A limited role for p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} in the loss of hematopoietic stem cells during proliferative stress

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It has long been known that prolonged culture or serial transplantation leads to the loss of hematopoietic stem cells (HSCs); however, the mechanisms for this loss are not well understood. We hypothesized that expression of p16\textsuperscript{Ink4a} or p19\textsuperscript{Arf} or both may play a role in the loss of HSCs during conditions of enhanced proliferation, either in vitro or in vivo. Arf was not expressed in freshly isolated HSCs from adult mice but was induced in phenotypically primitive cells after 10 to 12 days in culture. When cultured bone marrow cells from either Arf\textsuperscript{+/−} or Ink4a-Arf\textsuperscript{−/−} mice were compared to wild-type cells, no significant differences in HSC activity were seen. We then evaluated the role of p19\textsuperscript{Arf} and p16\textsuperscript{Ink4a} in the loss of HSCs during serial transplantation. Bone marrow cells from Ink4a-Arf\textsuperscript{−/−}, but not Arf\textsuperscript{−/−}, mice had a modestly extended life span and, on average, supported reconstitution of one additional recipient compared to wild-type cells. Mice given transplants of Ink4a-Arf\textsuperscript{−/−} cells eventually died of hematopoietic failure in the next round of transplantation. We conclude that mechanisms independent of the Ink4a-Arf gene locus play a dominant role in HSC loss during conditions of proliferative stress. (Blood. 2005;106:827-832)

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Introduction

Rapid turnover of mature hematopoietic cells requires production of tens of billions of cells every day during the life span of mammals. Hierarchical organization of the hematopoietic system helps to ensure the lifelong production of blood cells of different lineages. Hematopoietic stem cells (HSCs) have the ability to self-renew, differentiate into all hematopoietic lineages, and repopulate lethally irradiated hosts. Proliferative activity of HSCs is tightly regulated by incompletely understood mechanisms. HSCs are relatively quiescent and generally cytokine-resistant, with a more limited life span. Although the low proliferation potential of HSCs during conditions of enhanced proliferation, either in vitro or in vivo, is an obstacle in gene therapy applications and in clinical strategies to expand HSCs in vitro, several candidate genes have recently been implicated in the HSC self-renewal process. It has recently been demonstrated that HSC entry into the cell cycle is regulated by a cyclin-dependent kinase inhibitor (CKI), p21\textsuperscript{Cip1}, whereas proliferation of progenitors is regulated by another cell-cycle inhibitor, p27\textsuperscript{Kip1}. Deletion of an early G\textsubscript{1}-phase CKI, p18\textsuperscript{INK4C}, results in increased proliferation of progeny, have a robust proliferation potential, are highly cytokine-responsive, and have a more limited life span. Although the low proliferation potential of HSCs may play a role in maintenance of HSCs over time, it is an obstacle in gene therapy applications and in clinical strategies to expand HSCs in vitro.

Several candidate genes have recently been implicated in the HSC self-renewal process. It has recently been demonstrated that HSC entry into the cell cycle is regulated by a cyclin-dependent kinase inhibitor (CKI), p21\textsuperscript{Cip1}, whereas proliferation of progenitors is regulated by another cell-cycle inhibitor, p27\textsuperscript{Kip1}. Deletion of an early G\textsubscript{1}-phase CKI, p18\textsuperscript{INK4C}, results in increased self-renewal of the primitive hematopoietic cells and improved long-term engraftment. Low levels of telomerase in HSCs have been implicated in the replicative senescence of HSCs; however, overexpression of TERT, a catalytic reverse transcriptase component of telomerase, does not lead to enhancement of HSC self-renewal. Overexpression of the HOXB4 homeodomain transcription factor is able to induce expansion of HSCs both in vivo and in vitro. Overexpression of P-glycoprotein can also cause an

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there is an Arf/GFP knock-in mouse model where green fluorescent protein (GFP) is placed under control of the Arf promoter and results in an Arf-null phenotype.21

In mouse embryonic fibroblasts, both p19Arf and p16Ink4a proteins are induced during culture and play a role in limiting their life span.22,23 Based on these observations, we hypothesized that the Ink4a-Arf gene products may be induced during culture of bone marrow (BM) cells and play a role in the HSC loss seen during extended ex vivo cultures or during HSC exhaustion seen in serial transplantation assays. To address this question, we used mice lacking either the Arf gene alone or both the Ink4a and Arf genes to determine the role of the Ink4a-Arf locus in mouse HSCs.

**Materials and methods**

**Mice**

All transplant recipient mice were C57BL/6 females 8 to 12 weeks of age. Donor BM for competitive repopulation studies was obtained from B6.SJL-Ptprca Pep3b/B6 (C57BL/6J) congenic mice, purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in the Animal Resources Center with free access to sterilized food and water, and all experiments were approved by the Institutional Animal Care and Use Committee of St Jude Children’s Research Hospital.

**Preparation of BM cells**

Donor mice were humanely killed by CO2 inhalation, and the femurs and tibias were isolated. BM was flushed out in phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) using a 5-ml syringe and 25-gauge needle, and single-cell suspensions were prepared. An aliquot of the cells was spun down, and resuspended in the fresh media at the initial concentration of 0.5 × 10^6 BM cells. For CR analysis with sorted cells, 2 × 10^6 cells of Lin-GFP or Lin-GFP sorted cells after 10 days in culture were mixed with 2 × 10^5 freshly isolated B6.SJL-Ptprca Pep3b/B6 (C57BL/6-Ly5.1) BM cells as a competitor. Each CR experiment was repeated 2 to 3 times with 8 to 12 mice in each group. For the CFU-S experiments, mice received transplants of 1 × 10^5, 5 × 10^5, or 1 × 10^6 sorted cells (4 mice/group/dose) after 9 Gy whole-body irradiation. Spleens were harvested 13 days later, stained with Bouin solution (Sigma, St Louis, MO), and photographed.

**Measurement of donor-derived hematopoiesis**

Chimerism from Ly5.1 versus Ly5.2 donors was measured in 2 white blood cell compartments in the peripheral blood—myeloid (positive for granulocyte and macrophage markers Gr1 and Mac1) and nonmyeloid (Gr1- / Mac1-). Then, 75 μL peripheral blood was obtained from the retro-orbital plexus of anesthetized mice and mixed with 15 μL 10% EDTA (ethylenediaminetetraacetic acid) solution to prevent clotting. After lysing erythrocytes in hypotonic solution, the cells were costained with phycoerythrin (PE)-conjugated Gr-1 and Mac1 antibodies and fluorescein isothiocyanate (FITC)-conjugated Ly5.2 antibodies. Dead cells were excluded by propidium iodide staining. All samples were analyzed by flow cytometry and data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

**Serial transplantation**

BM was harvested from 2 to 5 mice from each of the following genotypes: C57BL/6 Arf-/-, C57BL/6, 129/C57BL/6 Ink4a-Arf-/-, and 129/C57BL/6. Primary recipients were given transplants with 2 × 10^5 BM cells of either genotype. For secondary transplantation, the BM was harvested from the animals in the primary transplant group 10 weeks later and transplanted into the secondary C57BL/6 animals at doses of 1 × 10^3, 2 × 10^3, 5 × 10^3, and 1 × 10^4. Complete blood count (CBC) analysis was done every 2 weeks starting 4 weeks after irradiation to detect animals with hematopoietic failure. For the following transplantations, BM was harvested separately from each group and transplanted into the next recipient at the same cell dose. Serial transplantation was repeated twice with similar results.

**Results**

Arf is not expressed in freshly isolated HSCs but is induced during culture

To analyze expression of Arf in fresh and cultured BM cells, we used ArfGFP+ mice, which express GFP under control of Arf transcriptional elements.21 GFP expression was absent in bulk BM, lineage-negative (Lin-), and c-kit+ Sca1+ Lin- (KSL) cell populations (Figure 1A). These data show that immature HSC-enriched cell populations in the BM do not express the Arf/GFP allele.

We next tested whether Arf/GFP could be induced during extended culture in serum and hematopoietic cytokines. BM cells from ArfGFP+ mice were cultured for 12 days in 15% FCS, supplemented with IL-3, IL-6, and SCF. We detected induction of GFP expression in about 12% to 20% of the Lin- and KSL cells (Figure 1B). It should be noted that many of the Lin- cells showed mature myeloid phenotypes in cytospin preparations (data not...
Figure 1. Expression analysis of the Arf/GFP allele in freshly isolated and cultured murine BM cells. (A) freshly isolated BM cells were stained with c-kit-allophycocyanin (APC), Sca1-PE, and a mixture of FITC-conjugated antibodies against mature lineage markers (CD4, CD8, B220, Gr1, and Mac1). The top row shows analysis of BM cells from wild-type BL6 mice and the bottom row from Arf\textsuperscript{GFP\textsuperscript{-}} mice. The left column shows GFP expression in a gated Lin\textsuperscript{-} fraction and the right from cells gated on the Lin\textsuperscript{-} c-kit\textsuperscript{-} Sca1\textsuperscript{-} phenotype. (B) After 12 days of culture, BM cells from Arf\textsuperscript{GFP\textsuperscript{-}} mice were stained with c-kit-APC, Sca1-PE, and Lin-FITC antibody cocktail. The top row shows analysis of BM cells from wild-type BL6 mice and the bottom row from Arf\textsuperscript{GFP\textsuperscript{-}} mice. As before, Lin\textsuperscript{-} and c-kit\textsuperscript{-} Sca1\textsuperscript{-} Lin\textsuperscript{-}(KSL) cells were gated and analyzed. Positions of gates and percentage of gated cells are shown.

shown), so these populations are not equivalent to those seen in fresh BM cells. BM cells from Arf\textsuperscript{GFP\textsuperscript{-}} mice have very similar kinetics of GFP induction in culture compared to cells from Arf\textsuperscript{GFP\textsuperscript{+}} mice (data not shown).

Induction of Arf in culture impairs CFU-S formation

To determine the functional effects of Arf induction on primitive hematopoietic cells, we sorted Lin\textsuperscript{-}GFP\textsuperscript{+} cells from 10-day cultures containing either Arf\textsuperscript{GFP\textsuperscript{+}} or Arf\textsuperscript{GFP\textsuperscript{-}} BM cells. It is important to note that Arf\textsuperscript{GFP\textsuperscript{-}} cells contain one copy of the wild-type p19\textsuperscript{Arf} gene so that GFP\textsuperscript{+} cells will also express wild-type Arf. In contrast, GFP\textsuperscript{+} cells from Arf\textsuperscript{GFP\textsuperscript{-}} mice do not express any Arf because the GFP allele disrupts the Arf coding sequence.\textsuperscript{21}

Increasing doses of sorted cells were assayed for their ability to form spleen colonies in sublethally irradiated mice. At the lowest cell doses, separate colonies were formed and counted visually. At higher cell doses, the whole spleen became confluent with CFU-Ss that could not be individually counted (Figure 2A). At cell doses in which CFU-Ss could be counted, the average of 3 experiments was determined and is shown in Figure 2B.

As expected, the number of CFU-Ss was equivalent using cultured Lin\textsuperscript{-}, GFP\textsuperscript{-} cells from either wild-type, Arf\textsuperscript{GFP\textsuperscript{+}}, or Arf\textsuperscript{GFP\textsuperscript{-}} mice. By contrast, there was significant reduction in CFU-Ss from Lin\textsuperscript{-}GFP\textsuperscript{+} cells obtained from Arf\textsuperscript{GFP\textsuperscript{+}} mice (Figure 2A-B). Sorted Lin\textsuperscript{-}GFP\textsuperscript{+} cells from Arf\textsuperscript{GFP\textsuperscript{-}} mice gave about 10-fold fewer day-13 CFU-Ss in comparison to Lin\textsuperscript{-} cells from wild-type mice, or Lin\textsuperscript{-}GFP\textsuperscript{-} cells from Arf\textsuperscript{GFP\textsuperscript{-}} mice (Figure 2B). This indicates that expression of the wild-type Arf allele in GFP\textsuperscript{+} cells from Arf\textsuperscript{GFP\textsuperscript{+}} mice resulted in a loss of CFU-Ss. It was also noted that Lin\textsuperscript{-}GFP\textsuperscript{-} cells from Arf\textsuperscript{GFP\textsuperscript{GFP\textsuperscript{-}}} mice gave somewhat fewer CFU-Ss than Lin\textsuperscript{-}GFP\textsuperscript{+} cells from any of the other genotypes. This may indicate that another gene is coinduced and leads to a relative depletion of CFU-Ss, or that mature cells are more likely to express the Arf/GFP gene than CFU-S cells.

Loss of Arf or Ink4a-Arf has no significant effect on hematopoietic engraftment using freshly isolated or cultured BM cells

To determine whether Arf or Ink4a-Arf deletion would alter the ability of HSCs to engraf and repopulate lethally irradiated mice, we performed CR experiments comparing BM cells from Arf\textsuperscript{-} or Ink4a-Arf-null and wild-type mice. Lethally irradiated recipient mice were given transplants of a 1:1 mixture of mutant to wild-type cells, which can be individually distinguished by their CD45.2 and CD45.1 surface markers, respectively. After transplantation, the chimism levels for mutant cells were assessed in peripheral blood leukocytes by flow cytometry. Using freshly isolated BM cells, equal contributions from the wild-type versus mutant cells were seen in peripheral blood myeloid and lymphoid cells (data not shown). Contribution of donor-derived cells did not change significantly between 8 weeks to 24 weeks after transplantation. The
experiments were done twice with the same results. These experiments show that loss of either Arf or Ink4a-Arf did not affect the repopulation abilities of freshly isolated BM HSCs, which is consistent with the lack of Arf expression in freshly isolated BM.

To assess the role of Ink4a-Arf locus in HSC loss during culture, we cultured Arf−/− or Ink4a-Arf−/− BM cells for 12 days and then performed a CR assay to determine whether loss of these genes would result in an increase in HSC numbers. At day 12 of culture, all the progeny of 0.4 × 10⁶ freshly isolated BM cells were mixed with 2 × 10⁶ fresh CD45.1+ competitor BM cells. This mixture was transplanted into lethally irradiated recipient mice. Total cell expansion at 12 days was 6 to 7 times greater for the mutant cells compared to wild-type cells.

Flow cytometry analysis of peripheral blood leukocytes at varying time points after transplantation showed that the chimera from cultured wild-type cells was between 6% and 10%, verifying the expected HSC loss in culture (Figure 3A-B). Reconstitution from the mutant BM cells was equivalently reduced, indicating that mutant HSCs were lost at the same rate as wild-type cells during culture. These results indicate that the loss of HSCs during culture does not depend on expression of the Ink4a-Arf locus. These experiments were done 3 times and gave equivalent results.

Arf−/− BM has no advantage in serial transplantation
We next tested the role of Arf in HSC proliferation using a serial transplantation protocol to induce enhanced HSC proliferation in vivo. In the first round of transplantation, recipient mice received 2 × 10⁵ BM cells from either wild-type or knock-out mice. Ten weeks after the first transplant, BM was transplanted into secondary recipients at different doses ranging from 1 × 10⁵ to 1 × 10⁶ cells (Figure 4A). In subsequent transplantations, BM from these groups was separately collected, pooled, and transplanted into subsequent recipients at the same dose. Blood was collected biweekly for complete CBC analysis to monitor the status of the hematopoietic system.

In all transplantation groups, the animals receiving the lowest dose of the BM cells were the first to develop signs of hematopoietic failure and HSC exhaustion. The first deaths were observed in secondary recipients that received 1 × 10⁵ BM cells. At each transplantation cycle, no difference was noted between wild-type and Arf−/− BM (Figure 4B). These results were confirmed in an independent second experiment. Consistent with the lack of effect of Arf deletion on HSC repopulation in serial transplantation, the ArfGFP−/+ allele was not expressed in vivo during the reconstitution phase (1.5-3 weeks after transplantation of 2 × 10⁵ cells) in bulk BM, Lin−, or KSL cell populations (Figure 4C).

Ink4a-Arf−/− HSCs have a modest survival advantage in serial transplantation
Mice receiving transplants of Ink4a-Arf−/− BM cells had a reproducible advantage compared to those given wild-type cells. Ten weeks after transplantation, 80% of secondary recipients given transplants of 1 × 10⁵ BM cells from wild-type mice died of hematopoietic failure, whereas all recipients given transplants of 1 × 10⁵ Ink4a-Arf−/− BM cells survived (Figure 5A). Tertiary recipients given transplants of 1 × 10⁵ BM cells from Ink4a-Arf−/− secondary recipients died of hematopoietic exhaustion within 5 weeks after transplantation (Figure 5B), indicating that the enhancement of self-renewal in mutant HSCs was limited. At each transplantation cycle, the survival advantage of Ink4a-Arf−/− HSCs was noted. For instance, in quaternary recipients given transplants of 1 × 10⁵ BM cells, 90% of the recipients who received transplants of wild-type BM cells died by 10 weeks after transplantation, whereas 100% of mice who received BM cells from Ink4a-Arf−/− BM survived (Figure 5C). CBCs performed on sick animals revealed severe anemia and leukopenia (data not shown), confirming that death was due to HSC exhaustion. These results were confirmed in an independent second experiment. Annexin V staining of the BM from primary recipients 2, 3, and 4 weeks after transplantation showed no difference in the apoptotic rates between wild-type and Ink4a-Arf−/− cells (Figure 5D).
Discussion

We tested the role of the Ink4a-Arf locus in HSC proliferation both in vitro and in vivo. We found that freshly isolated murine BM cells do not normally express p19Arf, whereas the p19Arf promoter was activated in bulk cultures. To test whether Arf expression could kill primitive cells in culture, we sorted cells expressing the Arf/GFP allele after culture and determined the number of CFU-Ss relative to controls. We found that the GFP+ fraction of cultured Arf/GFP+ BM cells had a greatly reduced ability to form CFU-Ss. To separate the activation of Arf promoter from effects of Arf expression, we sorted and transplanted cultured BM cells from Arf/GFP+ mice. The Lin- GFP+ fraction of Arf/GFP+ BM resulted in higher CFU-S activity in comparison with the Lin+ GFP- fraction from either genotype, indicating that Arf is preferentially expressed in mature cells that are depleted in CFU-S activity, or that another gene was coinduced with Arf and decreased CFU-S activity.

Consistent with absence of p19Arf expression, we found that freshly isolated HSCs from either Arf+/− or Ink4a-Arf−/− mice repopulate irradiated hosts with the same efficiency as wild-type HSCs. Activation of Arf promoter during in vitro culture suggested that the Ink4a-Arf locus may play a role in the loss of HSCs associated with extended culture; however, the deletion of either Arf or Ink4a-Arf showed no significant effect on HSC loss in culture. This is consistent with our observation that Arf is induced only in a small proportion of cultured cells and implies that most HSCs do not express Arf when cultured.

We also tested BM from Arf-null and Ink4a-Arf-null mice in serial transplantation assays. We observed that Ink4a-Arf−/− BM sustained repopulation activity for one additional round of transplantation compared to congenic wild-type BM cells. These data show that both p19Arf and p16Ink4a have a limited role in HSC self-renewal or only p16Ink4a is responsible for this effect. When a selective Ink4a-null mouse is available that is backcrossed on a pure C57BL/6 background, it will be possible to discriminate between these 2 possibilities.

Bmi1 is expressed in hematopoietic and neural stem cells, and its absence in Bmi1−/− mice leads to the rapid postnatal depletion of the stem cells in these compartments with subsequent failure of hematopoiesis.13-15 Bmi-1 deletion was associated with increased expression of p19Arf and p16Ink4a proteins, suggesting that the Ink4a-Arf locus may play a role in stem-cell regulation. Our analysis of the properties of HSCs containing deletions of the Arf gene alone, or of both the Ink4a and Arf genes, shows that although repression of Ink4a-Arf locus may be necessary for HSC proliferation, elimination of these genes results in only a modest increase in HSC self-renewal in serial transplantation assays and no effects during extended culture. Our results suggest that the well-characterized loss of HSCs during culture and during serial transplantation is not predominantly caused by induction of the Ink4a-Arf locus but is instead mediated by other as-yet-undefined mechanisms. Recent data show that another member of the Ink4 family of the proteins, p18Ink4c, appears to be a strong inhibitor limiting self-renewing capacity of HSCs in vivo. Its deletion results in improved long-term engraftment of the mutant cells, largely by increasing self-renewing divisions of the primitive cells.4 Therefore, p18Ink4c expression could have a role in HSC depletion during enhanced proliferation. Another possibility is that alternate targets of Bmi1, which have been described,13 could play a role in HSC loss during enforced proliferative stress.

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References


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