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A Limited Role for p21$^{Cip1/Waf1}$ in Maintaining Normal Hematopoietic Stem Cell Functioning

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ABSTRACT

Several studies have suggested that the cyclin-dependent kinase (CDK) inhibitor p21 plays a crucial role in regulating hematopoietic stem and progenitor pool size. To allow assessment of long-term stem cell functioning in vivo, we have backcrossed a p21 null allele to C57BL/6 (B6) mice, the most commonly used mouse strain in hematopoietic stem cell research. In various in vitro assays, the homozygous deletion of the p21 allele did not affect the number of hematopoietic cells in B6 mice. Furthermore, the competitive repopulation ability was not different between p21-deficient and wild-type stem cells from both young and aged (20-month-old) mice. These results show that p21 is not essential for regulation of stem cell number in steady state. When proliferative stress was applied on p21-deficient stem cells by serial transplantation of 1,500 Lin$^-$Sca-1$^+$c-kit$^+$ (LSK) cells, again no detrimental effect was observed on cobblestone area-forming cell (CAFC) frequency and competitive repopulating ability. However, when bone marrow cells from mice that received 2 Gy of irradiation were transplanted, p21 deficiency resulted in a more than fourfold reduction in competitive repopulation index. Finally, we did not find major differences in cell cycle status and global gene expression patterns between LSK cells from p21-deficient and wild-type mice. Our findings indicate that the background of mice used for studying the function of a gene by genetic modification may determine the outcome. Cumulatively, our data fail to support the notion that p21 is essential for stem cell function during steady-state hematopoiesis, but may be relatively more important under conditions of cellular stress. STEM CELLS *2007;25:836–843*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The genetic pathways that regulate the pool size of the hematopoietic stem cell compartment in the bone marrow (BM) have remained poorly understood. Whereas a large family of hematopoietic growth factors has been shown to be strongly involved in maintaining peripheral blood cell counts, only few cytokines, including stem cell factor (SCF), thrombopoietin (TPO), and Flt3-ligand, that have an effect on the number of stem cells in vivo have been identified [1–3]. Rather, intracellular proteins such as transcription factors and chromatin remodeling factors have been shown to intrinsically regulate stem cell numbers [4–7]. In addition, a series of studies has suggested that deficiency of cyclin-dependent kinase inhibitors, such as p27^{kip}, p18^{INK4C}, and p21$^{cip1/waf1}$, results in stem or progenitor cell abnormalities [8–10]. Cyclin-dependent kinase inhibitors have been shown to be associated with cellular senescence of fibroblasts [11, 12], and are believed to be involved in stem cell quiescence in the hematopoietic system [8–10]. Whereas absence of p27^{kip} appears to increase the number of progenitor cells [9], p18^{INK4C} and p21$^{cip1/waf1}$ deficiencies have been documented to induce stem cell expansion [8, 10]. In competitive repopulation assays in which wild-type bone marrow cells were competed with p18-deficient cells in equal ratios, the large majority of white blood cells in the recipients was of p18-deficient genotype [10].

It has been documented that p21 deficiency leads to an increased number of late-developing cobblestone area-forming cells [8]. Furthermore, serial transplantation using p21-deficient cells resulted in premature stem cell exhaustion, and p21-deficient animals were more sensitive to the cytotoxic drug 5-fluorouracil (5-FU). Because of the genetic background, long-term repopulation assays in which p21-deficient bone marrow cells were competitively transplanted with syngeneic wild-type bone marrow cells have not been performed yet. Because it was previously shown that different inbred strains of mice show widely divergent stem cell characteristics [13], the genetic background of donor and recipient strains may confound or obscure potential stem cell phenotypes. To assess the role of p21 in regulating stem cell activity during steady-state hematopoiesis, we have now backcrossed the p21 null allele to a B6 background and performed standard competitive repopulation assays at primary and secondary transplant. Our results show that p21 deficiency has a limited effect on stem cell behavior under steady-state conditions, but when cells are exposed to agents that cause cellular stress by inducing DNA damage, such as

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radiation, absence of p21 results in a substantial reduction of hematopoietic stem cell survival.

MATERIALS AND METHODS

Animals

B6;129S2-*Cdkn1a*^{tm1Tyj}/J (B6/129p21-deficient) and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (B6.SJL) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild-type female C57BL/6 (B6wt) mice were purchased from Harlan (Horst, the Netherlands) and were used to backcross B6/129p21-deficient mice, and as recipients for competitive repopulation. After 10 backcrosses, B6p21-deficient mice, selected by genotyping as described [8], were used in all experiments. Eight- to 12-week-old B6 mice were used for serial transplantation (Harlan). In one experiment, B6wt and B6p21-deficient mice were aged in our local animal facilities and analyzed at 20 months of age. For competitive repopulation assays, B6.SJL (CD45.1) congenic mice (bred locally at the animal facilities of University Medical Center Groningen) were used as a source of competitor bone marrow cells. Mice were fed ad libitum with food pellets and acidified tap water (pH = 2.8). All animal procedures were approved by the local animal ethics committee of the University Medical Centre Groningen.

Cell Collection, Culture, and Fluorescence-Activated Cell Sorting

Mouse embryonic fibroblasts (MEFs) were derived from 14-day-old embryos as described previously [14] and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; GibcoBRL, Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Cells were passaged every 3–4 days, and viable cells were counted to calculate the number of population doublings (PDL) with the formula: $\Delta PDL = \log(n_f/n_i)/\log 2$, in which n_f = final number of cells, and n_i is initial number of cells.

Bone marrow cells were harvested by flushing the femoral content with α-modified Eagle's medium (GibcoBRL) supplemented with 2% FBS and nucleated cell numbers were measured on a Coulter counter Model Z2 (Coulter Electronics, Hialeah, FL). For transplantation of unfractionated bone marrow cells, cells were diluted and transplanted into lethally irradiated (9.5 Gy single dose total-body irradiation [TBI], ^{137}Cs γ-rays) recipients. To purify the Lin^{-}Sca-1^{+}c-Kit^{+} (LSK) cell population from bone marrow, cells were stained with biotinylated lineage-specific antibodies (Mouse Lineage Panel, containing anti-CD45R, anti-CD11b, anti-TER119, anti-Gr-1 and anti-CD3e [BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com/], fluorescein isothiocyanate (FITC)-anti-Sca-1, and APC-anti-c-kit [BD Pharmingen]). Cells were washed twice with phosphate-buffered saline (PBS; Gibco, Invitrogen) supplemented with 0.2% bovine serum albumin (BSA; Gibco). Biotinylated antibodies were visualized with streptavidin- phycoerythrin (PE; BD Pharmingen). After antibody staining, cells were washed twice with PBS + 0.2% BSA and sorted by a MoFlow cell sorter (DakoCytomation, Fort Collins, CO, http://www.dakousa.com/). LSK cells were sorted and used in the cobblestone area-forming cell (CAFC) assay or transplanted into lethally irradiated recipients. The CAFC assay was performed as described previously [15] where early-appearing (day 7) cobblestone represent progenitors and late-appearing cobblestones (day 35) are a measure of the number of stem cells.

Irradiation and 5-FU Treatment of Mice

In some experiments, the response of B6wt and B6p21-deficient hematopoietic cells to total-body radiation or 5-FU was determined. To this end, mice were irradiated with 2 Gy (^{137}Cs γ-rays), given 5-FU (200 mg/kg i.p.), or left untreated. Twenty-four hours after TBI or 5-FU, mice were sacrificed, and bone marrow stem cell content was assessed in vitro by CAFC frequency analysis and in vivo by competitive repopulation analysis.

Cell Cycle Analysis

LSK BM cells were stained according to the staining method described herein and subsequently analyzed for DNA content using 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com/). After staining for lineage markers, c-kit and Sca-1, cells were fixed with 0.1% paraformaldehyde and permeabilized with 0.1% saponin for 15 minutes at room temperature. Cells were washed and left in PBS with 0.2% BSA containing 5 μg/ml DAPI and left at room temperature for at least 1 hour and then placed on ice. Samples were analyzed without further washing by flow cytometry (LSR II, Becton Dickinson, Palo Alto, CA).

Competitive Repopulation Assays

B6 recipients were lethally irradiated (9.5 Gy) 24 hours before transplantation. B6p21-deficient and B6wt were sacrificed, and bone marrow cells were isolated and transplanted in various cell doses in competition with CD45.1 congenic BM cells (two independent experiments with 4–6 mice per group). In addition, LSK cells were purified from BM cells of B6wt and B6p21-deficient mice as described previously [16]. Primary transplant was initiated with 1,500 LSK cells. Four months after primary transplant, recipients were sacrificed, and bone marrow cells were isolated, analyzed in vitro (LSK phenotyping and CAFC assay), and transplanted in various ratios with B6.SJL (CD45.1) competitor BM cells. Preirradiated, 5-FU-treated, or aged bone marrow cells were also tested in the competitive repopulation assay to determine the stem cell content of the bone marrow. At several time points after transplantation, leukocytes were stained with anti-CD45.1-PE and anti-CD45.2-FITC (BD Pharmingen) antibodies and analyzed using flow cytometry (FACSCalibur; Becton Dickinson) to assess chimerism. To quantify chimerism in the repopulation assays the competitive repopulation index can be calculated by taking the ratio of white blood cells derived from B6wt or B6p21-deficient cells to competitor bone marrow cells in the circulation and dividing it by the ratio of B6wt or B6p21-deficient cells to competitor bone marrow cells transplanted. From the same data, the number of repopulating units (RU) can be calculated according to the formula:

$$RU = (\%\ \text{chimerism} \times \text{No. of RU transplanted from } \textit{competitors})/(100 - \%\ \textit{chimerism}).$$

One RU represents the repopulating ability of 100,000 normal bone marrow cells, and RU data are especially useful when comparing bone marrow repopulating ability after cell depleting treatments such as total-body irradiation or 5-FU.

Gene Expression Analysis in B6wt and B6p21-Deficient LSK Cells

LSK cells were isolated according to the protocol described herein, RNA isolated, and cDNA synthesized according to the manufacturer's protocols (Invitrogen, Breda, the Netherlands). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to analyze expression of *Ccna1* (cyclin A1), *Ccnb1* (cyclinB1), *Ccnd1* (cyclin D1), *Ccne1* (cyclin E1), *Bcl2*, *Bax*, *Cdkna1* (p21), *Mdm2*, *Trp53* (p53), and *GAPDH* as a control housekeeping gene. We isolated total RNA derived from LSK

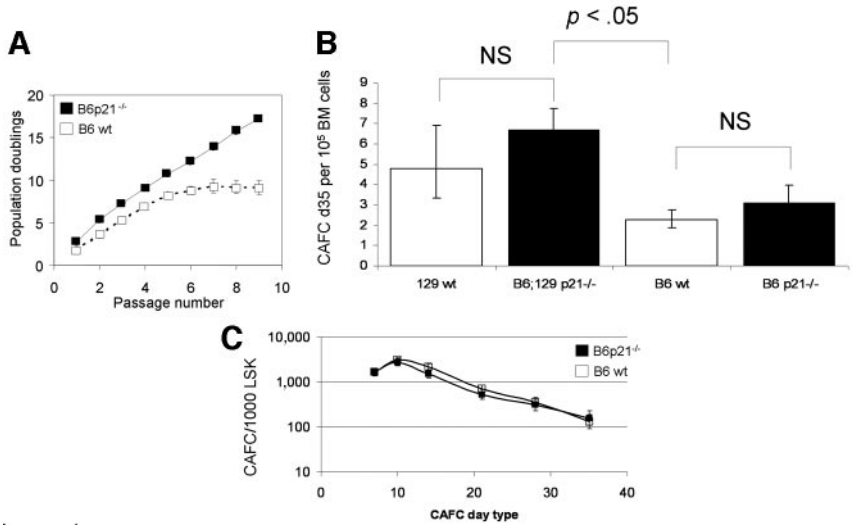


Figure 1. Characterization of p21-deficient MEFs and bone marrow cells. **(A):** Growth kinetics of MEFs derived from B6wt (open squares) and B6p21-deficient (closed squares) day-14 embryos. Cultures were performed in triplicate and the mean value ± 1 SEM is shown. **(B):** CAFCday35 frequency in bone marrow from 129 wt, B6;129p21-deficient, B6wt, and B6p21-deficient, cells. **(C):** The clonogenic ability of LSK cells isolated from the bone marrow of B6wt (white squares) and B6p21-deficient (black squares) mice as measured in the CAFC assay. Average ± 95% confidence intervals are shown from three pooled bone marrow samples from three independent experiments with 3–5 mice per experiment. Abbreviations: BM, bone marrow; CAFC, cobblestone area-forming cell; LSK, $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$; MEF, mouse embryonic fibroblast; NS, not significant; wt, wild type.

cells pooled from three mice using StrataPrep Total RNA Microprep kit (Stratagene, La Jolla, CA, http://www.stratagene.com) as described by the manufacturer.

RESULTS

Absence of Cellular Senescence in B6p21-Deficient MEFs

Deficiency of p21 is associated with absence of a senescence phenotype in fibroblasts [18]. To functionally verify the absence of the p21 allele, MEFs were grown from day-14 embryos of wild-type and p21-deficient mice to assess the number of population doublings (Fig. 1A). As expected, MEFs derived from B6 wt mice ceased proliferation after 5–6, passages suggesting cellular senescence. MEFs derived from B6p21-deficient mice, however, did not senesce, and grew continuously. These data confirm the established role of p21 in the senescence program of MEFs in vitro [18].

In Vitro Stem Cell Activity of p21-Deficient Bone Marrow Cells

No difference in peripheral blood cell values between B6wt and B6p21-deficient mice could be observed (data not shown). Next, we determined CAFCday35 frequencies in BM isolated from B6wt and B6p21-deficient mice. In addition, we measured CAFC frequencies in BM cells from the original B6/129p21-deficient mixed background strain and 129wt mice. We confirmed previously reported data [8] that B6/129p21-deficient mice had a threefold higher CAFCday35 frequency compared to B6wt (Fig. 1B). However, compared with 129 wt mice, B6/129p21-deficient had only a 1.4-fold higher CAFCday35 frequency. On a B6 background, p21-deficient mice had 1.4-fold more CAFCday35 per 10^5 bone marrow cells than did wild-type B6 mice. These values are comparable to the values reported by Cheng et al. [8], who found a 1.9-fold increase in CAFCweek5

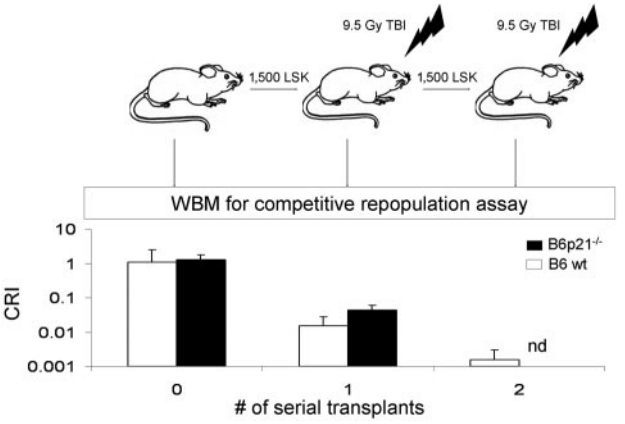


Figure 2. Serial transplantation similarly affects stem cell exhaustion in B6wt and B6p21-deficient mice. One thousand five hundred $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ cells from B6wt or B6p21-deficient mice were serially transplanted. Before each transplant, bone marrow cells were collected for analysis in vitro (Table 1). At each point in the serial transplantation procedure (in steady state, in primary recipients at the time of secondary transplantation, and in secondary recipients, 4 months after transplantation), 5×10^5 or 2×10^6 unfractionated bone marrow cells from the donors were transplanted, with 5×10^5 competitor cells, into lethally irradiated recipients to determine competitive repopualtion. Average ± one standard deviation CRI are shown for B6wt (white squares) and B6p21-deficient (black squares) bone marrow cells. (data from two pooled bone marrow samples from two independent experiments are shown). Abbreviations: CRI, competitive repopulation index; LSK, $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$; nd, not done; TBI, total-body irradiation; WBM, whole bone marrow; wt, wild type.

frequency. Comparison of several hematopoietic parameters revealed only a significantly higher number of progenitors as measured by the CAFCday7, whereas bone marrow cellularity, the percentage of LSK cells and CAFCday35 showed no differences (Table 1). Sorted LSK cells were plated in the CAFC assay, and frequency of all CAFC subtypes was calculated (Fig.

Table 1. Hematopoietic parameters in B6wt and B6p21-deficient mice in steady state and 4 months after primary and secondary transplantation

	B6wt	B6p21-deficient	*p* value
Steady state			
BMC[a]	25.3 ± 7.0 ($n = 17$)	31.8 ± 6.1 ($n = 6$)	NS
CAFC-7[b]	1206 (996–1461)	2215 (1589–3089)	$.01 < p < .05$
CAFC-35[b]	23 (19–28)	31 (25–40)	NS
% LSK[c]	0,19 ± 0.09 ($n = 2$)	0.23 ± 0.19 ($n = 2$)	
CAFC-35/1,000 LSK[d]	130 (90–187)	154 (106–224)	NS
Primary recipients[e]			
BMC[a]	23.2 ± 3.6 ($n = 27$)	21.4 ± 4.1 ($n = 14$)	NS
CAFC-7[b]	835 (671–1039)	1275 (971–1674)	NS
CAFC-35[b]	13 (10–17)	9 (6–13)	NS
% LSK[c]	0.11 ± 0.05 ($n = 2$)	0.06 ± 0.02 ($n = 2$)	
CAFC-35/1,000 LSK[d]	154 (108–219)	210 (146–302)	NS
Secondary recipients[f]			
BMC[a]	22.3 ± 5.0 ($n = 30$)	21.6 ± 3.7 ($n = 8$)	NS
CAFC-7[b]	388 (312–483)	787 (538–1151)	$.01 < p < .05$
CAFC-35[b]	22 (17–28)	1.9 (0.7–5.1)	$p < .001$
% LSK[c]	0.14 ± 0.10 ($n = 2$)	0.08 ± 0.02 ($n = 2$)	
CAFC-35/1,000 LSK[d]	182 (132–251)	18 (6–57)	$.002 < p < .01$

In all cases, data are shown from two pooled bone marrow samples from two independent experiments with 3–10 mice per experiment.
[a]Bone marrow cellularity is expressed as the number of cells ($\times 10^6$) per femur. Total number of mice is shown in parentheses.
[b]CAFC frequencies were determined from whole unfractionated bone marrow and is expressed as number of CAFC-7 or 35 per 10^6 bone marrow cells. The 95% confidence interval is indicated in parentheses.
[c]The percentage of LSK is determined from bone marrow cells pooled from 3–10 mice. The average of two separate experiments is shown, and no statistical test has been performed.
[d]LSK cells were sorted and plated as 1, 3, 10, 30 (and 100) cells per well, and the frequency of CAFCday35 per 1,000 LSK (with 95% confidence interval) was calculated.
[e]Primary recipients were transplanted with 1,500 freshly isolated LSK cells and analysed for BMC, CAFC, and percentage of LSK 4–5 months after transplantation.
[f]Secondary recipients were transplanted with 1,500 LSK cells isolated from primary recipients of 1,500 freshly isolated LSK cells, and analyzed for BMC, CAFC, and percentage of LSK 4–5 months after transplantation.
Abbreviations: BMC, bone marrow cellularity; CAFC, cobblestone area-forming cell; LSK, $Lin^-Sca\text{-}1^+c\text{-}kit^+$; NS, not significant; wt, wild type.

1C). The frequency of all CAFC subtypes per 1,000 LSK cells but most importantly, the CAFCday35, were similar in B6 wild-type and p21-deficient cells.

In Vivo Stem Cell Activity and Serial Transplantation of p21-Deficient Bone Marrow Cells

Backcrossing of the p21 null allele to a B6 background allowed us to perform competitive transplantation assays. When bone marrow cells from B6p21-deficient or B6wt mice were transplanted with an equal number of B6.SJL (CD45.1) competitors, chimerism levels of approximately 50% were found in both groups. This indicates equal repopulation ability, corresponding to a competitive repopulation index of 1 (Fig. 2). Thus, in vivo competitive repopulating activity of bone marrow cells was not affected by p21 deficiency. In addition, chimerism was similar in all lineages for B6wt and B6p21-deficient cells; red blood cells (TER119), myeloid cells (CD11b and Gr-1), lymphoid cells (CD3 and B220) (data not shown). A serial transplantation procedure was performed to evaluate the exhaustion of hematopoietic stem cells under conditions of proliferative stress (i.e., reconstitution after a stem cell transplant). To this end, 1,500 sorted LSK cells were transplanted into primary recipients, and, 4 months later, LSK were isolated from the primary recipients and transplanted into secondary recipients. At each point in the serial transplantation procedure, bone marrow cells were tested for femur cellularity, CAFC activity in whole bone marrow, percentage of LSK cells, and CAFC activity within the LSK population (results are given in Table 1). In addition, the in vivo competitive repopulation ability was evaluated by transplantation of serially transplanted bone marrow cells along with freshly isolated untreated congenic bone marrow cells (CD45.1). Figure 2 shows that the contribution of BM cells derived from primary transplantation competing with freshly isolated cells, dropped considerably compared to freshly isolated bone marrow cells (i.e., 0 serial transplants), but was similar for B6 wild-type and B6p21-deficient BM cells. The competitive repopulation index decreased from approximately 1 in untreated bone marrow to a value between 0.01 and 0.1, suggesting a more than 10-fold reduction in repopulating ability. However, there was no difference between wild-type and p21-deficient bone marrow cells. Table 1 shows the various hematopoietic parameters measured after zero (fresh material), one, and two serial transplants and revealed no difference between B6p21-deficient and B6wt. The only exceptions were an increased progenitor cell frequency (CAFC-7) and a 10-fold lower frequency of CAFCday35 in whole bone marrow as well as within the LSK population after two serial transplants.

Response of Hematopoietic Cells to 5-FU or 2-Gy TBI

Next, we investigated whether the response to DNA damage induction by 5-FU or 2-Gy irradiation of hematopoietic stem cells varied between B6p21-deficient and B6wt bone marrow cells. p21 is an important molecule in the arrest of cell cycle progression after DNA damage, allowing for DNA repair before the cell cycle proceeds [19]. Figure 3 shows that administration of 5-FU or 2 Gy of irradiation led to similar survival of both progenitor-like CAFCday7 and stem cell-like CAFCday35 in B6p21-deficient and B6wt mice (Fig. 3A–3B). Bone marrow cells from mice treated with 5-FU also showed similar numbers of repopulating units on transplantation in competitive repopulation assays (Fig. 3C). However, after a low radiation dose of

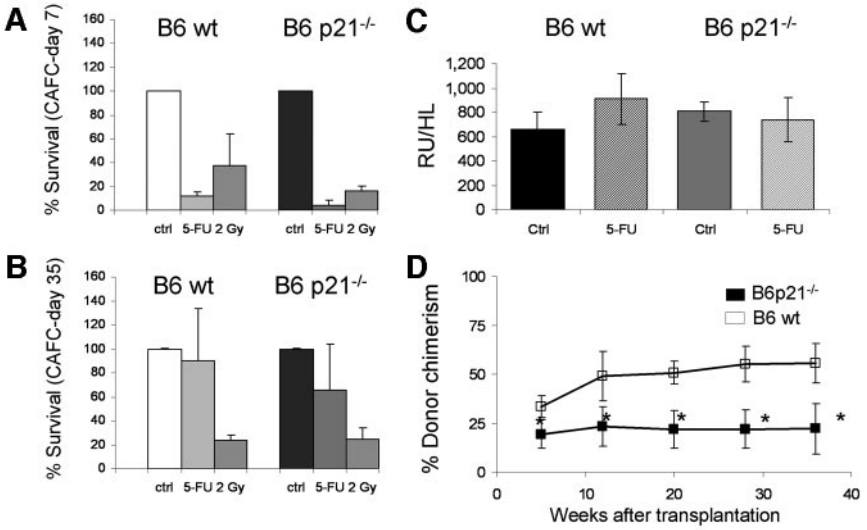


Figure 3. Sensitivity of bone marrow cells from B6wt and B6p21-deficient mice to 5-fluorouracil (5-FU) and radiation. Mice were given 200 mg/kg of 5-FU (i.p.) or 2 Gy total-body irradiation (TBI), and were sacrificed 24 hours later to analyze stem and progenitor cell survival. **(A):** Relative survival of progenitor cells as measured by CAFCday7 after 5-FU or 2-Gy TBI. Data are expressed as the percentage of the total number of surviving CAFC per hind limb in treated mice relative to untreated mice. Data from 2–4 animals per group. **(B):** Relative survival of stem cells as measured by CAFCday35 after 5-FU or 2-Gy TBI. Data are expressed as the percentage of the total number of surviving CAFC per hind limb in treated mice relative to untreated mice. Data from 2–4 animals per group. **(C):** The number of RU per hind limb in untreated and 5-FU treated mice was determined in B6wt and B6p21-deficient mice. Chimerism levels at 22 weeks after transplant were used to calculate RU. Average ± 1 standard deviation from two independent experiments with a total of 10–12 recipients. **(D):** Bone marrow cells isolated from 2 Gy-treated B6wt (white squares) or B6p21-deficient (black squares) mice were transplanted together with an equal number of 2 Gy-irradiated B6.SJL (CD45.1) competitor cells. Chimerism levels were measured at different times after transplantation. Average ± one standard deviation from 10 recipients per group from two independent experiments is plotted for the first two time points. Other time-point data from one experiment with five recipients per group is shown. ∗, significant difference ($p < .01$) between B6wt and B6p21-deficient cells, using Student's t test. Abbreviations: CAFC, cobblestone area-forming cell; ctrl, control; RU/HL, repopulating units/hind limb; wt, wild type.

2 Gy, p21-deficient bone marrow cells were not able to contribute to long-term hematopoiesis as well as wild-type bone marrow cells (Fig. 3D). Cotransplantation of 2-Gy irradiated p21-deficient cells with an equal number of 2-Gy irradiated competitor cells resulted in approximately 20%–25% chimerism, whereas 2-Gy irradiated wild-type cells competed well (50% chimerism) with the same competitors. A similar fourfold difference in competitive repopulation index was seen when irradiated wild-type and p21-deficient cells were transplanted with nonirradiated competitors (data not shown). This suggests an inherent defect in the response to DNA damage of p21-deficient cells.

Effect of Natural Aging on Stem Cell Content in p21-Deficient and Wild-Type Mice

Mice aged 20 months were analyzed for stem cell content in the in vitro CAFC assay and in a competitive repopulation assay with young competitor cells. As previously reported [20], the CAFCday35 frequency increased three- to fourfold in aged C57Bl/6 mice, but the frequency of CAFCday35 in aged p21-deficient mice remained unchanged (data not shown). However, the competitive repopulation index was similar in B6wt and B6p21-deficient animals when unfractionated bone marrow cells were transplanted in 1:1 or 4:1 (aged:young) ratios (Fig. 4).

Stem Cell Cycling and Gene Expression in B6wt and B6p21-Deficient Mice

Because the absence of p21 is expected to be associated with increased cell cycling, we determined the percentage of LSK cells in the G_0/G_1 phase of the cell cycle (Fig. 5). We observed no difference between B6wt (88.1 ± 3.9; $n = 6$), B6p21-deficient (84.8 ± 5.6; $n = 7$) in LSK cells but also in Lin^- cells (84.8 ± 3.5 vs. 85.3 ± 3.4).

In addition, we determined whether differences in gene expression exist between B6 wild-type and B6 p21-deficent LSK cells. To this end, specific genes involved in cell cycle regulation were investigated by RT-PCR. We tested for expression of several cell cycle regulators and found no difference in expression of genes involved in cell cycle regulation (cyclins B1, D1, and E1), and only an increased expression of mdm2 and p53 was found (Fig. 6). The expression of cyclin-dependent kinase inhibitors (with the exception of *Cdkn2c* [$p18^{INK4C}$]) and *Cdkn2d* [$p19^{INK4D}$]) was very low in LSK cells from both B6 wild-type and B6 p21-deficient mice (data not shown).

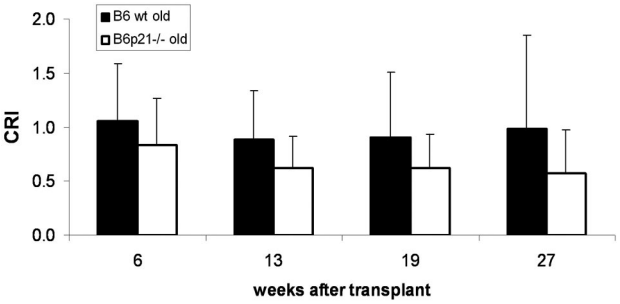
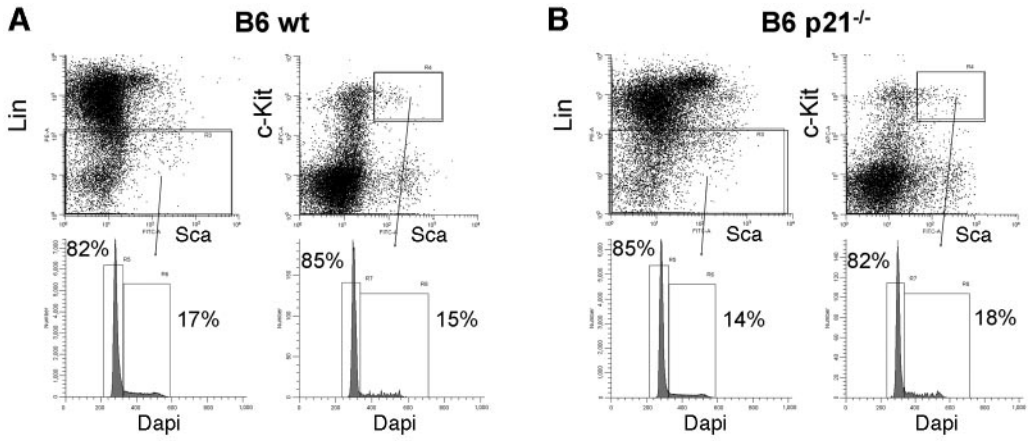


Figure 4. Competitive repopulation of aged B6wt and B6p21-deficient bone marrow cells is similar. Aged (20-month-old) mice bone marrow cells from wild-type and p21 deficient animals were transplanted with freshly isolated young B6.SJL competitors in two ratios (4:1 and 1:1) with a fixed number of competitor cells (5×10^5). Chimerism was determined at various times after transplantation and converted into competitive transplantation indexes. Averages ± standard deviations are shown for 9–10 mice recipients per group. Abbreviations: CRI, competitive repopulation index; wt, wild type.



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	B6 wt	B6 p21$^{-/-}$
Lin$^-$		
% G_0/G_1	84.8 ± 3.5	85.3 ± 3.4
Lin-Sca$^+$kit$^+$		
% G_0/G_1	88.1 ± 3.9	84.8 ± 5.6

Figure 5. Cell cycle analysis in bone marrow cells from B6wt and B6p21-deficient mice. Bone marrow cells were stained to visualize Lin$^-$ and Sca-1$^+$c-kit$^+$ cells, fixed and permeabilized to stain DNA with DAPI. A representative sample is shown for a B6wt **(A)** and a B6p21-deficient **(B)** mouse. The 5%–10% least positive cells for lineage markers were analyzed for DAPI staining and were used to identify Lin$^-$Sca-1$^+$c-kit$^+$ cells to allow measurement of the number of cells in G_0/G_1 in both populations. **(C):** Table comparing the percentage of cells in G_0/G_1 in both Lin$^-$ and Lin$^-$Sca-1$^+$c-kit$^+$ cells in B6wt and B6p21-deficient mice from 6 and 7 mice respectively ($p > .05$). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; wt, wild type.

DISCUSSION

Adult stem cells in general, and hematopoietic stem cells in particular, must have a tightly controlled regulation of cell division to maintain normal stem cell numbers throughout life and to sustain sufficient numbers of mature (blood) cells. Previously, it has been shown that various mouse strains differ in life expectancy, which could be correlated to the rate of cell division [20, 21]. It was shown that the cycling activity of colony forming-unit spleen and CAFCday7 varied among mouse strains, with longer-lived C57BL/6 mice having a lower cycling activity than, for instance, shorter-lived DBA/2 mice [20, 22]. In addition, BXD recombinant inbred mice, originating from a cross between DBA/2 and C57BL/6, revealed that progenitor cell cycling and mean life span are inversely correlated and genetically linked [21]. The relationship between life expectancy and cell cycling among the DBA/2 and C57BL/6 mouse strains suggests that a more active cycling stem cell compartment predisposes to earlier stem cell exhaustion. Therefore, genes regulating cell cycle may be important mediators of hematopoietic stem cell maintenance. Indeed, it was reported that the cyclin-dependent kinase inhibitor p21$^{cip1/waf1}$ (*Cdkn1a*) is critical for regulating the size of the stem cell pool because mice deficient for p21 displayed an increase in the number of late-appearing CAFCs, and their stem cells were suggested to undergo premature senescence upon serial transplantation [8]. However, these results were obtained in a mouse strain, 129/Sv, that excludes the use of the CD45 marker in competitive repopulation assays, although more cumbersome techniques are possible. Here, we made use of mice-deficient for p21 which were backcrossed to the C57BL/6 background. Extensive evaluation of various hematological parameters showed a very limited effect of p21 deficiency in hematopoietic stem cell regulation. Firstly, no differences were observed in in vitro surrogate stem cell assays (CAFC) when unfractionated bone marrow or purified LSK cells were evaluated. Secondly, we unexpectedly did not find any difference in cell cycle status, nor in the sensitivity to the S-phase-specific drug 5-FU. Finally, in vivo repopulation in competitive repopulation assays also failed to identify an effect of p21 deficiency in C57BL/6 mice. This was observed not only in primary but strikingly also after secondary transplantation. The only phenotypes that we observed in B6p21-deficient mice were the escape from senescence in cultured mouse fibroblasts and the increased sensitivity of hematopoietic stem cells to low-dose total body irradiation. These results are in agreement with previously published results on the increased radiation sensitivity of p21-deficient MEFs [19]. Also, cells lacking p21 fail to arrest the cell cycle in response to DNA damage, leading to bypass of senescence [18]. The same mechanism may be responsible for the increased sensitivity to radiation where the absence of a proper cell cycle checkpoint does not allow for sufficient DNA damage repair, leading to more radiation induced stem cell killing. Recently it was found that p16 deficient mice exhibit delayed stem cell aging and even increased stem cell functioning on a per cell basis [23]. In our experiments, naturally aged mice deficient for p21 did not show an effect on competitive repopulation when compared with wild-type B6 mice (Fig. 4). The cell cycle inhibitor p16^{INK4a} has recently been implicated in hematopoietic stem cell senescence [24] and its absence may lead to prevention of senescence in aged hematopoietic stem cells. Our results do not support a role for p21 in aging and stem cell exhaustion.

Other CDK inhibitors have been reported to be involved in hematopoietic cell regulation. For instance, p27^{kip} deficiency was shown to have no effect on stem cell numbers, stem cell cycling, or stem cell exhaustion, but progenitor cells exhibited increased cycling, and p27$^{-/-}$ stem cells outcompeted wild-type stem cells [9], suggesting increased proliferative capacity. Interestingly, p16^{INK4A} and p19^{ARF} were found to be downstream mediators of the polycomb group gene *Bmi-1*, which is strongly involved in the control of stem cell survival and pro-

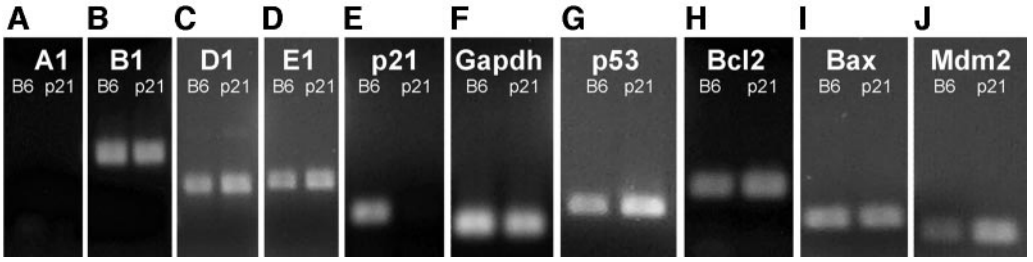

Figure 6. Gene expression in B6 wild-type (wt) and B6p21-deficient mice. **(A–J):** Reverse transcriptase polymerase chain reaction results for genes involved in cell cycle regulation. $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ cells were sorted from B6wt and B6p21-deficient mice, RNA isolated, cDNA synthesized, and analyzed for expression of *cyclin A1* (A1), *cyclin B1* (B1), *cyclin D1* (D1), *cyclin E1* (E1), *p53*, *Bcl2*, *Bax*, *MDM2*, *p21*, and *Gapdh* as quality control.

liferation; $p16^{INK4A}$ induced senescence and $p19^{ARF}$ induced apoptosis in hematopoietic stem cells [25]. *Bmi-1* downregulates $p16^{INK4A}$ and $p19^{ARF}$, allowing for more stem cell proliferation and survival. However, absence of $p16^{INK4A}$ and $p19^{ARF}$ does only modestly increase the self-renewal potential of hematopoietic stem cells under conditions of serial transplantation with low numbers of stem cells [26] and does not recapitulate the Bmi-1 phenotype. Deficiency of $p18^{INK4C}$ leads to enhanced self-renewal of stem cells [10], suggesting that p18-deficient cells can shift the balance of cell division from differentiation toward self-renewal or, alternatively, these cells can postpone exhaustion and senescence. The CDK inhibitors can be divided into two groups: the INK4 proteins ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$) inhibiting CDK4/6, and the Cip/Kip proteins ($p21^{cip1/Waf1}$, $p27^{kip1}$, $p57^{kip2}$) inhibiting CDK2. Collectively, the data support the notion that CDK4/6 is important for self-renewal divisions, because absence of INK4 inhibitors generally leads to enhanced stem cell engraftment. Deletion of CDK2 inhibitors often affects stem cell exhaustion when on a 129/Sv background. Recently, it was shown that combining deficiencies for $p18^{INK4C}$ and $p21^{cip1/Waf1}$ leads to a partial restoration of the premature exhaustion phenotype of $p21^{cip1/Waf1}$ knockout mice of mixed background (C57Bl/6;129/Sv) [27]. Overexpression of HoxB4, a molecule known to promote stem cell self-renewal [28, 29], enhanced stem cell expansion in p21-deficient 129/Sv bone marrow cells, probably by lack of cell cycle inhibition during the expansion period [30]. Because most of these studies were performed in 129/Sv mice or mice with mixed genetic background, and we found no apparent phenotype in steady state hematopoiesis when p21 deficiency was backcrossed to C57BL/6 mice, our results imply that cell cycle regulation in hematopoietic stem cells may vary among different mouse strains. In a preliminary screening for differences in gene expression between p21-deficient and wild-type C57Bl/6 LSK cells, only very minor variations were found, and only four genes showed a more than twofold increase or decrease in gene expression (data not shown). These results combined with the RT-PCR data presented in Figure 6 on expression of gene cells involved in cycle regulation suggest that either p21 deficiency has no effect on the transcriptional machinery in HSC or that is compensated by multiple subtle adaptations in gene expression of other gene family members.

There seems to be a contradiction between our results and the results published by Cheng et al. [8], but we believe that this may be attributed to the genetic background of the mice used in these studies. Apparently, when a p21-null allele is introduced in 129/Sv mice, hematopoietic stem cells exhaust faster in serial transplantation and show enhanced sensitivity to 5-FU. However, when C57BL/6 with the same mutation are investigated for hematopoietic stem cell cycling and exhaustion, a very limited effect is observed. This may be attributed to different modifier genes in these mouse strains. For instance, the C57BL/6 strain may express genes that mask or overwhelm the effect of p21 deficiency. A thorough determination of genetic linkage between strains with a hematopoietic phenotype (e.g., 129/Sv) and strains without a phenotype (C57BL/6) would be useful, but may require several years to complete. Therefore, until the issue on genetic modifiers has been resolved, researchers should be aware that the genetic background carrying a certain genetic modification may influence the outcome.

In summary, our data indicate that p21 deficiency in C57Bl/6 mice has a very limited, if any, effect on hematopoietic stem cell homeostasis, but may be more important under conditions of genotoxic stress. In addition, the difference in p21-deficient phenotype between 129/Sv [8] and C57Bl/6 mice (discussed herein), appears to result from modifier genes that either instigate or mask the effect of p21 deficiency in hematopoietic stem cells in these mouse strains.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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