STEM CELLS AND DEVELOPMENT
Volume 21, Number 10, 2012

DOI: 10.1089/scd.2011.0476

# Cyclin Dependent Kinase Inhibitors Differentially Modulate Synergistic Cytokine Responsiveness of Hematopoietic Progenitor Cells

Hal E. Broxmeyer,[1] David S. Franklin,[2] Scott Cooper,[1] Giao Hangoc,[1] and Charlie Mantel[1]

Cyclin dependent kinase inhibitors (CDKIs) influence proliferation of hematopoietic progenitor cells (HPCs), but little is known of how they influence proliferative responsiveness of HPCs to colony stimulating factors (CSFs), alone and in combination with other hematopoietically active factors, such as the potent co-stimulating cytokine stem cell factor (SCF), or inhibition by myelosuppressive chemokines. Using mice with deletions in $p18^{INK4c}$, $p21^{CIP1/WAF1}$, or $p27^{KIP1}$ genes, and in mice with double gene deletions for either *p18/p21* or *p18/p27*, we determined effects of absence of these CDKIs and their interactions on functional HPC numbers in vivo, and HPC proliferative responsiveness in vitro. There is a decrease in bone marrow HPC proliferation in $p18^{-/-}$ mice commensurate with decreased numbers of HPC, suggesting a positive role for *p18* on HPC in vivo, similar to that for *p21*. These positive effects of *p18* dominate negative effects of *p27* gene deletion. Moreover, the CDKIs differentially regulate responsiveness of granulocyte macrophage (GM) progenitors to synergistic cell proliferation in response to GM-CSF plus SCF, which is considered important for normal hematopoiesis. Responsiveness of HPCs to inhibition by myelosuppressive chemokines is directly related to the capacity of HPCs to respond to synergistic stimulation, and their cell cycle status. $P18^{INK4c}$ gene deletion rescued the loss of chemokine suppression of synergistic proliferation due to deletion of $p21^{CIP1/WAF1}$. These findings underscore the complex interplay of cell cycle regulators in HPC, and demonstrate that loss of one can sometimes be compensated by loss of another CDKI in both, a pro- or anti-proliferative context.

## Introduction

Cell cycle regulation is key to numbers, proliferative capacity, and function of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) in vivo and in vitro and to proper hematopoiesis [1,2]. The cell cycle is mediated in part by cyclin dependent kinases (CDKs) and CDK inhibitors (CDKIs). Seven CDKIs have been identified, belonging to the INK family of closely related ankyrin-repeat containing genes ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p18^{INK4d}$), or to the CIP1/KIP family ($p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$). Of these, $p21^{CIP1/WAF1}$ [1–7], $p27^{KIP1}$ [3,6,8–10], and $p18^{INK4c}$ [11,12] have been implicated in the regulation of different functions for HSC/HPC populations. Functional deletion of $p21^{CIP1/WAF1}$ gene in mice ($p21^{-/-}$) results in increases in HSC proliferation and absolute numbers in bone marrow. They also have impaired self-renewal capacity after serial transplantation [7], although recently a more limited role for $p21^{CIP1/WAF1}$ in maintaining normal HSC function has been reported [13]. In contrast, immature subsets of HPC of $p21^{-/-}$ mice have decreased HPC proliferation and reduced absolute numbers [3], whereas over expression has the opposite effect [4]. Deletion of $p27^{KIP1}$ ($p27^{-/-}$) results in enhanced proliferation and numbers of HPC [8] without effecting HSC number, cell cycling, or self-renewal [9]. Loss of $p18^{INK4c}$ ($p18^{-/-}$) results in increased long-term engraftment and increased self-renewal of HSC [9]. Interestingly, absence of $p18^{INK4c}$ counteracts HSC exhaustion of $p21^{-/-}$ cells after serial transplantation [11]. Thus, CDKIs differentially modulate HSC/HPC function in positive and negative ways.

Events mediating regulation of HPCs are perhaps as important as those that regulate HSCs, as these cells are intermediaries in the production of mature blood cells originating from the HSC compartment. Inherent in this regulation of hematopoietic progenitors is their response to cytokine stimulation of their proliferation [2]. Some colony stimulating factors (CSFs) stimulate one specific progenitor cell type, whereas others stimulate a number of different progenitors. Although granulocyte (G)-CSF will induce proliferation of mainly or only granulocyte progenitors (CFU-G), and macrophage (M)-CSF mainly or only stimulate macrophage progenitors (CFU-M), GM-CSF can stimulate

[1]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana.
[2]Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana.

proliferation of granulocyte macrophage (CFU-GM), in addition to CFU-G and/or CFU-M. Erythropoietin (EPO) is an example of a CSF which stimulates only colony formation by erythroid progenitors (BFU-E). HPCs responsive to a single cytokine are considered to be more mature subsets of HPCs [2]. In addition to the CSFs, there are also potent co-stimulating cytokines such as stem cell factor (SCF) and Flt3-ligand (FL), which act respectively through the tyrosine kinase receptors c-kit and Flt3, which have little or no CSF activity on their own. However, when a CSF is combined with one or both co-stimulating cytokines, SCF and/or FL, the responsive HPCs form larger colonies and are considered to be more immature HPCs than those stimulated by only a single CSF. We [2–4] and others [1] have been intrigued by the concept of cytokine synergy for proliferation of HPCs. Based on our previous interest in the role of the CDKI, $p21^{CIP1/WAF1}$ in cytokine synergy, we evaluated our hypothesis that $p18^{INK4c}$ plays a role in HPC proliferation and function, both alone, and also in combination with $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. We evaluated $p18^{-/-}$, and mice double-deleted for *p18/21* ($p18^{-/-}$-$p21^{-/-}$) and *p18/p27* ($p18^{-/-}$-$p27^{-/-}$), in comparison with control (+/+), $p21^{-/-}$, and $p27^{-/-}$ mice for: absolute numbers and cycling status of bone marrow and spleen granulocyte macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitor cells, responsiveness of CFU-GM to synergistic stimulation in vitro by the combination of granulocyte macrophage (GM) CSF and SCF, and to inhibition of HPC proliferation by selected members of the chemokine family.

## Materials and Methods

### Mice

These studies utilized normal control C57Bl/6 mice purchased from Jackson Laboratories (Bar Harbor, ME) and a number of CDKI knock-out ($^{-/-}$) mice: $p21^{-/-}$ [3], $p27^{-/-}$ [8], $p18^{-/-}$ [14,15], and dual $p18^{-/-}$-$p21^{-/-}$ [14], in addition to $p18^{-/-}$-$p27^{-/-}$ [15] and their littermate controls. The $p21^{-/-}$ mice were originally obtained from Chuxia Deng, NIDDK, NIH (Bethesda, MD), and bred at the Indiana University School of Medicine. All other CDKI knock-out mice were supplied by coauthor D.S. Franklin. All mouse studies followed IACUC guidelines.

### Cells

Femoral bone marrow and spleen cells were isolated and absolute numbers of HPC (CFU-GM, BFU-E, and CFU-GEMM) per femur and spleen, and the cycling status of the HPC (equals percent HPC in S-phase of the cell cycle as determined by high specific activity tritiated thymidine kill technique) were calculated as reported [16–18].

### Culture conditions

For studies evaluating absolute numbers and cycling status, bone marrow and spleen cells were plated respectively at $5\times10^4$ and $5\times10^5$ cells/mL in 1% methylcellulose culture medium in 30% fetal bovine serum (FBS; Hyclone, Inc., Logan, Utah) in the presence of 1 U/mL recombinant (r) human EPO (Epo; Amgen Corp., Thousand Oaks, CA), 5% vol/vol pokeweed mitogen mouse spleen cell conditioned medium [18], 50 ng/mL r mouse (m) SCF (R&D Systems, Minneapolis, MN), and 0.1 mM hemin (Eastman Kodak, Rochester, NY). For synergy studies, colony formation stimulated by rm GM-CSF (R&D Systems) was compared with that stimulated by the combination of GM-CSF and SCF. Chemokines (lymphotactin, XCL1; MCP-1, CCL2; IL-8, CXCL8; and MIG, CXCL9) were purchased from R&D Systems. The inhibitor effects of these myelosuppressive chemokines [19,20] were assessed on colony formation by bone marrow CFU-GM of normal C57Bl/6 mice at time zero, or after 24 h culture in suspension in the presence of rm FL (100 ng/mL; R&D Systems), rm GM-CSF (10 ng/mL), rm IL-6 (10 ng/mL; R&D Systems), and rm SCF (50 ng/mL) to increase the cycling of the CFU-GM. Cells in suspension culture for 24 h were washed prior to plating in semi-solid cultures. The cells were thus plated at time 0 or after 24 h in the absence or presence of the specified chemokine and in the presence of Epo, PWMSCM, and SCF plus hemin. All plates were incubated at lowered (5%) $O_2$ tension, to allow for optimal colony formation [21], in a humidified chamber at 5% $CO_2$.

### Statistics

Each experimental point was set up in triplicate and significant differences determined by 2 tailed students *t*-test, with a $P$ value of at least $<0.05$ considered statistically significant.

## Results

### Effects of CDKIs on HPC numbers and proliferation

Different CDKIs differentially effect proliferation of HPCs [1–13]. Functional deletion of $p21^{CIP1/WAF1}$ results in decreased numbers and cycling status of the immature subsets of mouse bone marrow HPC [4,5], those HPC that respond to synergistic proliferative stimulation when exposed to a CSF plus the potent co-stimulating/augmenting cytokine, SCF [21]. In contrast, loss of $p27^{KIP1}$ results in enhanced proliferation and numbers of HPCs [8].

In the mouse, the spleen is an active hematopoietic organ, but not all reports evaluate both marrow and spleen for hematopoiesis and hematopoietic effects, and usually not as side-by-side comparisons within the same mouse. Also, there are few reports of assessment of differential effects on subsets of HPCs such as CFU-GM, BFU-E, and CFU-GEMM, or whether the effects seen are the same for the immature subsets of each [responsive to CSF(s) plus SCF], or the more mature subsets of these cells (responsive to only a CSF) [2]. Since such evaluations can enhance information of effects of CDKIs, we assessed numbers and cycling of immature subsets of CFU-GM, BFU-E, and CFU-GEMM in marrow and spleen (Fig. 1). Consistent with what has been reported for total bone marrow HPCs of mice deleted of $p21^{CIP1/WAF1}$ [3], immature subsets of CFU-GM, BFU-E, and CFU-GEMM of $p21^{-/-}$ significantly decreased in bone marrow and spleen (Fig. 1). Moreover, the cycling status (% HPCs in S-Phase of the cell cycle) of these cells in bone marrow and spleen significantly decreased, even though cycling status of HPCs of control mice were already in a slow cycling state (Fig. 1). In contrast, absolute numbers of bone marrow and spleen HPC (CFU-GM, BFU-E, and CFU-GEMM) in the marrow and spleen of $p27^{KIP1-/-}$ mice significantly increased, and also the cell cycle status for the 3 HPC subsets was enhanced.

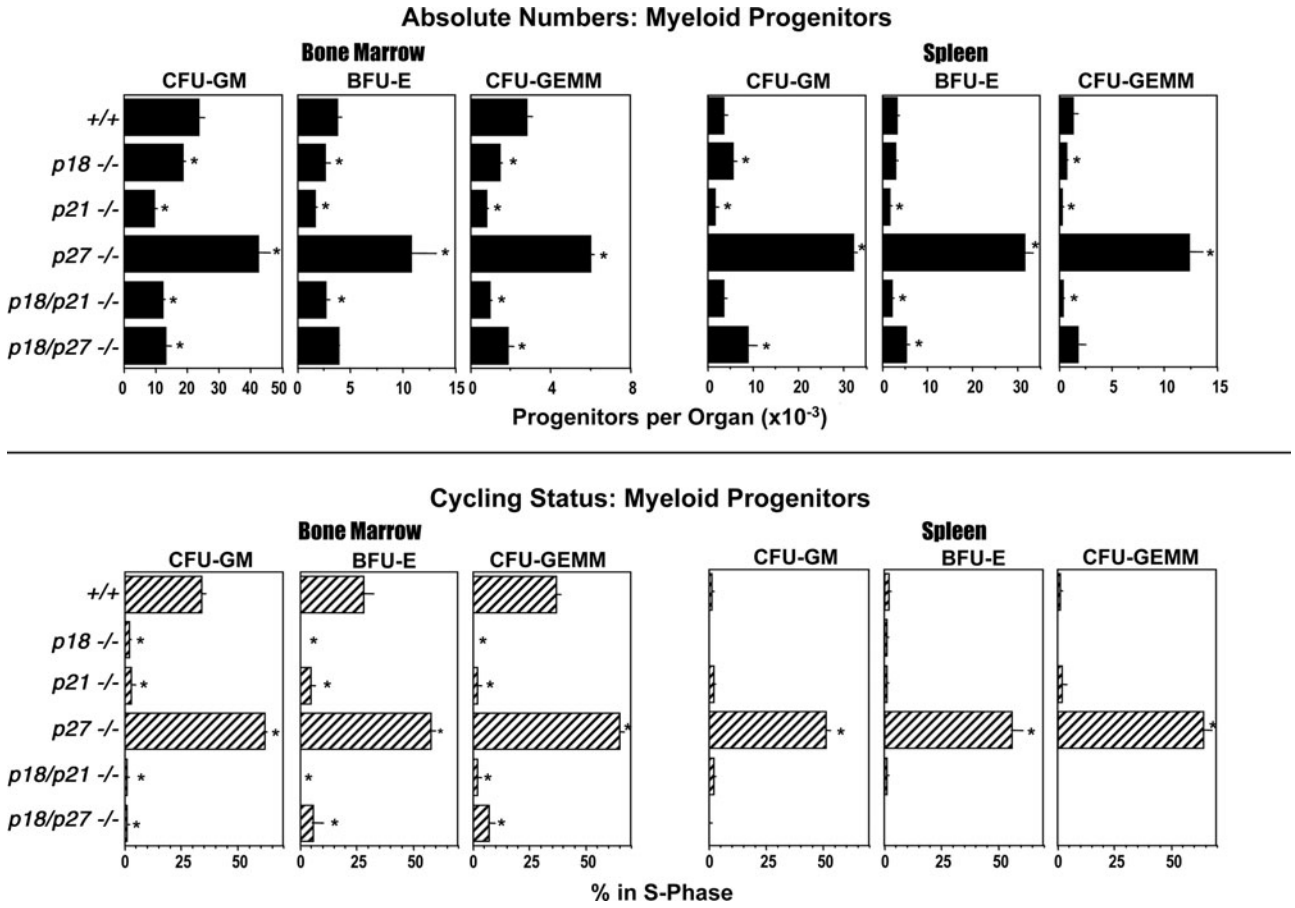


**FIG. 1.** Influence of CDKI$^{-/-}$ on absolute numbers and cycling status (% in S phase) of bone marrow and spleen CFU-GM, BFU-E, and CFU-GEMM. Results are shown as the mean ± 1SEM of 6 individually assessed mice, per point from a total of 2 experiments. *$P < 0.05$ compared to cells from +/+ mice. CDKI, cyclin dependent kinase inhibitor; GM, granulocyte macrophage.

Thus, p21$^{CIP1/WAF1}$ and p27$^{KIP1}$ act respectively as positive and negative regulators of immature subsets of HPC numbers and proliferation in vivo in bone marrow and spleen.

Characteristics of $p18^{-/-}$ bone marrow HPCs were quite similar to that of $p21^{-/-}$ HPCs in that absolute numbers and cycling status of bone marrow CFU-GM, BFU-E, and CFU-GEMM significantly decreased compared with control progenitors, although the decrease in numbers of $p18^{-/-}$ HPCs was not as great as that of the $p21^{-/-}$ HPCs (Fig. 1). Splenic $p18^{-/-}$ CFU-GEMM numbers decreased less than those from $p21^{-/-}$ mice; splenic $p18^{-/-}$ BFU-E did not decrease at all; and splenic $p18^{-/-}$ CFU-GM very modestly but significantly increased compared to +/+ and $p21^{-/-}$ cells. Thus, the effects of 3 CDKI family members (p21$^{CIP1/WAF1}$, p27$^{KIP1}$, and p18$^{INK4C}$) had not only overlapping but also unique effects on HPCs.

It has been reported that p18$^{INK4c}$ and p21$^{CIP1/WAF1}$ functionally interact with each other in terms of HSC exhaustion after serial transplantation [12]. We thus evaluated p18$^{INK4c}$ for interacting effects either with p21$^{CIP1/WAF1}$ or with p27$^{KIP1}$ in terms of numbers and proliferative status of immature subsets of marrow and spleen HPCs by using dual $p18^{-/-}$-$p21^{-/-}$ and dual $p18^{-/-}$-$p27^{-/-}$ mice in comparison with +/+, $p18^{-/-}$, $p21^{-/-}$, and $p27^{-/-}$ single gene-deleted mice (Fig. 1). Deletion of both the $p18^{INK4c}$ and $p21^{CIP1/WAF1}$ genes did not further effect decreased hematopoiesis noted in either of the single CDKI $^{-/-}$ genotypes. Decreased numbers of HPC of dual $p18^{-/-}$-$p21^{-/-}$ mice were either equal to that of the $p21^{-/-}$ mice or midway between that of the $p18^{-/-}$ and $p21^{-/-}$ mice, whereas the cells from these 3 groups of knock-out mice were in a slow or noncycling state. These results with HPC are different from the impressive effect of $p18^{-/-}$ on $p21^{-/-}$ that others have shown for serial repopulation of HSC [11,12], highlighting differences in effects of CDKIs on the HSC and HPC compartments. In contrast, the combination of deletion of both $p18^{INK4c}$ and $p27^{KIP1}$ had a strong modifying effect on HPC numbers and proliferation in the marrow and spleen. Dual $p18^{-/-}$-$p27^{-/-}$ mice significantly manifested suppressed numbers and cycling status of bone marrow CFU-GM, BFU-E, and CFU-GEMM compared with that of $p27^{-/-}$ mice with the marrow CFU-GM and CFU-GEMM numbers of the dual $p18^{-/-}$-$p27^{-/-}$ significantly decreased compared to both the +/+ and $p27^{-/-}$ mice. The very large enhancement in spleen CFU-GM, BFU-E, and CFU-GEMM numbers and cycling status seen in the $p27^{-/-}$ mice was greatly reduced in the dual $p18^{-/-}$-$p27^{-/-}$ mice. This demonstrates the profound modulatory influences of p18$^{INK4c}$ and p27$^{KIP1}$ on each other in terms of their effects on marrow and spleen HPC numbers and cell cycling.

### *Effects of CDKIs on synergistic proliferative responses of CFU-GM to GM-CSF and SCF*

The capacity of HPC to synergistically respond to the proliferative inducing effects of a CSF and a potent co-stimulating cytokine is a characteristic of immature subsets of HPC [2], events likely relevant to proper regulation of in vivo hematopoiesis where HPC can be simultaneously subjected to multiple cytokines. The combination of a CSF, such as GM-CSF, and a potent co-stimulating cytokine such as SCF, which has little capacity to stimulate colony formation on its own, results in enhanced numbers of colonies in vitro (Fig. 2; shown as a ratio of colony number stimulated by GM-CSF plus SCF, divided by the additive colony formation of cells stimulated by only GM-CSF or only SCF), and increased numbers of cells per colony (Data not shown). We previously reported that $p21^{CIP1/WAF1}$ has a role in HPC proliferation since $p21^{-/-}$ CFU-GM did not synergistically respond to the combined effects of GM-CSF and SCF [3], events reproduced in the current study (Fig. 2). Interestingly, here we found that $p27^{-/-}$, but not $p18^{-/-}$, CFU-GM respond in a manner similar to that of $p21^{-/-}$ cells (Fig. 2), suggesting that both $p27^{KIP1}$ and $p21^{CIP1/KIP1}$, but not $p18^{INK4c}$, are involved in the synergistic proliferative response of CFU-GM to the combination of GM-CSF and SCF. These differential effects were not due to the cell cycling characteristics of the CFU-GM prior to cytokine stimulation, as $p27^{-/-}$ cells, which were not responsive to synergistic stimulation (Fig. 2), had a large percentage of CFU-GM in cycle prior to stimulation (Fig. 1) compared with $p21^{CIP1/WAF1}$ cells which were in a slow or noncycling state (Fig. 1). The $p18^{-/-}$ CFU-GM that did synergistically respond to GM-CSF and SCF (Fig. 2) were in a slow or noncycling state before stimulation by cytokines (Fig. 1). That both $p21^{-/-}$ and $p18^{-/-}$ HPC could be induced into cycle by exogenous stimulation with cytokines is shown in Fig. 3, and is clear from the fact that these cells did form colonies (Fig. 1). The evidence for interactions between different CDKIs effects on proliferative responses of CFU-GM to synergistic stimulation is demonstrated by the responses of dual $p18^{-/-}$-$p21^{-/-}$ and $p18^{-/-}$-$p27^{-/-}$ HPC (Fig. 2). The dual $p18^{-/-}$-$p21^{-/-}$ HPC synergistically responded to GM-CSF and SCF, demonstrating that $p18^{INK4c}$ was dominant over $p21^{CIP1/WAF1}$ in its effects, whereas the dual $p18^{-/-}$-$p27^{-/-}$ HPC did not respond to the synergistic effects of GM-CSF and SCF, showing that the $p27^{KIP1}$ effects were dominant over the $p18^{INK4c}$ effects. These results were not due to percent of these dual $CDKI^{-/-}$ cells in S-phase before they were stimulated in vitro, as the cells of both dual $CDKI^{-/-}$ were in a slow or noncycling state before stimulation (Fig. 1).

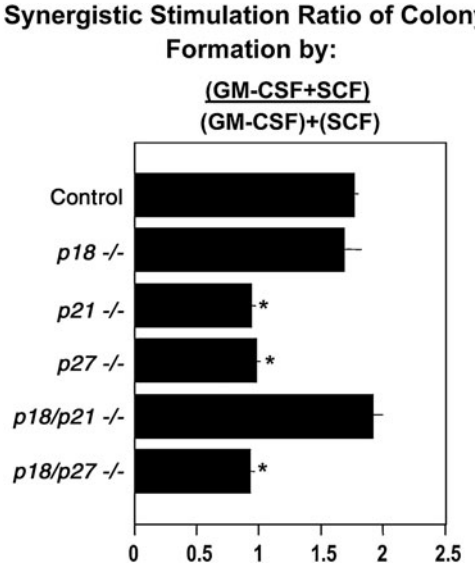


**FIG. 2.** Effect of $CDKI^{-/-}$ on the synergistic response of bone marrow CFU-GM to stimulation by the combination of GM-CSF plus SCF, compared with the additive effects of GM-CSF or SCF, each used alone to stimulate colony formation. Results are shown as the mean ± 1SEM as a stimulus ratio, and are based on studies from bone marrow cells of 6 individually assessed mice from a total of 2 experiments. *$P < 0.05$ compared to control cells. CSF, colony stimulating factors; SCF, stem cell factor.

### *Effects of CDKIs on responses of multi-cytokine stimulated CFU-GM to inhibition by suppressive chemokines*

A number of the members of the CC, CXC, and C chemokine families demonstrate suppressive effects on HPC in vitro and in vivo [19,20]. This includes the C chemokine XCL1 (lymphotactin), the CC chemokine CCL2 (MCP-1), and the CXC chemokines CXCL8 (IL-8) in addition to CXCL9 (MIG). As shown in Fig. 3 (left panel), CFU-GM from +/+, but not from $p18^{-/-}$, $p21^{-/-}$, $p27^{-/-}$, dual $p18^{-/-}$-$p21^{-/-}$, or dual $p18^{-/-}$-$p27^{-/-}$, responded to the suppressive effects of the 4 members of the chemokine family. However, HPC must be in active cell cycle before the addition of growth factors (GFs) and chemokines for them to be responsive to the inhibitory effects of suppressive chemokines [19,20]. Lack of chemokine suppression of $p27^{-/-}$ CFU-GM was not due to these CFU-GM not being in cycle, as these cells were in rapid cell cycle with a high percentage of cells in S-phase. To see if lack of responsiveness of $p18^{-/-}$, $p21^{-/-}$, and the dual $p18^{-/-}$-$p21^{CIP1-/-}$ and $p18^{-/-}$-$p27^{-/-}$ HPC to inhibition by chemokines was solely due to the percent of HPC in S-phase before the addition of cytokines and chemokines, we placed the CFU-GM from these $CDKI^{-/-}$ mice into rapid cell cycle by exposing them to a combination of cytokines for 24 h, before washing the cells and placing them in the presence of cytokines and chemokines. As shown in the right panel of Fig. 3, placing the $p18^{-/-}$ HPC into cycle restored their responsiveness to inhibition by these chemokines. However, enhancement of the percent of CFU-GM in S-phase did not restore the responsiveness of either $p21^{-/-}$ or $p27^{-/-}$ HPC to chemokine inhibition. Thus, having CFU-GM in active cell cycle before the addition of GFs and chemokines is alone not sufficient for the chemokines to cause their suppressive effects. However, the $p18^{-/-}$ component in the dual $p18^{-/-}$-$p21^{-/-}$ cells was capable of restoring the responsiveness of the $p21^{-/-}$ cells to chemokine inhibition, but was not capable of restoring the responsiveness to inhibition of $p27^{-/-}$ HPC in the dual $p18^{-/-}$-$p27^{-/-}$ cells. That the dual $p18^{-/-}$-$p21^{-/-}$, but not the $p18^{-/-}$-$p27^{-/-}$ HPC responded to synergistic stimulation suggest that the HPC must not only be in active cell

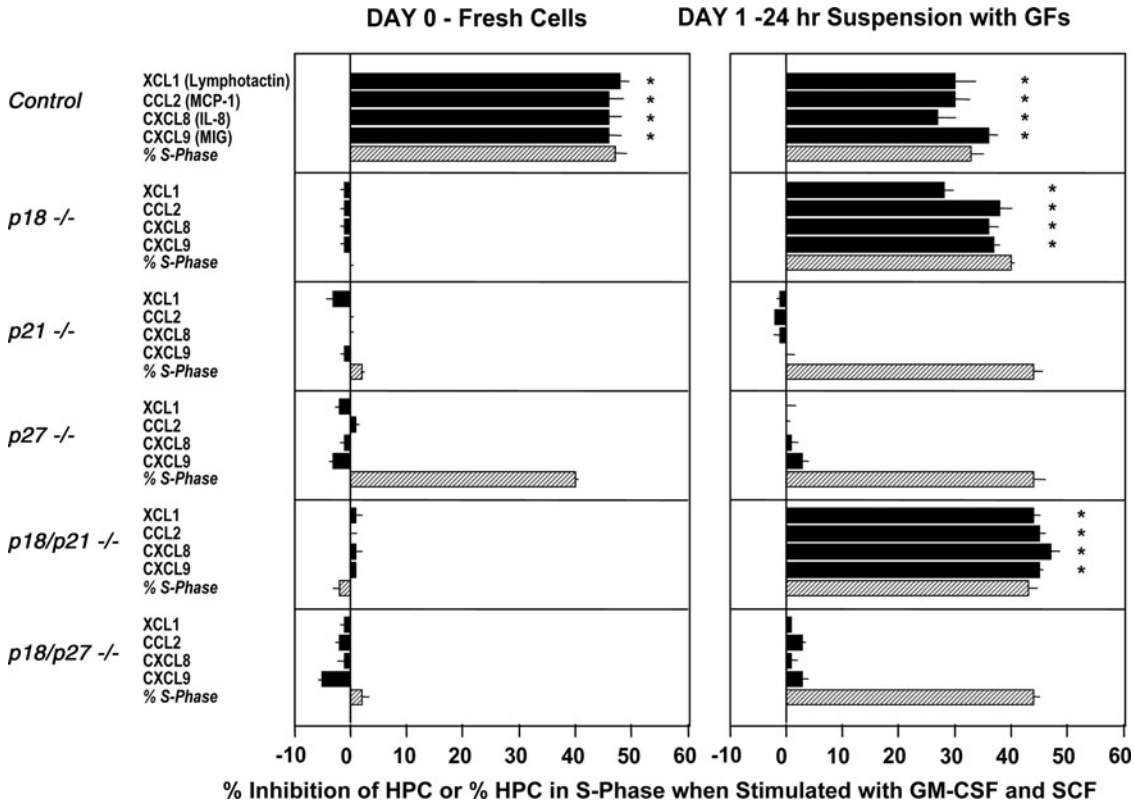


**FIG. 3.** Suppressive effects of chemokines on colony formation of control (+/+) and CDKI$^{-/-}$ bone marrow CFU-GM stimulated by GM-CSF and SCF, at time 0 (=freshly isolated cells), or after 24 h. suspension culture in the presence of SCF, GM-CSF, FL, and IL-6 (=growth factors [GFs]). Also shown is the cycling status of CFU-GM in the absence of chemokines at time 0, or after 24 h in suspension culture for 24 h with GFs. Cells in suspension culture were washed 2×, prior to plating with GM-CSF and SCF with or without chemokines. Results are shown as mean ± 1SEM for bone marrow cells of 6 individually assessed mice from a total of 2 experiments. *$P < 0.05$ compared to without chemokine.

**Summary of CDKI -/- Effects on HPC Proliferation, Response to Synergistic Stimulation of Proliferation, and Response to Inhibition by Chemokines**

| | TIME: 0 | | | TIME: 24 hours with growth factors* | |
|---|---|---|---|---|---|
| | HPC in Cycle | HPC response to Synergy | HPC Response to Inhibition by Chemokines | HPC in Cycle | HPC Response to Inhibition by Chemokines |
| +/+ | Yes | Yes | Yes | Yes | Yes |
| $p18^{INK4c}$ (-/-) | No | Yes | No | Yes | Yes |
| $p21^{cip/waf1}$ (-/-) | No | No | No | Yes | No |
| $p27^{kip1}$ (-/-) | Yes | No | No | Yes | No |
| $p18^{INK4c}$ / $p21^{cip/waf1}$ (-/-) | No | Yes | No | Yes | Yes |
| $p18^{INK4c}$/ $p27^{kip1}$ (-/-) | No | No | No | Yes | No |

* Cells preincubated in suspension culture with FLT3L, SCF, GM-CSF and FBS, or IL-6 plus SCF and FBS

**FIG. 4.** Summary of studies assessing CDKI$^{-/-}$ effects on proliferation of HPC (from Fig. 1), responsive of HPC to synergistic stimulation by GM-CSF plus SCF (from Fig. 2), and also to chemokine inhibition, on freshly isolated bone marrow cells or cells first incubated in suspension culture for 24 h with GFs and then washed (from Fig. 3). HPC, hematopoietic progenitor cell.

cycle for them to respond to suppression by chemokines, but these HPC must also be responsive to synergistic stimulation of their proliferation.

## Discussion

CDKIs have effects on the proliferation of a number of different cell systems. Some have been implicated in proliferation of HPCs and HSCs (2–13), T-cell proliferation [22], myogenesis [23], and tumorigenesis (14,15,24–28). There is still much to be learned regarding a role for CDKIs in hematopoiesis. This is especially true for the interacting/overlapping roles of CDKIs in this regulation. In the present paper, we focused on a role for CDKIs on HPCs, an intermediary cell between HSCs and more mature blood cells, for which little information is currently available. We compared effects of $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p18^{INK4C}$ on in vivo and in vitro proliferation of HPCs, and also the modifying effects of $p18^{INK4C}$ with either that of $p21^{CIP1/WAF1}$, or $p27^{KIP1}$ on this proliferation, by using CDKI $^{-/-}$ mice. Although there was previous information available on loss of $p21^{CIP1/WAF1}$, which results in decreased numbers of HPC [3], and $p27^{KIP1}$ which results in increased numbers of HPC [8], there was no information on $p18^{-/-}$ on these intermediary immature subsets of HPC. Nor was there any information available on how $p18^{-/-}$ interfaced with loss of $p21^{CIP1/WAF1}$ or $p27^{KIP1}$. Our present results demonstrate potent effects of $p18^{-/-}$ on HPC proliferation and responsiveness to stimulation by GFs, and of inhibition by certain members of the chemokine family. Additionally, there were modifying effects noted in these functions of HPCs when dual $p18^{-/-}$-$p21^{-/-}$ and $p18^{-/-}$-$p27^{-/-}$ genotypes were assessed. Although it is always difficult to translate findings in vitro to those in vivo, a number of the studies we show in vitro are consistent with our findings in the mice themselves. Figure 4 presents a summary of these effects which demonstrate the following: First, there was decreased HPC proliferation in $p18^{-/-}$ mice, suggesting a positive role for $p18^{INK4C}$ similar to that we previously reported for $p21^{CIP1/WAF1}$ [3,4]. Second, the positive effects of $p18^{INK4C}$ dominated over the negative effects of $p27^{KIP1}$ on HPC proliferation. Third, CDKIs differentially regulated responses of CFU-GM to synergistic proliferation in response to GM-CSF and SCF, an observation of potential in vivo relevance as synergy between cytokines in vivo is likely to be an important physiological response since HPCs in vivo can be subjected to a number of different cytokines at the same time. In this context, it is possible that the sequence of specific receptor occupancies and receptor cross-talk in the cell may dictate the intracellular signals of relevance and ultimate HPC response(s). Fourth, the responsiveness of HPC to inhibition by myelosuppressive chemokines from 3 different subfamilies of chemokines was directly related to the capacity of HPC to respond to synergistic stimulation. Also, the cycling status of HPC was apparently under control by CDKIs. Exactly how the different CDKIs mechanistically regulate these different responses of HPCs to respective positive and negative cytokine or chemokine effects remains to be determined. A structure-based approach, as noted for $p18^{INK4C}$ [29], may be helpful in this regard in the future. Once we know exactly how CDKIs act downstream, the upstream events that modulate CDKIs, each alone, and in combination through possible cross-talk with each other in both normal homeostatic nonstressed, and in stressed conditions, we may eventually be able to either accelerate or suppresses proliferation of HPC and/or HSCs for clinical advantage. It is possible that CDKIs may play a role in the progression of nonmalignant and malignant hematological disorders that interfere with the balance needed for normal hematopoiesis under nondisease homeostatic conditions. If so, intervention at the level of CDKIs may also be useful in this context. Further studies on the CDKIs, HPCs, HSCs, and hematopoiesis under normal and disease conditions are thus warranted.

## Acknowledgments

These studies were supported by the U.S. Public Health Service Grants R01 HL056416, R01 HL067384, and P30 DK090948 from the National Institutes of Health to H.E.B.

## Author Disclosure Statement

The authors have no conflicts of interest to disclose.

## References

1. Steinman RA. (2002). Cell cycle regulators and hematopoiesis. Oncogene 21:3403–3413.
2. Shaheen M and HE Broxmeyer. (2009). The humoral regulation of hematopoiesis. In: *Hematology: Basic Principles and Practice,* 5th edition. R Hoffman, EJ Benz Jr., SJ Shattil, B Furie, LE Silberstein, P McGlave, H Heslop and J Anastasi, eds. Elsevier Churchill Livingston, Philadelphia, PA. Part III, Chapter 24, pp 253–275.
3. Mantel C, Z Luo, J Canfield, S Braun, C Deng and HE Broxmeyer. (1996). Involvement of p21cip-1 and p27kip-1 in the molecular mechanisms of steel factor-induced proliferative synergy *in vitro* and of p21cip-1 in the maintenance of stem/progenitor cells *in vivo*. Blood 88:3710–3719.
4. Braun SE, C Mantel, M Rosenthal, S Cooper, L Liu, KA Robertson, R Hromas and HE Broxmeyer. (1998). A positive effect of p21cip1/waf1 in the colony formation from murine myeloid progenitor cells as assessed by retroviral-mediated gene transfer. Blood Cells Mol Dis 24:138–148.
5. Mantel C, SE Braun, S Reid, O Henegariu, L Liu, G Hangoc and HE Broxmeyer. (1999). p21(cip-1/waf-1) deficiency causes deformed nuclear architecture, centriole overduplication, polyploidy, and relaxed microtubule damage checkpoints in human hematopoietic cells. Blood 93: 1390–1398.
6. Taniguchi T, H Endo, N Chikatsu, K Uchimaru, S Asano, T Fujita, T Nakahata and T Motokura. (1999). Expression of p21(Cip1/Waf1/Sdi1) and p27(Kip1) cyclin-dependent kinase inhibitors during human hematopoiesis. Blood 93:4167–4178.
7. Cheng T, N Rodrigues, H Shen, Y Yang, D Dombkowski, M Sykes and DT Scadden. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science 287: 1804–1808.
8. Fero ML, M Rivkin, M Tasch, P Porter, CE Carow, E Firpo, K Polyak, LH Tsai, V Broudy, et al. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell 85:733–744.
9. Cheng T, N Rodrigues, D Dombkowski, S Stier and DT Scadden. (2000). Stem cell repopulation efficiency but not pool size is governed by p27(kip1). Nat Med 6:1235–1240.

10. Walkley CR, ML Fero, WM Chien, LE Purton and GA McArthur. (2005). Negative cell-cycle regulators cooperatively control self-renewal and differentiation of haematopoietic stem cells. Nat Cell Biol 7:172–178.
11. Yuan Y, H Shen, DS Franklin, DT Scadden and T Cheng. (2004). *In vivo* self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. Nat Cell Biol 6:436–442.
12. Yu H, Y Yuan, H Shen and T Cheng. (2006). Hematopoietic stem cell exhaustion impacted by p18 INK4C and p21 Cip1/Waf1 in opposite manners. Blood 107:1200–1206.
13. van OR, LM Kamminga, A Ausema, LV Bystrykh, DP Draijer, PK van, B Dontje and HG de. (2007). A Limited role for p21Cip1/Waf1 in maintaining normal hematopoietic stem cell functioning. Stem Cells 25:836–843.
14. Franklin DS, VL Godfrey, DA O'Brien, C Deng and Y Xiong. (2000). Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. Mol Cell Biol 20:6147–6158.
15. Franklin DS, VL Godfrey, H Lee, GI Kovalev, R Schoonhoven, S Chen-Kiang, L Su and Y Xiong. (1998). CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. Genes Dev 12:2899–2911.
16. Christopherson KW, G Hangoc, CR Mantel and HE Broxmeyer. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 305:1000–1003.
17. Broxmeyer HE, CM Orschell, DW Clapp, G Hangoc, S Cooper, PA Plett, WC Liles, X Li, B Graham-Evans, et al. (2005). Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med 201:1307–1318.
18. Cooper S and HE Broxmeyer. (1996). Measurement of interleukin-3 and other hematopoietic growth factors, such as GM-CSF, G-CSF, M-CSF, erythropoietin and the potent costimulating cytokines steel factor and Flt-3 ligand. In: *Current Protocols in Immunology*. JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober and R Coico, eds. John Wiley & Sons, Inc., New York, Suppl 18, pp 6.4.1–6.4.12.
19. Kim CH, CK Qu, G Hangoc, S Cooper, GS Feng and HE Broxmeyer. (1999). Abnormal chemokine-induced responses of immature and mature hematopoietic cells from motheaten mice implicates the protein tyrosine phosphatase SHP-1 in chemokine responses. J Exp Med 190:681–690.
20. Broxmeyer HE, BS Youn, CH Kim, G Hangoc, S Cooper and C Mantel. (2001). Chemokine regulation of hematopoiesis and the involvement of pertussis toxin-sensitive Gαi proteins. NY Acad Sci 938:117–128.
21. Broxmeyer HE, S Cooper, L Lu, ME Miller, CD Langefeld and P Ralph. (1990). Enhanced stimulation of human bone marrow macrophage colony formation *in vitro* by recombinant human macrophage colony stimulatng factor in agarose medium at low oxygen tension. Blood 76:323–329.
22. Kovalev GI, DS Franklin, VM Coffield, Y Xiong and L Su. (2001). An important role of CDK inhibitor p18(INK4c) in modulating antigen receptor-mediated T cell proliferation. J Immunol 167:3285–3292.
23. Franklin DS and Y Xiong. (1996). Induction of p18INK4c and its predominant association with CDK4 and CDK6 during myogenic differentiation. Mol Biol Cell 7:1587–1599.
24. Damo LA, PW Snyder and DS Franklin. (2005). Tumorigenesis in p27/p53- and p18/p53-double null mice: functional collaboration between the pRb and p53 pathways. Mol Carcinog 42:109–120.
25. Dib A, TR Peterson, L Raducha-Grace, A Zingone, F Zhan, I Hanamura, B Barlogie, J Shaughnessy Jr. and WM Kuehl. (2006). Paradoxical expression of INK4c in proliferative multiple myeloma tumors: bi-allelic deletion vs increased expression. Cell Div 1:23.
26. Joshi PP, MV Kulkarni, BK Yu, KR Smith, DL Norton, VW van, JW Hoppener and DS Franklin. (2007). Simultaneous downregulation of CDK inhibitors p18(Ink4c) and p27(Kip1) is required for MEN2A-RET-mediated mitogenesis. Oncogene 26:554–570.
27. van VW, CJ van Gasteren, DS Acton, DS Franklin, R Berger, CJ Lips and JW Hoppener. (2008). Synergistic effect of oncogenic RET and loss of p18 on medullary thyroid carcinoma development. Cancer Res 68:1329–1337.
28. Kool J, AG Uren, CP Martins, D Sie, RJ de, G Turner, UM van, K Matentzoglu, W Lagcher, et al. (2010). Insertional mutagenesis in mice deficient for p15Ink4b, p16Ink4a, p21Cip1, and p27Kip1 reveals cancer gene interactions and correlations with tumor phenotypes. Cancer Res 70:520–531.
29. Venkataramani RN, TK MacLachlan, X Chai, WS El-Deiry and R Marmorstein. (2002). Structure-based design of p18INK4c proteins with increased thermodynamic stability and cell cycle inhibitory activity. J Biol Chem 277:48827–48833.

Address correspondence to:
*Dr. Hal E. Broxmeyer*
*Department of Microbiology and Immunology*
*Indiana University School of Medicine*
*950 West Walnut Street, Room 302, R2 Building*
*Indianapolis, IN 46202-5181*

*E-mail:* hbroxmey@iupui.edu

Received for publication August 22, 2011
Accepted after revision September 20, 2011
Prepublished on Liebert Instant Online September 21, 2011