Retinoblastoma Gene Deficiency Has Mitogenic but not Tumorigenic Effects on Erythropoiesis

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ABSTRACT

The retinoblastoma protein (Rb), an important ubiquitous cell cycle regulator, was initially identified as the retinoblastoma tumor suppressor. To further address the activities of Rb in proliferation and tumorigenesis in the hematopoietic lineage, we transplanted Rb−/− fetal liver cells into sibling mice and assessed the outcome of Rb−/− hematopoietic cells in both short-term and long-term studies. Rb−/− hematopoietic cells rescued lethally irradiated mice with an efficiency comparable to that of wild-type cells. In spleen colony-forming unit assays, proliferation rates of the Rb−/− cells were greater than those of the wild-type cells. Similarly, in vitro burst-forming unit-erythroid and colony-forming unit-erythroid assays showed increased erythroid colony numbers from Rb−/− embryonic livers. Recipients of Rb−/− cells lived for more than 15—18 months, and most blood cell lineages matured normally with the expected switch from fetal to adult hemoglobin. However, the continued presence of nucleated erythrocytes in the peripheral blood and extensive extramedullary erythropoiesis indicated that the Rb−/− erythrocytes were not completely normal. No erythroleukemia developed during the 15—18 month period following transplantation. These results demonstrate the mitogenic effect but not tumorigenic transformation in erythocyte lineage in the absence of Rb, which is distinct from the effect of Rb deficiency in neuroectodermal cells. The study supports the prevalent model that loss of the ubiquitously expressed tumor suppressor gene predisposes to only a limited spectrum of tumors.

INTRODUCTION

The human retinoblastoma susceptibility gene is one of the best studied tumor suppressors that is mutated in multiple tumor types (reviewed in Refs. 1—3). Mutational inactivation of the retinoblastoma gene is the rate-limiting step in Rb3 formation (4—8). Rb is also mutated in other tumors, such as small-cell lung carcinoma and breast carcinoma (9—11). The heterogeneous expression of the Rb protein in the breast carcinoma suggests that inactivation occurs during progression of the tumor (10, 12).

Rb plays an important regulatory role in G1-S progression (13). It is a substrate for cyclin-dependent kinases and an inhibitor of the transcription factor E2F (reviewed in Refs. 1—3, 14, and 15). Hypophosphorylated Rb inhibits G1-S progression, and phosphorylation of Rb by cyclin-dependent kinases abrogates the inhibitory activities of Rb (13, 16). The hypophosphorylated form of Rb binds to E2F (17—19) and represses its transcription activator function, which is required for the expression of S-phase genes (reviewed in Ref. 15).

Two Rb-related genes, p107 and p130, have been identified (20, 21). p107 and p130 result in enhanced proliferation (22). Inactivation of both Rb and p107 promotes proliferation of the neuroretina cells (23). Therefore, effects of Rb deficiency on specific cell types may depend on the activities of Rb-related genes in these cells. Homozygous Rb−/− embryos die between E12.0 and E16.0 with major defects in neurogenesis, lens development, erythropoiesis, and myogenesis (24—27). However, chimeric mice with Rb−/− and wild-type cells are viable, and Rb−/− cells variably contribute to all tissues examined, suggesting that the effect of Rb inactivation may be cell type specific or non-cell autonomous (28, 29).

All heterozygous Rb+/− mice are predisposed to pituitary tumors but not retinoblastoma (24—26, 30, 31). Inactivation of the wild-type Rb allele occurs prior to hyperplastic transformation (31). Chimeric mice consisting of wild-type and Rb mutant cells invariably develop pituitary tumors and die prematurely of metastatic tumors (28, 29). Therefore, the long-term effect of Rb inactivation cannot be followed in these chimeric mice. The transplantation of fetal liver cells was undertaken to address the outcome of Rb−/− hematopoietic cells in both short-term and long-term studies.

MATERIALS AND METHODS

Animals. Recipients of blood cell transplantation were 3—4-month-old wild-type mice obtained from heterozygous Rb+/− mouse crosses (129/ C57BL). Hematopoietic stem cell donors were E11.5—E13.5 embryos from Rb+/− heterozygotes crosses. The generation of the Rb−/− strain has been described (26). All animals were housed in microisolator cages and provided with sterilized food and acidified water.

Transplantation of Embryonic Hematopoietic Stem Cells. Embryonic liver-derived cells were dissociated by gentle pipetting of wild-type and Rb−/− E11.5—E13.5 embryos from Rb−/− heterozygotes crosses. The generation of the Rb−/− strain has been described (26). All animals were housed in microisolator cages and provided with sterilized food and acidified water.

In Vivo CFU Assay. The mice were sacrificed by cervical dislocation 7—10 days after transplantation, and the number of macroscopic and microscopic colonies in the spleen were evaluated. Spleens were fixed in paraformaldehyde, and paraffin sections were prepared for histological analysis.

In Vitro Erythrocyte Progenitor Cell Assays. The procedure was similar to that of Wu et al. (32). Briefly, individual fetal livers from E12.5 embryos were removed into IMDM, dissociated, and passed through a metal mesh to obtain single-cell suspensions. After three washes with IMDM, aliquots of cell suspension were diluted 1:20 in 2% acetic acid to lyse nonnucleated mature erythrocytes and counted. A 1.5 × 105 cells/ml cell suspension in IMDM and 2% FBS was prepared. Cell suspensions from each liver (0.1, 0.3, and 0.6 ml) were mixed with 3 ml of growth medium containing methylcellulose (Stem Cell Technologies Inc.), and 1.1 ml of the mixture was plated into 35-mm dishes in triplicate. Colony formation was assessed by benzidine staining for erythroid and colony-forming unit-erythroid assays. Two Rb-related genes, p107 and p130, have been identified (20, 21). In chondrocytes, inactivation of these two Rb-related proteins

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3 The abbreviations used are: Rb, retinoblastoma protein; Rb, retinoblastoma gene; BFU-E, burst-forming unit; BFU-E, BFU-erythroid; CFU, colony-forming unit; CFU-E, CFU-erythroid; IMDM, Iscove’s modified Dulbecco’s medium; E, embryonic day.
anticoagulant EDTA (Becton Dickinson, San Jose, CA). The blood samples were vortexed and divided into 30–50 μl aliquots in 12 × 75-mm plastic tubes containing appropriate antibodies. After incubation in the dark for 20 min, 1.5 ml of red cell lysis solution (FACS lysis solution, Becton Dickinson) were added, and the mixture was incubated for 5 min. The blood cells were pelleted by centrifugation, washed once with PBS containing 1% sodium azide, and resuspended in 0.3 ml of PBS. Samples were analyzed immediately on a flow cytometer (FACScan, Becton Dickinson). The distribution of major blood cell types and the percentage of undifferentiated cell populations were monitored by a panel of antimonocyte monoclonal antibodies (PharMingen, San Diego, CA): CD3 (pan T), CD4 (T-helper), CD8 (T-suppressor), B220 and slg (B cell), GR1 (granulocyte), and R-PE (erythrocyte). To further identify lymphocytes at different stages of differentiation, CD5, 6C3, heat-stable Ag, and CD43 were used as the markers.

Histopathological Analysis. Mouse tissues, including spleen, liver, salivary gland, and kidney, were fixed in 4% paraformaldehyde in PBS, dehydrated, processed, and embedded in Paraplast (Fisher Scientific) following standard histological procedures. The paraffin tissue blocks were cut into 4–6 μm thin sections, which were deparaffined and stained with H&E or Wright-Giemsa stain. Blood smears were stained with SureStain Wright-Giemsa (Fisher Scientific) according to manufacturer’s instructions. A 3'-dime-thoxybenzidine staining was also performed on blood smears as described by McLeod et al. (33). Photomicrographs were taken under a Zeiss Axioskop microscope with Kodak Ektar 25 film (ASA 100).

Southern Blot Analysis. Genomic DNA was isolated from tail, spleen, liver, and salivary gland tissues of transplant recipient mice. All DNA was digested with EcoRI, fractionated on a 0.8% agarose gel, and transferred to nylon membranes (Hybond Amersham). Southern blots were hybridized with a 0.3 kb probe from intron 19 of the mouse Rb-1 gene, which distinguishes wild-type alleles by a 10.0 kb band from mutated alleles with an 11.5 kb band (26).

In Situ Hybridization. Digoxigenin-labeled antisense Neo 50-mer probe (5'-CCAAGCGCCGGAGAACCCTGTGCAATCCATCTCCAGTTC-AATTGCCCAGATC-3') was prepared according to manufacturer’s instructions (Genius 6 oligonucleotide tailing kit, Boehringer Mannheim). Six months after transplantation, bone from recipients were dissected, paraffin embedded, and cut in 4–6 μm sections. Hybridization and color development were according to the manufacturer’s guide (Boehringer Mannheim).

Switching of Hemoglobin. mRNA was extracted from wild-type and mutant livers at E13.5 and E14.5 and from the spleens of transplantation recipients, using Micro-Fast Track mRNA isolation kit (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized from 100–200 ng of mRNA using SuperScript II RNase H− reverse transcriptase (Life Technologies, Inc.) with oligo(dT) as the primer. Oligonucleotide primer pairs were used in PCR to amplify specific hemoglobin chains: 5'-primer, 5'-GTGAC AGATGCTCTCUGGG-3' and 3'-primer, 5'-CACAA CCCCAGAAAC-3' (β-major globin); 5'-primer, 5'-GGAG A GTCC A TTAGA AACCT AGCA A-3' and 3'-primer, 5'-CCGTG TATTCG CAAAG TGAC-3' (α-globin; Y-Z); and 5'-primer, 5'-GTGAC GAGGCCCAGAGA AAAGC TCTAC-3' and 3'-primer, 5'-AGGGG CCGGA CTCAT CGTAC TC-3' (β-actin as internal control; Ref. 34). Briefly, each PCR reaction mixture contained 2 μl of cDNA mixture, reaction buffer containing 2 μM Mg2+, 0.2 mM oligonucleotide primers, 200 μM dNTPs, and 3 units of Taq DNA polymerase (Boehringer Mannheim). Reactions were cycled at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s for 40 cycles using a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). For quantitative comparison, β-actin was used as control.

Recombinant Blood Cells from Recipients of Rb−/− Cells into Nude Mice. The spleens of transplantation recipients of the Rb−/− cells were removed, and 5 × 107 to 40 × 107 dissociated splenic cells were injected into tail vein of four nude and three wild-type mice. Blood was withdrawn from these mice posttransplantation at 1-month intervals for FACS analysis, and blood smears were prepared and analyzed by light microscopy.

RESULTS

Efficient Rescue of Lethally Irradiated Mice by Rb-deficient Hematopoietic Cells. One million to 5 million cells were found to be sufficient to rescue lethally irradiated mice, regardless of the origin of donor cells.

Variation in Proliferative Activity of Rb-deficient Hematopoietic Cells in Vivo. To further examine Rb−/− blood stem cells, we compared their repopulation ability to those from the wild-type embryos in a CFU assay. CFUs were observed in the spleens of mice transplanted with wild-type, heterozygous, and homozygous cells on day 7 (Fig. 1, A and B) but not in control irradiated animals. Because no difference was found between the wild-type and heterozygous donor cells in all assays performed, cells from mice of these two genotypes will not be distinguished. On day 7, CFUs in mice receiving wild-type donor cells were uniform in size and included colonies of multiple lineages (i.e., CFU-E, megakaryocytic, and myeloid). Larger CFU-Es were identified in mice receiving Rb−/− cells (Fig. 1B). Because individual CFUs are clonally derived, variation in the size of CFUs in recipients of Rb−/− cells suggests differences in the proliferation rate of their stem cells. On day 10, in contrast to the distinct CFUs seen in the recipients of wild-type cells, there were no distinct erythroid colonies in the spleens of mice receiving Rb−/− cells. Instead, the spleens were filled with immature erythroid cells at different stages of maturation (data not shown). Differences in erythroid colonies between wild-type and Rb−/− recipients on days 7 and 10 suggest that Rb−/− erythroid cells have accelerated growth. Interestingly, no differences were found in megakaryocyte and myeloid colonies (data not shown), consistent with previous findings in the Rb−/− embryos (24, 25, 26).

Variation in Proliferative Activity of Rb-deficient Hematopoietic Cells in Vitro. To further determine which stage(s) of erythrocyte development is affected by Rb deficiency, in vitro erythrocyte progenitor colony assay was performed. The number of CFU-Es, a rapid dividing cell that gives rise to an erythroblast colony of 8–64 cells in 2 days, and BFU-Es, a more immature, slowly dividing cell that gives rise to larger colonies of erythroblasts in 7–10 days, was determined. In Rb−/− hematopoietic cells, there were increased numbers of both the rapidly dividing CFU-Es (Fig. 1C) and the more immature and less frequently dividing BFU-Es (Fig. 1D). This effect was most pronounced (~2-fold) in the 8-day culture of Rb−/− BFU-E. These results are consistent with the in vivo finding of increased growth of erythrocyte progenitor cells.

Rb−/− Donor Cells Persist in Bone Marrow of the Recipients. Because we were interested in the long-term effect of Rb inactivation in the hematopoietic cells, we tested whether Rb−/− donor cells were sustained in the recipients. A Neo probe that specifically hybridizes to transcripts from the mutant Rb allele but not the wild-type allele was used in situ hybridization. Six months after transplantation, all bone marrow cells but not the cartilage cells in the Rb−/− recipients were shown to be derived from the donor (Fig. 2).

Extramedullary Erythropoiesis. Blood smear and flow cytometric analysis revealed the presence of nucleated erythrocytes in the peripheral blood of transplant mice receiving Rb−/− cells as early as 3 weeks posttransplantation. To determine whether the abnormal erythrocytoblasts seen in the recipients were derived from Rb−/− donor cells, DNA was isolated from various tissues of two 8-month-old recipients. Southern blot analysis was carried out using a probe spanning intron 19 of the Rb-1 gene. A predominant band at 11.5 kb representing the mutant Rb-1 allele was seen in the spleens of mice that received Rb−/− cells (Fig. 3). In addition, Rb−/− cells were also present in the liver and salivary gland. Extensive erythropoiesis was detected histologically by 6 months posttransplantation. Spleen, liver, salivary gland, and kidney of recipient mice of Rb−/− cells were filled with immature erythroid cells. Wright-Giemsa stain indicated that these were likely intermediate and late stage normoblasts (data not shown). Some of the cells had abundant cytoplasm and clumped chromatin similar to immunoglobulin-producing plasma cells. However, they stained negative by periodic acid-Schiff and positive by

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benzidine (see below), confirming that they were immature erythrocytes. Thus, histological examination of \( Rb^{-/-} \) recipient mice indicated extensive extramedullary erythropoiesis.

**Flow Cytometric Analysis Confirms the Presence of Circulating Immature Erythrocytes.** A systematic analysis of the hematopoietic cells in recipient mice by flow cytometric analysis using a panel of mouse monoclonal antibodies specific for different stages of T-cells, B-cells, granulocytes, and erythrocytes revealed differences between wild-type and \( Rb^{-/-} \) recipients (Table 1). At different times, 10-30 weeks after transplantation, comparable populations of mature T lymphocytes (as indicated by staining of CD3, or CD5 or by double staining of CD4 and CD8) were present in the recipients. Similarly, the granulocyte numbers were comparable. Although there appeared to be a decrease in the mature B-cell population, as indicated by 6C3 and B220 staining, such a decrease was not consistently seen at 1 month posttransplantation (data not shown). On the other hand, an elevated percentage (36-38%) of nucleated erythrocytes persisted in the blood of \( Rb^{-/-} \) recipients (Table 1). This phenomenon was confirmed morphologically by blood smear examination that showed 33-56% nucleated erythrocytes. The nucleated erythrocyte population persisted in all \( Rb^{-/-} \) recipients regardless of the stage of donor cells (E11.5-E13.5). Therefore, it appears to be an inherent property of the \( Rb^{-/-} \) stem cells. On the basis of the flow cytometric and blood smear results, we conclude that \( Rb \) deficiency specifically affects the erythrocyte lineage.

**Normal Hemoglobin Switching of \( Rb \)-deficient Erythrocytes.** Despite the continued presence of peripheral nucleated erythrocytes, all mice transplanted with \( Rb^{-/-} \) stem cells also harbored morphologically normal red cells in peripheral blood, although there was a propensity for increased polychromasia in the erythrocytes. To assess differentiation of \( Rb^{-/-} \) erythrocytes, we determined the hemoglobin type of \( Rb^{-/-} \) hematopoietic cells at different stages. Reverse transcription-PCR was carried out using mRNAs isolated from embryonic livers and spleens of the transplant recipients. Both adult \( \beta \)-major and embryonic \( \gamma \)-hemoglobin were produced in the livers of E13.5 and E14.5 wild-type and mutant embryos (Fig. 4). In the spleens of recipients of wild-type and \( Rb^{-/-} \) cells, a complete switch to the adult \( \beta \)-major hemoglobin was observed (Fig. 4). Thus, consistent with the chimera analysis (28, 29), \( Rb \)-deficient erythrocytes undergo normal hemoglobin chain switching.
Mice Are Anemic after Transplantation of Rb-deficient Cells. The aberrant phenotypes in the recipients of Rb-deficient cells described thus far indicate deregulation of proliferation in the erythrocytes. It is also plausible that such deregulated erythropoiesis manifested by polychromasia and increased numbers of immature erythroid precursors in the peripheral blood accompanied by widespread extramedullary hematopoiesis may be a physiological response to anemia. To evaluate this, cell count and blood profile were performed in older transplant recipients. One year after transplantation, a decrease in hemoglobin amount from 10.8 g/dl in the wild-type recipient to 6.8 g/dl in the Rb<sup>−/−</sup> recipient and a decrease in hematocrit from 31.9% in the wild-type recipient to 18.2% in the Rb<sup>−/−</sup> recipient was seen. On the basis of the blood analysis, it is concluded that mice transplanted with Rb<sup>−/−</sup> cells but not wild-type cells were anemic.

Increased Fragility of Rb-deficient Erythrocytes. The efficient rescue of lethally irradiated mice by Rb<sup>−/−</sup> hematopoietic cells and long life span of the transplant recipients suggested that Rb<sup>−/−</sup> erythrocytes are functional. However, the persistent anemia suggested that they are not completely normal. A plausible cause for anemic phenotype of Rb<sup>−/−</sup> recipients was revealed by blood smear. Increased lysis of the Rb<sup>−/−</sup> nucleated erythrocytes was consistently observed in blood smear preparations (Fig. 5A), suggesting elevated membrane fragility. Addition of albumin prior to blood smear preparation greatly decreased cell lysis (Fig. 5B). Benzidine staining demonstrated that increased populations of nucleated blood cells were indeed immature erythrocytes (Fig. 5C). An additional osmotic fragility test also indicated that Rb<sup>−/−</sup> nucleated erythrocytes are more fragile compared to wild-type erythrocytes (data not shown).

Transplantation of Blood Cells from Recipients of Rb<sup>−/−</sup> Cells to Nude Mice. Morphologically, the peripheral nucleated erythrocytes in recipients of Rb<sup>−/−</sup> cells do not resemble transformed erythroleukemia cells. To test whether peripheral nucleated erythrocytes seen in recipients of Rb<sup>−/−</sup> cells could continue to divide, we transplanted spleen cell preparations containing infiltrated hematopoietic cells from recipients of Rb<sup>−/−</sup> cells into nude mice. The Rb<sup>−/−</sup> cells did not persist in these nude mice. In addition, no nucleated erythrocytes or abnormal hematopoietic cells were seen in the peripheral blood of the nude mice (data not shown).

DISCUSSION

In this study, we have addressed the short-term and long-term effects of Rb deficiency in hematopoietic cells. We demonstrated that Rb<sup>−/−</sup> cells could rescue lethally irradiated mice as efficiently as wild-type cells. The donor fetal liver cells were taken from E11.5–E13.5 embryos, the stages at which little liver pathology was observed in Rb-deficient mice (26). Because a consistent phenotype was observed in Rb<sup>−/−</sup> recipients receiving a wide range of doses of donor cells (1 × 10<sup>6</sup> to 5.0 × 10<sup>6</sup>), it is likely that the abnormal phenotype seen is intrinsic to the Rb<sup>−/−</sup> erythroblasts.
Effects of Rb Deficiency in Various Blood Cell Lineages. Rb appears to have predominant effects on erythrocyte lineage based on FACS analysis, blood smear, and histopathological studies. Consistent with earlier work on chimeric mice by injection of Rb-/- embryonic stem cells into RAG-2-deficient blastocysts (35) in which all T and B cells derived from Rb-/- embryonic stem cells are normal (35), there is no gross abnormality in T and B cells in the recipient mice. It is plausible that p107 and p130 complement Rb function in T and B cells. Indeed, the neuroretinal defect seen in the p107-/-Rb-/- mice is consistent with overlapping functions between these two genes in vivo (23). Erythrocyte lineage in chimeric mice of Rb-/- and wild-type embryos appeared normal, although transient presence of nucleated erythrocytes was noticed (28, 29). The lack of a persistent abnormality in the erythrocyte lineage in the chimeric mice may be due to short life spans caused by metastatic pituitary tumors and the presence of wild-type erythrocytes in these chimeric animals (see below).

It is well accepted that myeloid cells, including platelets, neutrophils, and erythrocytes, are all derived from common precursor cells. In these studies, the platelets appear normal in Rb-/- cell recipients (data not shown). Additionally, no aberrant phenotype was observed in the neutrophil lineage. Therefore, defects due to Rb deficiency are mainly limited to the CFU-E compartment. Both morphology and histological staining suggested that the peripheral nucleated erythrocytes were normoblasts at intermediate and late stages of differentiation, indicating that no single specific stage of erythrocyte development was preferentially affected.

<table>
<thead>
<tr>
<th>Donor cells</th>
<th>Age (wk)</th>
<th>% of nucleated erythrocytes in total nucleated blood cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb+/+</td>
<td>10</td>
<td>By FACS: 4, By morphology: &lt;1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>By FACS: 3, By morphology: &lt;1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>By FACS: 4, By morphology: &lt;1</td>
</tr>
<tr>
<td>Rb-/-</td>
<td>10</td>
<td>By FACS: ND, By morphology: 33</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>By FACS: 36, By morphology: 38</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>By FACS: 38, By morphology: 56</td>
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* ND, not determined.

Rb in the Acceleration of Erythrocyte Proliferation and Differentiation. Both spleen CFU assay in vivo and erythrocyte progenitor assay in vitro demonstrate accelerated growth of Rb-/- erythrocyte progenitor cells (Fig. 1). Because CFU-E in spleen was compared 7–10 days after transplantation, and colonies were scored 2 or 8 days after plating in the in vitro progenitor assays, the data indicate initial proliferation rates of the primary cells prepared from mouse embryos. These results differ from that of the extensively cultured cell lines in that stable cell lines established after replacement of wild-type Rb have growth rate comparable to that of the parental Rb-deficient cells (36). Similarly, the doubling time of Rb-deficient fibroblast cell lines is comparable to that of the wild-type fibroblasts despite a short G1 phase in the former cells (37). The discrepancy may reflect a favorable selection of fast-growing cell population in culture upon continuous passage.

Increased proliferation of Rb-/- cells seen in the spleen CFU-E may reflect the intrinsic growth suppression function of Rb in the erythrocyte (1–3). On the other hand, there may be compound causes for the anemic phenotype and persistent extramedullary erythropoiesis. Fragility testing indicated that the Rb-/- erythrocytes were much more fragile than normal erythrocytes (Fig. 5 and data not shown). The molecular basis of the fragility is unknown, but it is likely that deregulated expression of membrane-associated proteins may be involved. There are abundant examples in which Rb may activate or repress the expression of downstream target genes through its interaction with transcription factors (2). Fragility of the Rb-/- nucleated erythrocytes may shorten their life spans and prevent further accumulation of mutations that promote their malignant transformation. In addition, the decreased hemoglobin levels in Rb-/- animals may stimulate erythropoiesis to maintain blood homeostasis. The anemic phenotypes seen in the recipients of Rb-/- cells may result from these combined factors.

The presence of morphologically normal mature red cells and the normal switching of hemoglobin genes indicate that Rb-/- erythrocytes undergo normal differentiation processes. This is consistent with the finding from chimera analysis (28, 29). However, the anemic phenotype seen in the recipients of Rb-/- cells suggests a suboptimal function of Rb-/- erythrocytes. The lack of such a phenotype in the

Fig. 4. Embryonic and adult hemoglobin expression in wild-type and Rb-mutant mice. mRNA was extracted from livers of E13.5 and E14.5 wild-type and mutant embryos and from spleens of wild-type and mutant cell recipients. Reverse transcriptase-PCR analysis was performed using specific primer pairs to amplify adult (β-major) and embryonic (ε) hemoglobin and β-actin as control.

Liver
E13.5   E14.5
-/-      +/-   -/-      +/-   -/-      +/-

Spleen
adult

β-major
ε
β-actin
RB IN ERYTHROPOIESIS

Fig. 5. Fragility of the Rb<sup>−/−</sup> nucleated erythrocytes. A, blood smears prepared from Rb<sup>−/−</sup> transplant recipient. ine, lysed nucleated erythrocyte; l, lymphocyte; m, monocyte; n, neutrophil. B, same as A except that 1 drop of bovine albumin was added prior to preparation of blood smear. ine, nucleated erythrocyte. C, nucleated erythrocytes and mature RBCs are benzidine stain positive. D, blood smear of wild-type transplant recipient. Bar, 20 µm.

hematopoietic lineages in the chimera may be in part due to the fact that fully functional erythrocytes and Rb<sup>−/−</sup> erythrocytes coexist in the chimera. On the other hand, recipients having complete replacement of hematopoietic cells in our study had only functionally subnormal Rb<sup>−/−</sup> hematopoietic cells that may have led to the anemic phenotype described. Roles for Rb in differentiation have been demonstrated and implicated in several cell types. Neuronal differentiation is incomplete in the absence of Rb (38). Studies of muscle cells and adipocytes have also shown that Rb is required for their differentiation (39, 40). Rb-deficient mice partially rescued by a low level of Rb expression encoded by a transgene were shown to have shorter myotubes with fewer myofibrils and reduced muscle fibers (27). Therefore, Rb is required for full differentiation in many cell lineages. Although Rb<sup>−/−</sup> erythrocytes are morphologically normal and express β-globin, the level of β-globin may not be optimal, similar to that of the decreased myosin in Rb<sup>−/−</sup> muscle.

Mitogenic but not Tumorigenic Effects of Rb in Erythrocyte Lineage. The absence of erythroleukemia despite persistent presence of peripheral immature nucleated erythrocytes and increased proliferation in CFU-E assay in vivo and in vitro in Rb<sup>−/−</sup> recipients is intriguing. Although mutation of the Rb gene has been found in many tumor types, only specific cancer risks (i.e., retinoblastoma and osteosarcoma) are highly elevated in the Rb heterozygotes (1). It is plausible that Rb mutation may facilitate tumor progression in multiple cell types, but its role in tumor initiation may be cell type specific. Such specific tumor predisposition is also true in heterozygous Rb<sup>+/−</sup> mice in that they are 100% predisposed to pituitary tumors of the intermediate lobe (30). Inactivation of genes in the Rb pathway have been shown to be tumorigenic (reviewed in Ref. 41). Deregulation of cell proliferation does not always lead to tumorigenesis. Underlying mechanisms of the cell-type specific effects and the different consequences of the dysfunction of a cell cycle regulator in a given cell type are yet to be addressed.

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