% in Rb<sup>−/−</sup> animals (Figure 2A). We observed the rapid development of a myeloproliferative-like disease within the bone marrow. The phenotype was fully penetrant and was characterized by myeloid hyperplasia (predominantly neutrophilia) and suppression of both B-lymphopoiesis and erythropoiesis (Figures 2B, S1, and S2). Phenotypic stem and primitive progenitor populations (LKS<sup>−</sup> and LKS+) were increased significantly in the BM of Rb<sup>−/−</sup>
animals (Figure 2C); however, the number of phenotypic HSCs per femur was not significantly altered (LKS+CD34+/CD38lo; Osawa et al., 1996; Yang et al., 2005). In addition, RbD/D animals exhibited striking changes in the architecture of the bone, evidenced by loss of trabecular bone (Figure 2D). Trabecular bone is thought to represent an important niche for HSCs within the BM (Calvi et al., 2003). Quantitative histomorphometric analysis of the bone at 2 weeks post-pIpC, a time point correlating with the presence of progenitors in the peripheral blood and spleen, demonstrated a significant reduction in trabecular volume as a proportion of total marrow volume, a 40% reduction in the number of trabeculae, and a doubling of the separation of trabeculae (Figures 2E–2H).

In parallel with BM myeloproliferation, RbD/D animals developed extensive extramedullary hematopoiesis. Spleen weight increased rapidly by 5.5-fold relative to controls due to expanded numbers of myeloid cells, megakaryocytes, and erythrocyte cells (Figures 2I and 2J). B- and T cell lymphopoiesis were present at comparable levels in RbD/D and control spleens. Phenotypic stem and progenitor populations (LKS+ and LKS−) increased progressively in the spleens of RbD/D animals, and by 12 weeks were increased 45- to 50-fold (Figures 2K, S3, and S4). Splenic architecture was effaced as a result of myeloid and erythroid elements (Figure 2J). Hematopoietic foci were also observed in the liver but not in the kidney (data not shown). Despite chronic myeloproliferation, no hematopoietic tumors have developed during the lifespan of RbD/D mutant animals. RbD/D animals survive for approximately 8 months post-pIpC; heterozygous animals are normal (Figures S5 and S6). Eight-month-old RbD/D animals present with a phenotype reminiscent of hematopoietic failure, characterized by a significant reduction in

Figure 1. Rapid Mobilization of Primitive Cells into the Peripheral Blood Following Deletion of Rb
(A) Genomic PCR on whole BM from control (Mx°pRbf/f) and Rb-deficient animals (MxpRbD/D) at 6 and 12 weeks post-pIpC.
(B) qRT-PCR for pRb, p107, and p107 on cDNA of control and Rb-deficient animals (n = 3 independent samples) 12 weeks post-pIpC.
(C) Platelets and (D) leukocytes in PB following Rb deletion (time 0 = final dose of pIpC); n ≥ 4/time point; *p < 0.05.
(E) Day 12 CFU-GEMM and CFU-GM/M from the PB of at 12 weeks post-pIpC; n ≥ 9/genotype; *p < 0.01. Value inside bars represents fold increase.
(F) FACS profile and mean number of Lin−c-Kit+Sca−1+ (LKS+) in the PB; n > 4/genotype; *p < 0.01. Methylcellulose plates from day 12 of culture. Data expressed as mean ± SEM.

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Figure 2. Myeloproliferation Following Rb Deletion

(A) Femoral cellularity, n ≥ 3/genotype/time point.

(B) Number of cells of each lineage/femur at 12 weeks post-pIpC: Granulocytes CD11b<sup>+</sup>Gr-1<sup>+</sup>, Macrophages CD11b<sup>+</sup>F4/80<sup>+</sup>, Immature B lymphoid IgM<sup>+</sup>‘B220<sup>+</sup>’, Mature B lymphoid IgM<sup>+</sup>‘B220<sup>+</sup>’, Mature Erythroid CD71<sup>+</sup>‘Ter119<sup>+</sup>’, and Immature Erythroid CD71<sup>+</sup>‘Ter119<sup>+</sup>; n ≥ 6/genotype; *p < 0.01.

(C) Number of phenotypic HSCs (LKS<sup>+</sup>CD34<sup>+</sup>/low) and primitive progenitors/femur; 12 weeks post-pIpC; n ≥ 5/genotype; *p < 0.05.

(D) Representative sections of tibiae at 12 weeks post-pIpC.

(E) Volume of marrow space occupied by bone (BV/TV); 2 weeks post-pIpC; n ≥ 13/genotype; *p < 0.05.

(F) Trabecular number/mm; *p < 0.05.

(G) Separation of trabeculae; *p < 0.05.

(H) Representative longitudinal sections of tibiae stained with Von Kossa technique (mineralized bone stained black).

(I) Spleen cellularity; n ≥ 3/genotype/time point; *p < 0.01.
spleen weight and replacement of BM by granulocytes; however, pituitary tumors are also observed (Figure S5 and data not shown).

**HSCs Are Lost from BM following RB Deletion**

RB and other negative cell-cycle regulators have been postulated to play an important role in the regulation of HSCs and in the subsequent hematopoiesis. However, neither the myeloproliferative disease nor defective HSC function was observed when RB was deleted from HSCs in the context of a wild-type microenvironment (Walkley and Orkin, 2006). Given the striking phenotype we observed when RB was deleted from both hematopoietic cells and the BM microenvironment, as occurs with Mx-Cre (Zhang et al., 2003), we sought to determine the consequences of RB loss on HSCs in these animals.

Within the BM we observed a significant increase in the frequency of mature day 7 colony-forming cells but a decrease in the frequency of the more primitive in vivo day 12 colony-forming unit-spleen (CFU-S12, Figures 3A and 3B). The numbers of both in vitro colony-forming cells (68-fold increase in CFU-GEMM) and CFU-S8 in the spleen were increased by 8-fold (p = 0.0005). When normalized to reflect the increased cellularity of the BM (2 x 10^6 or 2 x 10^5) we observed high levels of circulating progenitors and substantial levels of progenitor activity in the spleen, we sought to determine if latent HSC activity was also present in extramedullary sites. Whole spleen cells from RB^−/−^ animals (either 1 x 10^6 or 2 x 10^5) were transplanted with competitor whole BM (2 x 10^5) into congenic recipients. At 17 weeks post-transplant, significant multilineage repopulating activity derived from the spleens of RB^−/−^ animals was present, demonstrating that functional HSCs were present in the periphery of RB^−/−^ mice (Figures 3E and 3F).

To determine the HSC content of the BM, we performed limit-dilution competitive repopulation analysis with whole BM from donor animals treated 12 days earlier with pIpC (Purton et al., 2006; Szilvassy et al., 1990; Walkley et al., 2005). Whole BM from control (RB^+/+^) or RB-deficient (RB^−/−^), both CD45.2+ mice was mixed at varying doses with a fixed number of competitor BM cells (CD45.1+/CD45.2+) and transplanted into congenic recipient animals (CD45.1+). The frequency of long-term repopulating HSCs in the RB^−/−^ BM at 6 months posttransplant was reduced by 8-fold (p = 0.0005). When normalized to reflect the increased cellularity of the RB^−/−^ BM, this represents a 5-fold decrease in the absolute number of HSCs per femur (Figure 4A). Secondary transplantation demonstrated that RB^−/−^ HSCs were serially transplantable and capable of stable multilineage contribution for at least 3 months. Importantly, we did not observe a progressive decline in contribution from RB^−/−^ HSCs to hematopoiesis, thereby demonstrating that self-renewal-mediated maintenance of HSCs over time is not affected by the absence of RB (Figure S7).

When 1000 freshly isolated lin^−^c-Kit^+^Sca-1^+^ cells were competetively transplanted, RB^+/+^ LKS+ displayed a 20-fold reduction in long-term repopulating potential on a per-cell basis (Figure 4A). The reduced repopulating potential of the RB^−/−^ LKS+ fraction, despite a marked increase in this phenotypic population observed in the bone marrow (see Figure 2), demonstrates that the surface phenotype of the cells does not faithfully reflect their functional potential. We, and others, have previously observed a lack of fidelity of phenotypic markers both in mutant mice and following perturbation of homeostasis in wild-type animals (Purton et al., 2006; Spangrude et al., 1995; Tajima et al., 2000; Walkley et al., 2005).

HSCs may be lost from the BM for several reasons, including a failed capacity of RB^−/−^ HSCs to home and engraft following transplantation, an increased rate of apoptosis, or a mobilization/redistribution to extramedullary sites. As cell-cycle status correlates with engraftment capacity of HSCs (Gothot et al., 1998; Passegue et al., 2005), we directly assessed the cell-cycle status of phenotypic RB^−/−^ progenitors (LKS−), primitive progenitors (LKS+), and HSCs (LKS+CD34^-/lo^). All three populations displayed a comparable cell-cycle profile, with HSCs from RB^−/−^ displaying the same distribution of cells in the G0/G1, or S phase of the cell cycle as control cells (flu: flu: G0/G1 = 86.2 ± 5.7%; G1/S: G0/G1 = 85.1 ± 2.4%, p = 0.85, flu/flu: S = 6.7 ± 1.9%; G1/S: S = 7.8 ± 0.7%, p = 0.54, n = 4/flu, 7 G1/S; expressed as mean ± SEM). There was no difference in the rate of cell-cycle entry of LKS+ cells between control and RB^−/−^ cells as determined by BrdU-incorporation rates at either 2 or 4 weeks post-pIpC (Figure S9). Furthermore, analysis of the in vivo homing of RB^−/−^ BM did not reveal a difference compared to control BM at either 2 or 12 weeks post-pIpC (Figure 4B and data not shown). RB^−/−^ LKS+ cells exhibited decreased apoptosis, as assessed by annexin-V staining, at 2 weeks and exhibited normal levels at 12 weeks post-pIpC compared to control LKS+ cells (Figure 4C). As we had observed significantly increased progenitors and HSCs in extramedullary sites, these data are consistent with the loss of HSCs from the BM as a result of both enhanced differentiation of HSCs within the BM and a redistribution to extramedullary sites as a consequence of the changes in the niche.

**Myeloid-Restricted Inactivation of RB Does Not Result in Myeloproliferation**

To determine the contribution of myeloid-derived cells (granulocytes, macrophages, and osteoclasts) to the myeloproliferation observed in RB mutants, we generated Lysosome-M-Cre pRbfl/fl mice to achieve myeloid-restricted deletion of RB (Figure 5). Deletion of RB with Lys-M-Cre did not lead to myeloproliferation or extramedullary hematopoiesis, consistent with the results.
obtained by deletion of Rb from hematopoietic cells in a wild-type environment (Figure 5A; Walkley and Orkin, 2006). We observed a subtle increase in the numbers of granulocytes in the BM and slight reduction in erythroid cells, but no change in either lymphoid or phenotypic progenitor and HSC-enriched fractions (Figures 5A and 5B). Lineage distribution within the spleen or PB was largely comparable to controls (Tables S1 and S2; data not shown). Thus, deletion of Rb from myeloid-derived populations does not recapitulate the phenotype observed in the Mx-Cre model. Collectively, these results suggest that RB may regulate HSCs and hematopoiesis in an extrinsic manner, possibly through regulating the competence of the bone marrow niche.

An Rb-Dependent Interaction between Myeloid-Derived Cells and the BM Microenvironment Results in Myeloproliferation

We next sought to determine the relative contributions of the hematopoietic cells and the nonhematopoietic (non-transplantable) elements of the BM microenvironment to the observed phenotype. Hematopoietic cells alone were not capable of inducing either myeloproliferation or the loss of HSCs from the BM that we observed in the
Mx-CreRb<sup>D/D</sup> model (Walkley and Orkin, 2006). Consistent with this conclusion we did not observe myeloproliferation when previously excised Rb<sup>D/D</sup> HSCs were supported by a wild-type microenvironment, even at high cell doses (described in Figure 3).

To ascertain if RB loss from the niche was responsible for the myeloproliferation and loss of BM HSCs, reciprocal transplants of wild-type hematopoietic cells into lethally irradiated Mx-Cre<sup>+/C0</sup>pRb<sup>fl/fl</sup> and Mx-Cre<sup>+/C0</sup>pRb<sup>fl/fl</sup> recipients were performed, and, following establishment of hematopoiesis, recipients were injected with pIpC to delete Rb from the hematopoietic microenvironment. This strategy was successfully used to demonstrate a role for BMP receptor type 1 in the regulation of the HSC niche (Zhang et al., 2003), and a similar approach demonstrated that a RAR<sub>γ</sub><sup>−/−</sup> microenvironment alone could induce myeloproliferation (Walkley et al., 2007 [this issue of Cell]). Having observed significantly increased neutrophils and monocytic-derived osteoclasts in the Mx-Cre<sup>D/D</sup> model (Figure 5C), we hypothesized that deletion of Rb from myeloid-derived cells together with an Rb-deficient microenvironment might recapitulate the phenotype observed in the Mx-Cre<sup>D/D</sup> model.

Hematopoietic cells from sex-mismatched Lysozyme-M-Cre pRb<sup>fl/fl</sup> animals were transplanted into lethally irradiated Mx-Cre<sup>pRb<sup>fl/fl</sup></sup> and Mx-Cre<sup>pRb<sup>fl/fl</sup></sup> recipients, and, following establishment of hematopoiesis, recipients were injected with pIpC to delete Rb from the BM microenvironment. Analysis of Y chromosome levels by quantitative PCR on peripheral blood leukocytes confirmed engraftment and high-level chimerism of all recipients prior to pIpC and at the time of analysis (data not shown). This transplant strategy resulted in Rb deficiency in myeloid-derived cells (granulocytes, macrophages, and osteoclasts) and an Rb-deficient niche. In addition, RB expression is retained within the HSC compartment.

We observed synergistic interaction between the pRb<sup>D/D</sup> myeloid cells and the pRb<sup>D/D</sup> microenvironment (Figure 6). Mx-Cre<sup>pRb<sup>D/D</sup></sup> recipients rapidly developed signs of distress as early as 2 weeks after the completion of plpC were markedly increased (Figures 5C and 5D). Osteoclasts and macrophages derived from plpC-treated Mx-Cre mice showed efficient deletion of Rb, as did osteoclasts and macrophages derived from Lysozyme-M-Cre pRb<sup>fl/fl</sup> mice (Figure 5E). Osteoclasts have been proposed to contribute to the release of HSCs from the bone marrow during mobilization (Kollet et al., 2006). Having observed significantly increased neutrophils and monocyctic-derived osteoclasts in the Mx-Cre<sup>pRb<sup>D/D</sup></sup> model (Figure 5C), we hypothesized that deletion of Rb from myeloid-derived cells together with an Rb-deficient microenvironment might recapitulate the phenotype observed in the Mx-Cre<sup>pRb<sup>D/D</sup></sup> model.

Bone homeostasis is maintained through balanced activities of mesenchymal-derived osteoblasts and myeloid-derived osteoclasts (Martin and Sims, 2005). As we observed a rapid loss of trabecular bone following Mx-Cre-mediated deletion, we quantitated the numbers of osteoclasts present in the BM and spleen. The numbers of osteoclasts in both the BM and spleen by 6 weeks post-
pIpC. No Mx-Cre·pRbΔ/Δ recipient survived beyond 11 weeks post-pIpC. Greater than 70% of recipients were moribund by 5 weeks, in contrast to recipients of wild-type BM cells that survived at least 20 weeks post-pIpC with no significant changes in hematopoiesis (Figure 6). We observed rapid development of a completely penetrant myeloproliferative disorder in the BM, characterized by myeloid cell hyperplasia and suppression of lymphopoiesis.
and erythropoiesis. Splenomegaly, accompanied by myeloid and erythroid hyperplasia and extramedullary hematopoiesis, was also observed, demonstrating a striking similarity to the full Mx-Cre model (see Figure 2 and Tables S1 and S2). The increased spleen size of Mx-Cre<sup>−</sup> recipients of LysM-Cre<sup>pRb<sup>fl/fl</sup></sup> BM can be accounted for by the LysM-Cre<sup>pRb<sup>fl/fl</sup></sup> BM itself, rather than a contribution from the recipient environment (Tables S1
and S2). These data demonstrate that the observed myeloproliferation is the consequence of an RB-dependent interaction between myeloid-derived cells and the BM microenvironment and that it develops independent of the HSC RB status. Our data provide direct experimental evidence that myeloproliferation may ensue from aberrant interactions between myeloid-derived cells and the BM microenvironment, revealing hematopoietic extrinsic contribution to myeloproliferation.

DISCUSSION

We sought to determine the role RB plays in the regulation of hematopoiesis and stem cell function. Recent studies suggest that cell-cycle regulation is an important determinant of stem cell fate; however, none have discriminated between intrinsic or extrinsic contributions (Cheng et al., 2000; Janzen et al., 2006; Walkley et al., 2005; Yuan et al., 2004). RB was implicated as an important regulator of stem cell maintenance in Arabidopsis; however, the limitations of the experimental system did not allow for the clear demonstration of a stem cell intrinsic role for RB (Wildwater et al., 2005). Here we demonstrate that RB extrinsically regulates HSCs by maintaining the competence of the adult bone marrow to support HSCs and, in turn, normal homeostatic hematopoiesis.

Rb and Stem Cell Self-Renewal

Understanding the regulation of cell cycle in stem cells is important from several perspectives. Stem cells must enter the cell cycle to self-renew; hence, induction of cycling may be desirable to achieve HSC expansion. Engraftment of transplanted HSCs is cell cycle dependent (Gothot et al., 1998; Passegue et al., 2005). The slow cycling of HSCs may spare them from acute toxicity (such as chemotherapy) but may also prevent neoplastic cells from eradication (Hodgson and Bradley, 1979; Lerner and Harrison, 1990). Our understanding of the normal regulation of self-renewal will also provide insight into tumorgenesis, where self-renewal pathways are thought to be active (Krivtsov et al., 2006).

The importance of cell-cycle regulation in HSC fate decisions has been suggested by the analysis of animals deficient in negative cell-cycle regulators such as p21\(^{Cip1}\), p27\(^{Kip1}\), and p16\(^{INK4a/p19ARF}\) (Cheng et al., 2000; Stepanova and Sorrentino, 2005; Walkley et al., 2005). However, these studies have not revealed if such HSC defects are cell intrinsic or extrinsic in nature. The “RB pathway” has also been implicated in phenotypes observed in both the Bmi1\(^{-/-}\) and ATM\(^{-/-}\) HSCs (Ito et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003). Surprisingly, we did not observe an intrinsic requirement for Rb in HSCs. If provided with a wild-type niche, Rb\(^{−/−}\) HSCs contribute normally to multilineage hematopoiesis and display serial transplant potential comparable to wild-type HSCs. Furthermore, we failed to detect alterations in numerous cell-cycle- or self-renewal-associated genes in Rb-deficient HSCs and progenitors isolated from both a wild-type and mutant microenvironment, consistent with our interpretation that Rb is dispensable in the HSCs (Figure S8). Our observations, taken together with those from analysis of p27\(^{Kip1}\) mutant mice, reveal that cell-cycle regulation is a novel extrinsic regulator of hematopoiesis (Chien et al., 2006; Walkley and Orkin, 2006). The loss of BM HSCs in the Mx-Cre model is a secondary consequence of the disrupted environment within the BM and was not observed in the context of a wild-type niche, demonstrating that myeloproliferative-like disorders may deplete HSCs from the BM. A reanalysis of cell-cycle mutants proposed to harbor HSC defects is needed to clarify the intrinsic and extrinsic roles that cell-cycle regulation plays in these phenotypes.

It has been documented that self-renewal of embryonic stem cells occurs in an RB independent manner (Stead et al., 2002); however, the RB pathway is thought to be near universally targeted in human cancer cells (Hanahan and Weinberg, 2000). We have described that self-renewal of nontransformed HSCs occurs independent of RB, highlighting the important question of what role RB plays in the regulation of the process of cellular self-renewal in both normal and oncogenic settings. It may be that the requirement for RB and the RB pathway in self-renewal is developmentally and lineage dependent, with progenitor cells having a greater dependence on RB for their division than bona fide stem cells. One prediction of such a hypothesis is that mutation of the RB pathway is of greater benefit to a progenitor cell than a stem cell during tumor formation.

Rb and Hematopoiesis

Previous studies examining the role of RB in hematopoiesis have raised conflicting evidence regarding intrinsic and extrinsic effects, particularly in erythropoiesis (Clark et al., 2004; Iavarone et al., 2004; Spike et al., 2004; Whyatt and Grosveld, 2002). Our study utilized compartment-restricted somatic mutagenesis to analyze the role of RB in adult hematopoiesis and HSCs. Compartment restricted deletion enables a direct assessment of the contribution of hematopoietic and nonhematopoietic cells to the observed phenotype. We have not observed a progressive failure of hematopoiesis as reported by Spike et al. (2004) when RB-deficient HSCs were supported by a wild-type environment, nor was this observed in a separate study utilizing germline-deficient fetal liver hematopoietic cells (Hu et al., 1997). In contrast to in vitro findings (Iavarone et al., 2004), deletion of Rb from myeloid-derived cells using Lys-M-Cre did not result in anemia in vivo. Further studies utilizing lineage-restricted deletion of Rb will be required to clarify the role of RB in erythropoiesis.

Interactions between Hematopoietic Cells and Their Microenvironment Regulate HSCs and Hematopoiesis

The phenotype of Mx-Cre\(^pRb^{−/−}\) animals is due to an RB-dependent interaction between myeloid-derived cells (most probably macrophages and osteoclasts) and the
bone marrow microenvironment (summarized in Tables 1 and S1). Evidence supports a direct role for the bone marrow microenvironment, but we cannot entirely exclude a contribution from other sites of Cre activity in the Mx-Cre model. Myeloproliferation is generally considered to be hematopoietic intrinsic, and evidence from the overexpression of activated kinase receptors in mouse models is consistent with this view (Araki et al., 2004; Chan et al., 2004; Le et al., 2004). In light of the data derived from Mx-Cre-pRb<sup>fl/fl</sup> mice, the BM microenvironment may play an active role in the promotion and/or maintenance of myeloproliferative disorders. Additional studies are required to define the cell(s) within the BM niche that are responsible for this interaction. The BM microenvironment is composed of numerous nonhematopoietic cell types including osteoblasts, endothelial cells, adipocytes, and nerve cells. Histomorphometry demonstrated a significant disruption to bone homeostasis in the Rb-deficient animals, correlating with the observed mobilization and extramedullary hematopoiesis (Figures 2E–2H). Myeloproliferation in Mx-Cre-pRb<sup>-/-</sup> mice is dependent on concomitant deletion of Rb from both myeloid-derived cells and the environment. In other situations, myeloproliferation may result directly from an aberrant niche and may be independent on mutation(s) within hematopoietic cells (Walkley et al., 2007)

Evidence of the role of stroma and the microenvironment in oncogenesis is accumulating, notably from analysis of solid tumors. Moreover, mathematical modeling of tumor behavior predicts that the environment is a major selective modifier of tumor morphology and phenotype (Allinen et al., 2004; Anderson et al., 2006; Balkwill, 2004; Hill et al., 2005; Kurose et al., 2002; Oh et al., 2004; St Croix et al., 2000). Somatic mutations divergent from those found in the tumor have been identified in stromal cells. In prostate cancer, results suggest that such mutations may contribute nonautonomously to tumor behavior (Hill et al., 2005; Kurose et al., 2002). Understanding the interactions between hematopoietic cells and their microenvironment is directly relevant to hematopoietic disease. Mutations in the Rb pathway occur in ~75% of cases of multiple myeloma (Kramer et al., 2002). Multiple myeloma clearly demonstrates that the interaction of hematopoietic cells—in this case B cells—and the BM microenvironment is a major contributor to disease (Hideshima and Anderson, 2002; Mitsiades et al., 2006). These studies have focused on mutations present in established disease but have not addressed the role of the microenvironment or stroma in the initiation of the disease process.

In addition to our data focused on RB loss, the significance of the hematopoietic microenvironment to disease initiation has been suggested by recent studies. Mx-Cre<sup>Pten<sup>fl/fl</sup></sup> mice (Pten-deficient hematopoietic cells and microenvironment) develop rapid and aggressive myeloproliferation that progresses to overt leukemia/lymphoma in 4 to 5 weeks postdeletion (Yilmaz et al., 2006; Zhang et al., 2006b). However, when Pten deletion

Table 1. Summary of the Phenotype Observed Following Loss of Rb

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hematopoietic Cells</th>
<th>Niche / Microenvironment</th>
<th>HSC</th>
<th>Myeloid</th>
<th>Lymphoid</th>
<th>Erythroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mx-Cre pRb&lt;sup&gt;fl/fl&lt;/sup&gt; model</td>
<td>Δ/Δ</td>
<td>Δ/Δ</td>
<td>↓↓↓</td>
<td>↑↑↑</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Δ/Δ HSC into WT niche&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Δ/Δ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>WT</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>WT HSC into Δ/Δ niche</td>
<td>Δ/Δ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Δ/Δ</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Δ/Δ myeloid cells</td>
<td>Δ/Δ (myeloid)</td>
<td>Δ/Δ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↑↑↑</td>
<td>↓</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>Δ/Δ myeloid cells into Δ/Δ niche</td>
<td>Δ/Δ (myeloid)</td>
<td>Δ/Δ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↔</td>
<td>↑</td>
</tr>
</tbody>
</table>

<sup>a</sup> Summary of data previously described (Walkley and Orkin, 2006).

<sup>b</sup> Indicated compartment was nondeleted (Mx-Cre pRb<sup>fl/fl</sup>) at time of transplant and deleted 5 weeks after transplant with pIpC injection.

<sup>c</sup> HSC and Progenitors as determined by flow cytometry (Lin<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> and Lin<sup>-</sup>c-Kit<sup>-</sup>Sca-1<sup>-</sup>) and in vitro progenitor analysis.

In Table 1 and S1, we show evidence for a nonautonomous role for the microenvironment in hematopoietic disease. Evidence suggests that mutations in the Rb pathway may contribute nonautonomously to tumor behavior (Hill et al., 2005; Kurose et al., 2002). Understanding the interactions between hematopoietic cells and their microenvironment is directly relevant to hematopoietic disease. Mutations in the Rb pathway occur in ~75% of cases of multiple myeloma (Kramer et al., 2002). Multiple myeloma clearly demonstrates that the interaction of hematopoietic cells—in this case B cells—and the BM microenvironment is a major contributor to disease (Hideshima and Anderson, 2002; Mitsiades et al., 2006). These studies have focused on mutations present in established disease but have not addressed the role of the microenvironment or stroma in the initiation of the disease process.

In addition to our data focused on RB loss, the significance of the hematopoietic microenvironment to disease initiation has been suggested by recent studies. Mx-Cre<sup>Pten<sup>fl/fl</sup></sup> mice (Pten-deficient hematopoietic cells and microenvironment) develop rapid and aggressive myeloproliferation that progresses to overt leukemia/lymphoma in 4 to 5 weeks postdeletion (Yilmaz et al., 2006; Zhang et al., 2006b). However, when Pten deletion
was activated in the context of a wild-type BM microenvironment, phenotypic and functional HSCs were lost without evidence of myeloproliferation or transformation (Yilmaz et al., 2006). This striking result suggests that \( \text{Pten}^{+/+} \) hematopoietic cells alone are not intrinsically susceptible to myeloproliferation and subsequent malignant transformation in the presence of a wild-type microenvironment. Mutations in PTEN have been reported in the stroma of human breast tumors, suggesting a broader role for this pathway in the microenvironment and stroma of diverse organ systems (Kurose et al., 2002). Intriguingly JunB, Bmi-1, and ATM, implicated in HSC regulation and myeloproliferation, also have roles in regulating the bone marrow microenvironment (Kenner et al., 2004; Oguro et al., 2006; Passegue et al., 2004; Rasheed et al., 2006). JunB-deficient mice develop severe osteopenia due to intrinsic defects in osteoclasts and osteoblasts, cellular constituents of the HSC niche, while ATM mutants develop osteoporosis as a result of defective osteoblast differentiation. The contribution of these microenvironmental defects to the HSC phenotypes in these mutants has yet to be described. Such results demonstrate the need for further analysis of the interaction between the hematopoietic cells and their environment. This reconsideration will further our understanding of normal homeostatic hematopoiesis and the development of hematopoietic disease.

Our finding that the myeloproliferative-like disorder in the \( \text{Rb} \) mutants is the result of an interaction between myeloid-derived cells and the bone marrow microenvironment, together with the microenvironment-induced myeloproliferative-like disorder that develops in the \( \text{RAR} \gamma^+ \) mice (Walkley et al., 2007), underscores a previously unrecognized role for the hematopoietic microenvironment in the development of myeloid disease. These data further suggest that mutations within the hematopoietic niche might also serve as initiating events in the development of hematopoietic disease. In contrast to previous reports of the importance of cell-cycle regulation in HSC fate determination, we find scant evidence for an intrinsic requirement for RB in HSCs and that, indeed, RB is a novel extrinsic regulator of hematopoietic stem cells. As our findings underscore, interactions between hematopoietic cells and the bone marrow niche/microenvironment profoundly affect hematopoietic homeostasis and the behavior of HSCs.

**EXPERIMENTAL PROCEDURES**

A detailed version of the Experimental Procedures can be found in the Supplemental Data.

**Experimental Animals**

\( \text{pRb}^{fl/fl} \) mutant mice were generously provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, MA, USA; Sage et al., 2003). \( \text{Mx-Cre} \) transgenic mice have been previously described (Kuhn et al., 1995; Walkley and Orkin, 2006). \( \text{Lys-M-Cre} \) animals were purchased from The Jackson Laboratory (Clausen et al., 1999). All experiments were performed with approval of the respective Institute Animal Ethics Committees (DFCI or CHB).

**Flow Cytometry Analysis**

All antibodies and clone numbers are listed in Supplemental Experimental Procedures. Flow cytometry was performed on a FACS Calibur, and sorting was performed on a FACS Aria; all data were analyzed using Cell Quest Pro software (Becton Dickinson).

**Progenitor Cells Assays: CFC and CFU-S**

BM, spleen cells, and PB leukocytes were assessed for in vitro colony-forming cell (CFC, defined as >50 cells/colony) potential at either day 7 CFC (DME/agar media) or day 12 CFC (IMDM/methylcellulose media) as described in Supplemental Experimental Procedures. Colony-forming unit-spleen (CFU-S) was performed using the CFU-S assay of Till and McCulloch with both WBM (CFU-S day 12) and spleen (CFU-S day 8)-derived cells (Purton et al., 1999; Till and McCulloch, 1961).

**Long-Term Repopulating Cell (HSC) Analysis**

Limit-dilution competitive repopulation analysis was performed as previously described (Purton et al., 2000; Szilvassy et al., 1990; Walkley et al., 2005) using test cell doses of \( 3 \times 10^3, 2 \times 10^3, \) and \( 2 \times 10^2 \) cells competed against \( 2 \times 10^3 \) WT WBM (CD45.1+/CD45.2−). Four to five recipients/cell dose-genotype/experiment were transplanted, and the experiment was performed in duplicate. Recipients were analyzed at 3 and 6 months posttransplant. HSC frequencies were calculated using L-Calc software (StemCell Technologies Inc.) using Poisson statistical analysis (Taswell, 1981). Secondary recipients were analyzed at 3 months posttransplant. One thousand freshly isolated LKS+ were injected with \( 2 \times 10^5 \) competitor WBM into five recipients per genotype.

Whole spleen cells were isolated from \( \text{Rb}^{+/−} \) 8 weeks post-plpC and competitively transplanted (\( 1 \times 10^7 \) or \( 2 \times 10^7 \)) with \( 2 \times 10^7 \) competitor WBM into five recipients per genotype.

For transplant of WT or \( \text{Lys-M-Cre}^{+/−} \text{pRb}^{−/−} \) WBM into \( \text{Mx-Cre}^{−/−} \) or \( \text{Mx-Cre}^{−/−} \text{pRb}^{−/−} \) recipients, recipients were transplanted with \( 3 \times 10^5 \) WBM from sex mismatched animals. Chimerism was confirmed either by CD45.1/CD45.2 allele analysis or by Y chromosome qPCR as indicated.

**Analysis of Transplant Recipients**

PB from each individual recipient was obtained from the retro-orbital plexus at the indicated time points posttransplant and was analyzed as described for chimerism and lineage contribution of test cells (Purton et al., 2000; Walkley et al., 2005).

**Statistical Analyses**

Statistical analyses were performed using the paired and unpaired Student’s t test. Calculation of HSC frequency was performed using Poisson statistical analysis using L-Calc software (StemCell Technologies Inc.). Histomorphometric data was analyzed by ANOVA followed by Fisher’s PLSD Test.

**Supplemental Data**

Supplemental Data include Experimental Procedures, References, two tables, and nine figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/6/1081/DC1/.

**ACKNOWLEDGMENTS**

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REFERENCES


