Chapter 5
Normal hematopoietic stem cell functioning in B6p21<sup>−/−</sup> mice

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Abstract

Recently, several studies have suggested that p21, a prominent member of the family of cyclin-dependent kinase inhibitors, plays a crucial role in regulating hematopoietic stem and progenitor pool size. However, a rigorous test analyzing p21−/− stem cells in competitive repopulation assays has not been performed. In the present study we have backcrossed a p21 null allele from mice with a mixed genetic background to inbred C57BL/6 mice. As expected, mouse embryonic fibroblasts derived from B6p21−/− mice failed to undergo senescence. In contrast, homozygous deletion of the p21 allele did not affect the percentage of Lin− Sca-1+ c-kit+ (LSK) cells in S-phase, and did not result in any alterations of in vitro cobblestone area forming cell activity. Most importantly, B6p21−/− stem cells had completely normal competitive repopulating activity for up to 1 year after transplant. Also, no difference in stem cell pool size or competitive repopulating ability was observed after serial transplantation. Our data fail to support the notion that p21 is essential for stem cell function during steady state hematopoiesis.
Introduction

The genetic pathways that regulate the pool size of the hematopoietic stem cell compartment in the bone marrow 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 have been identified that have an effect on the number of stem cells in vivo \(^1\)-\(^3\). Rather, intracellular proteins such as transcription factors and chromatin remodelling factors have been shown to deregulate stem cell numbers\(^4\)-\(^6\). Recently, a series of studies has suggested that deficiency of cyclin-dependent kinase inhibitors, such as p27, p18, and p21, also results in stem or progenitor cell abnormalities\(^7\)-\(^9\). Cyclin-dependent kinase inhibitors have been shown to be associated with cellular senescence of fibroblasts\(^10\),\(^11\), and are believed to be involved in stem cell quiescence in the hematopoietic system\(^7\)-\(^9\). Whereas absence of p27 appears to increase the number of progenitor cells\(^8\), p18 and p21 deficiency has been documented to induce stem cell expansion\(^7\),\(^9\).

In competitive repopulation assays in which wildtype bone marrow cells were competed with p18\(^-/-\) cells in equal ratios, the large majority of white blood cells in the recipients was of p18\(^-/-\) genotype. It should be noted, however, that competitive repopulation assays using p18 deficient bone marrow cells were performed with mice of mixed 129/Sv (129) and C57BL/6 (B6) background. As it is well known that different inbred strains of mice show widely divergent stem cell characteristics\(^12\), the mixed genetic background of donor and recipient strains may confound, or indeed obscure, potential stem cell phenotypes.

It has been documented that p21 deficiency leads to an increased number of late developing cobblestone area forming cells\(^7\). Furthermore, serial transplantation using p21\(^-/-\) cells resulted in premature stem cell exhaustion, and p21\(^-/-\) animals were more sensitive to the S-phase specific cytotoxic drug 5-fluorouracil. Because of the mixed genetic background, long-term repopulation assays in which p27 or p21 deficient bone marrow cells were competitively transplanted with syngeneic wildtype bone marrow cells have not been performed yet. To assess the role of p21 in regulating stem cell activity during steady state hematopoiesis, we have backcrossed the p21 null allele to a B6 background and performed standard competitive repopulation assays in primary and secondary transplantations, in which various cell doses of CD45.2 B6p21\(^-/-\) cells were co-transplanted with congeneric CD45.1 B6 cells.
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Materials and methods

Animals
B6;129S2-Cdkn1a^{tm1Tyj} (B6/129p21^{-/-}) were purchased from The Jackson Laboratory (Bar Harbor, ME). Wildtype female C57BL/6 (B6^{wt}) mice were purchased from Harlan (Horst, The Netherlands) and were used to backcross B6/129p21^{-/-} mice, and as recipients for competitive repopulation. After 5 backcrosses littermate B6p21^{+/+}, B6p21^{+-}, and B6p21^{-/-} mice were selected by genotyping as described. Eight to twelve weeks old C57BL/6 mice were used for serial transplantation (Harlan). For competitive repopulation assays B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1) congenic mice (bred locally) were used as competitors.

Cell culture
Mouse Embryonic Fibroblasts (MEFs) were derived from 14-day-old embryos as described previously. The number of population doublings (PDL) was calculated by the formula: \( \Delta \text{PDL} = \log(n_f/n_i)/\log2 \) in which \( n_f \) = final number of cells and \( n_i \) is initial number of cells. Bone marrow (BM) cells were isolated and cobblestone area forming cell (CAFC) assays were performed as described previously.

Cell cycle analysis
Lin^{+}Sca-1^{+}c-kit^{+} (LSK) bone marrow cells were stained according to a four color method for cell selection and DNA analysis with some modifications. Briefly, after erythrocyte lysis cells were stained with a cocktail of lineage specific antibodies, anti-c-kit-Allophycocyan (APC), and anti-Sca-1-phycoerythrin (PE) antibodies (Pharmingen, San Diego, CA). After staining with Streptavidin Alexa 488 (Molecular Probes, Eugene, OR) cells were fixed with 0.1% para-formaldehyde and permeabilized with 0.1% saponine for 15 min at room temperature. Cells were subsequently resuspended in 0.5 ml PCB solution (6.1 M phosphate-citrate, 0.15 M NaCl, 5mM sodium EDTA, 0.5% BSA and 0.02% saponin, pH 4.8) and 10 \( \mu \)g/ml 7-AAD (Sigma-Aldrich, St. Louis, MI) for DNA staining for 45 minutes. Cells were resuspended in PCB containing 10 \( \mu \)g/ml AD (Sigma-Aldrich), and placed on ice protected from light for at least 10 minutes. Samples were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Palo Alto, CA).

Competitive repopulation assays
B6^{wt} recipients were lethally irradiated (9.5 Gy, IBL 637 Cesium-135 \( \gamma \) source, CIS Biointernational, Gif-sur-Yvette, France) 24 hours prior to transplantation. B6p21^{-/-} and B6p21^{+/+} littermates were sacrificed, bone marrow (BM) cells were isolated and transplanted in various cell doses in
competition with $2 \times 10^6$ CD45.1 congenic BM cells ($n = 4$ mice per group). In addition, LSK cells were purified from BM of B6wt- and B6p21−/− mice as described previously. Primary transplants were initiated with 1,500 LSK cells ($n = 8$ for B6wt and $n = 5$ for B6p21−/−). Four months after primary transplant, recipients were sacrificed, BM cells were isolated and serially transplanted in a 1:1 ratio with $5 \times 10^5$ competitor BM cells ($n = 6$ for B6wt and $n = 5$ B6p21−/−). At several time points after transplantation leukocytes were stained with anti-CD45.1-PE and anti-CD45.2-fluorescein isothiocynate (FITC) (Pharmingen) antibodies. Chimerism was assessed using flow cytometry (FACSCalibur, Becton Dickinson).

Results and Discussion

Absence of senescence in B6p21−/− mouse embryonic fibroblasts (MEFs)

Deficiency of p21 is associated with absence of a senescence phenotype in fibroblasts. In order to phenotypically confirm the absence of the wildtype p21 allele (Figure 5.1A), MEFs were grown from d14 littermate embryos of each genotype and the number of population doublings was assessed (Figure 5.1B). MEFs derived from B6p21+/+ mice showed similar growth kinetics compared to B6wt MEFs, and ceased proliferation after 5 to 6 passages. MEFs derived from B6p21+/− mice showed an intermediate phenotype, whereas MEFs derived from B6p21−/− mice did not senesce and grew continuously. These data confirm the established role of p21 in the senescence program of MEFs in vitro.

In vitro stem cell activity of B6p21 deficient bone marrow cells

We determined CAFC d35 frequencies in BM isolated from normal B6wt, B6p21−/−, and B6p21+/+ mice and in addition in B6wt and B6p21−/− BM isolated four months after primary transplant. We also measured CAFC frequencies in BM cells isolated from the original B6/129p21−/− mixed background strain, both at young (~3 months) and old (~21 months) age. We confirmed the data by Cheng et al., who showed that young B6/129p21−/− mice have a substantial higher CAFC d35 frequency compared to B6wt (Figure 5.1C). Strikingly, when B6/129p21−/− mice were aged for 20 months, CAFC d35 frequency increased even further. We have previously shown that a similar increase can also be observed in aged B6wt mice.

In contrast, when we backcrossed B6/129p21−/− animals to a B6 background, there was no effect of the absence of p21 on CAFC frequencies (Figure 5.1C). Moreover, B6wt and B6p21−/− stem cells also show similar CAFC frequencies four months after transplantation (Figure 5.1C). The observed (4.5-fold) decrease in CAFC frequency after transplantation was similar to values in

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previous studies of serial transplantation. In addition, we were unable to document any difference in peripheral blood cell values between B6\(^{\text{wt}}\) and B6p21\(^{-/-}\) mice (data not shown). As absence of p21 would be expected to be associated with increased cell cycling, we determined the percentage of LSK cells in the S-phase of the cell cycle (Figure 5.1D). We failed to document any difference between B6\(^{\text{wt}}\) (9.4 ± 0.3, \(n = 25\)), B6p21\(^{-/-}\) (5.4 ± 0.4, \(n = 3\)), B6p21\(^{+/+}\) (4.3, \(n = 1\)), and B6p21\(^{+/+}\) (5.4 ± 2.4, \(n = 2\)) mice.

**In vivo competitive repopulating activity of B6p21 deficient bone marrow cells.**

To conclusively assess the effect of p21 on hematopoietic stem cell functioning, we performed competitive repopulation assays in which various cell doses of CD45.2 B6p21\(^{-/-}\) and B6p21\(^{+/+}\) bone marrow cells were competed with \(2 \times 10^6\) CD45.1 congenic bone marrow cells. After transplant, B6p21\(^{-/-}\) and B6p21\(^{+/+}\) bone marrow cells contributed equally well to long-term hematopoiesis (Figure 5.2A), and, observed levels of chimerism were in the expected range (Figure 5.2B). Finally, BM cells isolated from primary recipients transplanted with 1,500 LSK cells four months earlier were competed in a 1:1 ratio with freshly isolated BM cells (CD45.1). After a temporary advantage for B6p21\(^{-/-}\) BM cells 5 weeks after secondary transplantation, competitive repopulating ability was similar for B6p21\(^{-/-}\) and B6\(^{\text{wt}}\) stem cells (Figure 5.2C).

Collectively, our data fail to support the notion that p21 is an essential stem cell gene for steady state hematopoiesis. The in vitro p21\(^{-/-}\) stem cell phenotype, as originally documented in 129p21\(^{-/-}\) mice, appears to result from modifier genes present in the mixed genetic background. However, we cannot exclude that a p21 stem cell phenotype is masked by modifying genes in B6\(^{\text{wt}}\) mice, or that p21 deficiency does play a role in perturbed hematopoiesis, such as after chemotherapy.
Figure 5.1. In vitro phenotype of B6\textsuperscript{wt}, B6p21\textsuperscript{-/-}, B6p21\textsuperscript{+/-}, and B6p21\textsuperscript{+/+} cells. A. Littermate mice were genotyped as described\textsuperscript{7}, using an allele-specific PCR which results in a 750 bp product for the p21\textsuperscript{-/-} allele and a 900 bp product for the p21\textsuperscript{+/-} allele. B. Growth kinetics of MEFs derived from B6\textsuperscript{wt} (closed triangles), B6p21\textsuperscript{-/-} (closed circles), B6p21\textsuperscript{+/-} (grey triangles), and B6p21\textsuperscript{+/+} (open squares) day-14-embryos. As expected, B6p21\textsuperscript{+/-} derived MEFs showed equal growth patterns as B6\textsuperscript{wt} MEFs, whereas B6p21\textsuperscript{-/-} MEFs failed to display any signs of senescence. B6p21\textsuperscript{+/-} MEFs showed an intermediate phenotype. Cultures were performed in triplicate and the mean value ± 1 SEM is shown. C. CAFC d35 frequencies in the bone marrow of B6\textsuperscript{wt}, B6p21\textsuperscript{-/-} (both prior and after transplant), B6p21\textsuperscript{+/-}, B6p21\textsuperscript{+/+}, and original B6/129p21\textsuperscript{-/-} mice are shown. B6/129p21\textsuperscript{-/-} mice showed increased CAFC d35 frequencies, as has been previously reported\textsuperscript{7}. However, in backcrossed B6p21\textsuperscript{-/-} mice no difference compared to B6\textsuperscript{wt} could be observed. Also, no difference in CAFC frequencies four months after transplantation of 1,500 LSK cells was observed between B6\textsuperscript{wt} and B6p21\textsuperscript{-/-} mice (after 1\textsuperscript{st} tpx). Aged (>20 months) B6/129p21\textsuperscript{-/-} mice showed a 4-fold increase of CAFC d35 frequencies compared to young B6/129p21\textsuperscript{-/-} mice. Error bars indicate 95% confidence intervals. D. Representative DNA histogram used to assess cell cycle status of Lin\textsuperscript{-}Sca-1\textsuperscript{-}c-kit\textsuperscript{+} (LSK) stem cells. The percentage of LSK cells in S-phase for all strains used in this study is indicated. No significant differences between strains were observed.
Figure 5.2. In vivo phenotype of B6p21−/− stem cells. A. B6p21−/− (closed symbols) and B6p21+/+ (open symbols) BM cells were mixed in a 1:4 (squares), 1:1 (triangles), or 2:1 (circles) ratio with 2 × 10^6 CD45.1 congenic BM cells and transplanted in lethally irradiated recipient mice (B6 wt, n = 5 in all groups). Chimerism levels remained stable for 1 year, over all cell doses tested. Moreover, identical competitive repopulation potential was observed using B6p21−/− or B6p21+/+ bone marrow cells, arguing against the presence of a competitive defect of B6p21−/− stem cells. Mean chimerism levels ± 1 SEM are shown.

B. Closed bars indicate the expected levels of chimerism calculated from transplanted cell ratios, assuming equal repopulating ability of CD45.1, B6p21−/− and B6p21+/+ stem cells. For each cell ratio the mean observed level of chimerism (± 1 SEM) was calculated from the individual levels of chimerism over the entire time course of the experiment, as shown in panel A. No significant difference between observed and expected chimerism was observed, again indicating similar repopulating potential of B6p21−/− and B6p21+/+ stem cells.

C. Chimerism after secondary transplantation of B6 wt and B6p21−/− BM cells. Four months after transplantation of 1,500 B6 wt or B6p21−/− LSK cells, primary recipients were sacrificed and unfractionated BM cells were transplanted in a 1:1 ratio with 5 × 10^5 freshly isolated BM cells. Shortly after secondary transplantation a minor competitive advantage of B6p21−/− BM cells can be observed, but this is rapidly lost after 10 weeks after transplantation and donor contribution is equal for B6p21−/− and B6 wt in competitive repopulation.
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