RNA Interference Reveals a Requirement for Both p18\textsuperscript{INK4c} and p27\textsuperscript{Kip1} in B Lymphopoiesis

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Cyclin-dependent kinase inhibitors (CKIs) p18\textsuperscript{INK4c} (p18) and p27\textsuperscript{Kip1} (p27) were reported to be able to modulate self-renewal and differentiation of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells, and regulate the lineage cell proliferation and maturation into the terminal elements; however, whether p18 and p27 in HSCs affect the development of lineage cells, especially B lymphocytes, in the reconstituted blood is unknown. Here we employed small-interference RNA (siRNA) technique, which provides a powerful tool for tissue-targeted knockdown of genes, to evaluate the biological functions of the p18 and p27 in the hematopoiesis process. We knocked down the expression of p18, p27 alone or both in the isolated sca-1\textsuperscript{+} bone marrow cells by lentiviral vector-based siRNA system, and transplanted these cells into lethally irradiated C57BL/6j mice to evaluate the effect of these two genes on reconstituted lymphocyte development. The knockdown of p18 or p27 alone or both was proved to be effective as verified by western blotting. FACS analysis results showed that compared with the control group, the B lymphocytes were both significantly lower in p18, p27 alone and especially in both p18 and p27 knockdown group in reconstituted peripheral blood; and the B lymphocytes showed similar trend in bone marrow. Interestingly, the differentiation to T cells was not greatly changed, only with the dramatic decrease of the CD4/CD8 ratio. Overexpression of the antiapoptotic protein Bcl2 could not rescue the B lymphopoiesis. All these results demonstrate that p18 and p27 are collaboratively involved in B lymphopoiesis, and simultaneous knockdown of p18 and p27 probably blocks the differentiation from HSCs to B lymphocytes, but not triggers apoptosis of B cell precursors.

Keywords: RNA interference (RNAi), hematopoietic stem cells, lymphopoiesis, p18\textsuperscript{INK4c} (p18), p27\textsuperscript{Kip1} (p27)

Introduction

Multiple genes are involved in the lineage development in hematopoiesis. Self-renewal of stem cells and lineage development are regulated by different cell cycle regulators (Della Ragione et al., 1997; Furukawa et al., 2000; Schrantz et al., 2000; Yuan et al., 2004; Walkley et al., 2005). The hematopoietic cell cycle should be strictly regulated during normal blood development, otherwise cells may be transformed into malignant cells (Drexler, 1998; Furukawa et al., 2000), fail to differentiate or be eliminated by apoptotic mechanism so that the failure of blood production may occur (Yoshida, 1993; Furukawa et al., 2000). The components involved in the regulation of cell cycle are cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs). There are evidences that CKIs play crucial roles in cell cycle regulation and blood development (Della Ragione et al., 1997; Wang et al., 2008a, b). These inhibitors belong to two different families: one is the INK4 family, consisting of p16\textsuperscript{INK4a} (p16), p15\textsuperscript{INK4b} (p15), p18\textsuperscript{INK4c} (p18) and p19\textsuperscript{INK4d} (p19), and the other is the Cip/Kip family, consisting of p21\textsuperscript{Cip1/Waf1/Sdi1} (p21), p27\textsuperscript{Kip1} (p27) and p57\textsuperscript{Kip2} (p57; Schrantz et al., 2000). The CKI expression profile shows that p15, p18, p19, p27 but not p16 are expressed in all the lineages including CD34\textsuperscript{+} cells, BFU-E, CFU-E, CFU-GM and lymphocytes (Della Ragione et al., 1997). It has been demonstrated that p18 and p27 play roles in B cell differentiation and activation (Schrantz et al., 2000; Tourigny et al., 2002). But the cooperative effect of p18 and p27 on lymphopoiesis, especially B lymphopoiesis, is unknown, which is partially due to the limitation of methodology. Gene knockout is the common method to study the gene function, but the effect of gene knockout is systemic, which will lead to impair animal’s health. For example, simultaneous knockout of p18 and p27 resulted in formation of pituitary adenomas and death soon after birth (Franklin et al., 1998). To study the effect of loss of p18 and p27 on hematopoiesis, the optimal choice is loss of the two gene expression only in hematopoietic cells. The application of small-interference RNA (siRNA) technology to hematopoietic stem cells (HSCs) can help us to achieve this goal.

In this paper, we show that simultaneous knockdown of p18 and p27 in HSCs using siRNA technology results in deficiency of B cells in hematopoiesis process. And overexpression of the
antiapoptotic protein Bcl2 in both p18- and p27-knocked down HSCs could not rescue B lymphopoiesis.

Results

Knockdown of p18 and p27 alone or both via lentiviral vector-based siRNA

In the preliminary experiments, the effectiveness of lentiviral vector-based siRNA-mediated gene knockdown was assessed. Here we obtained CopGFP+ cell lines in which p18 and p27 alone or both were stably knocked down as previously described (Figure 1; Wang et al., 2008b).

Simultaneous knockdown of p18 and p27 only in HSCs blocks B lymphopoiesis

We transduced the isolated sca-1+ bone marrow cells with the lentivirus-bearing p18 siRNA, p27 siRNA, control siRNA, or both p18 and p27 siRNA, respectively. The transduction efficiencies ranged from 27 to 46% (%GFP+ cells). Then these transduced cells were transplanted into lethally irradiated mice. The transplanted mice lived normally under normal care conditions. After the blood was reconstituted, the CopGFP+ cells were isolated from the spleen cells by FACS and the expression of p18 or p27 in the CopGFP+ cells was detected by western blotting. The expression of p18 and p27 was markedly knocked down (Figure 1C), indicating the siRNAs in the CopGFP+ cells worked well. Next we detected the lymphocyte subsets in the reconstituted peripheral blood 3 or 6 months after transplantation. In the gated lymphocytes in forward scatter (FSC) vs. side scatter (SSC) flow cytometric dot plot, we found the percentages of B cells [CD19+ (Figure 2A) or B220+ cells (Figure 2B)] in peripheral blood lymphocytes were high (around 69%) in control C57BL/6 mice, which is consistent with Chen and Harrison’s (2002) report. Interestingly, we found there were lower proportions of B cells in the CopGFP+ reconstituted lymphocytes from mice transplanted with the HSCs transduced with lentivirus-bearing p18 siRNA alone (n = 7) (P < 0.05 or 0.01), p27 siRNA alone (n = 6) (P < 0.01), or both p18 and p27 siRNA (n = 6) (P < 0.01) than that in the CopGFP+ reconstituted lymphocytes from mice transplanted with the HSCs transduced with lentivirus-bearing control siRNA (n = 6) (Figure 2). And surprisingly, knockdown of both p18 and p27 via siRNA almost totally prevented B lymphopoiesis, whereas knockdown of single p18 or p27 just partially reduced the proportion of B cells in the CopGFP+ reconstituted lymphocytes (Figure 2A and C). Furthermore, we also performed the detection of CD19+ and B220+ cells in CopGFP+ bone marrow cells 6 months after transplantation, there were the same trends in the change of CD19+ and B220+ cell proportion in bone marrow as in the peripheral blood for these knockdown mice (data not shown). These results indicate that p18 or p27 can affect B lymphopoiesis, and p18 and p27 have additive or synergistic effects on B lymphopoiesis because the p18 or p27 single knocked down mice displayed phenotypes intermediate between p18 and p27 double knockdown mice and control mice.

Simultaneous knockdown of p18 and p27 only in HSCs decreases CD4/CD8 ratio in T cell subsets

Nevertheless, T cells (CD3+ cells) could develop under single knockdown of p18 or p27, or both knockdown of p18 and p27 (Figure 3A and B), only with drastic decrease in the CD4/CD8 ratio in the CopGFP+ reconstituted T cells (CD3+ cells) from mice transplanted with the HSCs transduced with lentivirus bearing both p18 and p27 siRNAs (Figure 3B and C), suggesting knockdown of both p18 and p27 affects the T lymphopoiesis. RNAi phenotype does not result from off-target effects of RNAi

To confirm that the phenotype observed above resulted from siRNA-induced simultaneous knockdown of p18 and p27 genes, but did not result from off-target effects of RNA interference (RNAi), the HSCs were introduced with both p18 and p27 siRNAs and the RNAi-resistant p18 or p27 transgene to rescue the RNAi phenotype (due to the technical limitations, p18 and

![Figure 1](https://academic.oup.com/jmcb/article-abstract/2/4/209/866509/download) Sufficient knockdown of genes via lentiviral vector-based siRNA strategy. (A) The effectiveness of p18 siRNA-mediated knockdown was assessed by western blot analysis of p18 expression in NIH-3T3 cells, which were transduced with lentivirus-bearing p18 alone or both p18 and p27 siRNA or control siRNA. (B) The effectiveness of p27 siRNA-mediated knockdown was assessed by western blot analysis of p27 expression in the 10-day hematopoietic differentiating murine embryonic stem cells (CCES), which were transduced with the lentivirus-bearing p27 alone or both p18 and p27 siRNA or control siRNA. (C) Western blot analysis of expression of p18 or p27 in the CopGFP+ spleen cells after the blood was reconstituted.
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Figure 2 Knockdown of single or both of p18 and p27 affected development of reconstituted B lymphocytes. (A) FACS analysis of CD19\textsuperscript{+} cells in the peripheral lymphocytes obtained from mice transplanted with the HSCs transduced with lentiviruses bearing control siRNA, p18 siRNA, p27 siRNA or both p18 and p27 siRNAs (p18 + p27 siRNA) 3 (upper panel) or 6 months (lower panel) after transplantation. (B) FACS analysis of B220\textsuperscript{+} cells in the peripheral lymphocytes obtained from mice transplanted with the HSCs transduced with lentiviruses bearing control siRNA or both p18 and p27 siRNAs (p18 + p27 siRNA) 3 months after transplantation. (C) Percentage of CD19\textsuperscript{+} cells in CopGFP\textsuperscript{+}-reconstituted lymphocytes. Analyses on samples were carried for 6–7 mice in each group, and were presented as means ± S.D. (*P < 0.01 \#P < 0.05 compared with control siRNA; \&P < 0.01 compared with control siRNA or p18 siRNA or p27 siRNA).

p27 cDNA could not be simultaneously constructed in one lentiviral vector. To confirm if p18 or p27 was expressed in the reconstituted blood cells, the CopGFP\textsuperscript{+} cells were isolated from the spleen cells by FACS and the expression of the RNAi-resistant p18 or p27 in the CopGFP\textsuperscript{+} cells was detected by western blotting. Results showed that the reconstituted CopGFP\textsuperscript{+} cells expressed the RNAi-resistant p18 or p27 transgene (Figure 4A). The B cells (CD19\textsuperscript{+} cells) were detected in CopGFP\textsuperscript{+}-reconstituted lymphocytes, which expressed both p18 and p27 siRNAs and the RNAi-resistant p18 or p27 transgene (Figure 4B). The proportion of B cells in CopGFP\textsuperscript{+}-reconstituted lymphocytes, which expressed both p18 and p27 siRNAs and the RNAi-resistant p18 or p27 transgene is similar to the proportion in the CopGFP\textsuperscript{+}-reconstituted lymphocytes expressed p27 siRNA alone or p18 siRNA alone (Figure 4B vs. 2A), indicating the RNAi phenotype was rescued. These results suggest the RNAi phenotype results from siRNA-induced simultaneous knockdown of p18 and p27 genes, but not from off-target effects of RNAi.

Overexpression of Bcl2 in the HSC with p18 and p27 knockdown fails to rescue B lymphopoiesis

Based on the phenomenon above that simultaneous knockdown of p18 and p27 leads to undetectable B lymphocytes, we hypothesized this phenomenon results from either apoptosis of B cell precursors triggered by simultaneous knockdown of p18 and p27, or blockade of differentiation of HSCs to B cell precursors due to loss of p18 and p27. If simultaneous knockdown of p18 and p27 triggered the apoptosis of B cell precursors, the B lymphopoiesis should be at least partially rescued by overexpression of antiapoptotic molecule Bcl2. Therefore, we introduced the
antiapoptotic molecule Bcl2 into the HSCs to prevent apoptosis of B cell precursors. To confirm if the Bcl2 was overexpressed in the reconstituted blood cells, the CopGFP+ spleen cells were isolated from the spleen cells by FACS and the expression of Bcl2 in the CopGFP+ cells was detected by western blotting. Results showed that the introduced HA-Bcl2 overexpressed (Figure 5A). To verify the anti-apoptotic effect of overexpressed HA-Bcl2 on reconstituted blood cells, the HSCs transduced with lentiviruses bearing HA-Bcl2 and control siRNA (p18 + p27 siRNA) 3 months (upper panel) or 6 months (lower panel) after transplantation. Analyses on samples were carried for 6–7 mice in each group, and were presented as means ± S.D. (*P < 0.01 compared with control siRNA or p18 siRNA or p27 siRNA).

Figure 3 Knockdown of single or both of p18 and p27 affected development of reconstituted T lymphocytes. (A) FACS analysis of CD3+ cells in the peripheral lymphocytes obtained from mice transplanted with the HSCs transduced with lentiviruses bearing both p18 and p27 siRNAs (p18 + p27 siRNA) 3 months (upper panel) or 6 months (lower panel) after transplantation. (B) FACS analysis of CD3+ and CD4+ cells in CopGFP+ reconstituted lymphocytes 6 months after transplantation. (C) CD4/CD8 ratio in CopGFP+ CD3+-reconstituted lymphocytes 6 months after transplantation. Analyses on samples were carried for 6–7 mice in each group, and were presented as means ± S.D. (*P < 0.01 compared with control siRNA or p18 siRNA or p27 siRNA).

Figure 4 Rescue of the RNAi phenotype. (A) The expression of RNAi-resistant p18 or p27 was assessed by western blot analysis of reconstituted CopGFP+ spleen cells obtained from mice transplanted with the HSCs transduced with lentiviruses bearing RNAi-resistant p18 or p27 transgene cassette and both p18 and p27 siRNA, or bearing only both p18 and p27 siRNA. (B) FACS analysis of CD19+ cells in the peripheral lymphocytes obtained from mice transplanted with the HSCs transduced with lentiviruses bearing RNAi-resistant p18 or p27 transgene cassette and both p18 and p27 siRNAs, or bearing only both p18 and p27 siRNA 2 months after transplantation.
knockdown of p18 and p27 by siRNA might block the differentiation of HSCs to B cells, but not trigger apoptosis of B cell precursors.

**Discussion**

We show here that simultaneous knockdown of p18 and p27 in HSCs leads to deficiency of B cells and alter the CD4/CD8 ratio in the reconstituted blood. In this study, knockdown of single p18 or p27 partially reduced the percentage of B cells (Figure 2A), which is consistent with the report that loss of p27 in p27<sup>−/−</sup> mice reduce the percentage of B cells (Wolfraim and Letterio, 2005), whereas simultaneous knockdown of both p18 and p27 almost totally prevented B lymphopoiesis. Moreover, this RNAi phenotype is proven not to result from off-target effects of RNAi by RNAi phenotype rescue experiments.
Therefore, it is obvious that p18 and p27 have the additive or synergistic effects on control of B lymphopoiesis.

Declined proportion of B cells also was caused by loss of other genes, for example, hemoglobin (Lipovsky et al., 2003), interleukin-2 (IL-2) (Chen et al., 2002), Trp53 (TeKippe et al., 2003). However, what are their target molecules in the signal pathway that regulate this deficiency of B cells has not been known. The fact that p18 and p27 have the additive or synergistic effects on B lymphopoiesis suggests that cell cycle entry might cause this B cell proportion decline. p18 and p17 are required for myogenic differentiation of an immature proliferating myoblast into a fully differentiated myotube, this differentiation involves escape from the cell cycle, enhanced resistance to apoptosis, synchronized muscle-specific gene expression (Myers et al., 2004). Therefore, it is reasonable that differentiation of HSCs to B cells and B cell precursors also involves these events. Once cell cycle exit cannot take place due to loss of the cell cycle inhibitors p18 and p27, HSCs then might not be able to differentiate into B cells or HSCs are able to differentiate into B cells but these differentiated B cell precursors might be more likely to undergo apoptosis.

Nevertheless, knockdown of both p18 and p27 does not lead to T cell deficiency, just altered the CD4/CD8 ratio, but we did not find that knockdown of p18 or p27 alone altered the ratio, though Wolfram et al. (Wolfram and Letterio, 2005) reported that loss of p27 in p27−/− mice altered the CD4/CD8 ratio. This might result from non-complete loss of p18 or p27 expression via siRNA. The reason why loss of p27 alters the CD4/CD8 ratio is that p27 acts to restrict the proliferative capacity of naive CD8+ T cells to a much greater extent than it does of naive CD4+ T cells (Wolfram and Letterio, 2005). Therefore, we guess that the additive or synergistic effects of p18 and p27 make the alteration of CD4/CD8 ratio detectable.

Because we could not detect the B (CD19+ or B220+) cells in mice transplanted with HSCs with both p18 and p27 knockdown, which stage that these two CKIs act on is not known. But we can infer it. Because p18 and p27 are not expressed in pro-B cells (Fink and LeBien, 2001), the action site for p18 and p27 should not be in pro-B cell stage. In B cell developmental stages, up-regulated p27 leads to clonal deletion (Wu et al., 1999), which indicates that the action site could not be in clonal deletion stage. Therefore, we guess it should be before pro-B stage. However, the mechanism by which p18 and p27 synergistically regulate B lymphocytes development is not clear entirely.

It has been demonstrated that apoptosis plays an important role in maintaining homeostasis and quality control in the renewing cell systems of the body. During B lymphopoiesis in bone marrow, apoptosis is the main reason for negative selection. The brief period between onset of apoptosis and clearance by macrophages (apoptotic transit time) is similar for most precursor B cells. Apoptosis-modulating factors produce substantial changes in apoptotic activity among pro-B and pre-B cells, associated with altered expression of Bcl2 family proteins (Lu and Osmond, 2000). Overexpression of Bcl2 in lymphocytes or fibroblasts antagonizes apoptosis and delays their transition from the quiescent state into the cell cycle. Lymphocyte progenitors in mice bearing a human Bcl2 transgene were protected from dexamethasone treatment (Igarashi et al., 2005). The apoptotic rates of precursor B cells, but not of more mature B cells, are enhanced by IL-7 gene deletion, associated with increased intracellular content of Bax and decreased Bcl2, while, conversely, an IL-7 transgene suppresses precursor B cell apoptosis and produces low Bax and high Bcl2 levels (Lu et al., 1999). Moreover, it was reported that B cell-specific transgenic expression of Bcl2 rescues early B lymphopoiesis but not B cell responses in BOB.1/OBF.1-deficient mice (Brunner et al., 2003). These results suggest that the antiapoptotic protein Bcl2 can protect pro-B and pre-B cells from apoptosis. However, our results showed that overexpression of Bcl2 could not rescue any of the B cells when the p18 and p27 were knocked down by siRNA in HSCs. One of the explanations for this phenomenon might be the possibility that the simultaneous knockdown of p18 and p27 totally blocks HSCs to differentiate into B cells, therefore this loss of B lymphocytes has nothing to do with apoptosis so that Bcl2 cannot enforce B cells to appear. This possibility may be reasonable because the antiapoptotic protein Bcl2 should have been able to rescue the B cells (McDonnell et al., 1989; Brunner et al., 2003), at least part of the B cells if the deficiency of B cells resulted from apoptosis due to simultaneous loss of p18 and p27.

In summary, this study has shown that both p18 and p27 are required in lymphopoiesis, especially in B lymphopoiesis. Moreover, this study also provides a possibility to study gene collaboration in hematopoietic cells using siRNA strategy.

### Materials and methods

#### Lentiviral constructs and lentivirus preparation

The pFIV-H1/U6-CopGFP lentiviral vector (System Biosciences (SBI), Mountain View, CA) containing double H1 and U6 RNA pol III promoters was used for lentiviral vector-based gene knockdown. The construction of lentiviral plasmids containing p18 or (and) p27 siRNA templates has been described (Wang et al., 2008b).

For expression of Bcl2 transgene, or the RNAi-resistant p18 or p27 transgene in the HSCs with simultaneous knockdown of p18 and p27, the transgene cassette including an insulator [chicken hypersensitive site 4 (CHS4)], CAG promoter, Bcl2 or the RNAi-resistant p18 or p27 cDNA, and hGH (human growth hormone) poly A was cloned upstream of the CopGFP cassette at antisense orientation in the pFIV-H1/U6-CopGFP plasmids (Tian and Andreadis, 2009). The Bcl2 was fused to HA so that the transgene Bcl2 could be discriminated from the endogenous Bcl2. The RNAi-resistant p18 or p27 transgene cDNA was achieved by mutating the siRNA-targeted sequence taatgtaaacgtcaacgct or ttggaatttcgactttcag into taatgtaaacgtcaacgct or ttggaatttcgactttcag, respectively (Ma et al., 2007).

The modified pFIV-H1/U6-CopGFP plasmids and the packaging plasmids (SBI) were transfected into the packaging cell line 293T/17 (ATCC) to produce the lentiviruses according to the manufacturer’s instructions. The virus titer was determined by infection of NIH 3T3 cells. The effectiveness of p18 and p27
siRNA-mediated knockdown was assessed by western blot analysis of p18 or p27 expression in the cell lysate of cell lines known to express p18 or p27.

**Isolation and lentiviral transduction of HSCs**

Bone marrow cells were obtained by flushing the tibias and femurs of 8-week-old male C57BL/6j mice with PBS. Sca-1+ bone marrow cells were collected using MACS (Miltenyi Biotec) according to the instructions provided by the manufacturer. The sca-1+ BM cells were cultured in the medium [high glucose DMEM medium (Gibco), 15% fetal bovine serum (ES specific, Gibco), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acid (Stem Cell Technologies), 1% Pen/Strep (100x, Gibco)] supplemented with 50 ng/ml SCF, 20 ng/ml IL-3 and 50 ng/ml IL-6. After 48 h, the sca-1+ BM cells were transduced with the lentivirus. Briefly, the sca-1+ BM cells were suspended in the 2 ml lentiviral supernatants supplemented with 4 µg/ml polybrene in the six-well plate, then the plate was spinoculated at 900 g for 50 min at room temperature. After spinoculation, the lentiviral supernatants were replaced with the fresh culture media. After 24 h, the lentiviral infection procedure was repeated again. After 24 h, the lentiviral infected sca-1+ BM cells were transplanted into the lethally irradiated mice. Animal Care and Use Committee of Guizhou University approved all studies.

**Transplantation of HSCs**

The lentiviral infected cells were transplanted by retroorbital injection into the lethally irradiated (950 rads) 8-week-old female C57BL/6j mice. The transplanted mice were maintained on antibiotic water for 1 month. Two to 6 months after transplantation, peripheral blood or bone marrow cells were obtained from each recipient mouse for determination of lymphocytes using FACS.

**Blood and BM analyses using FACS**

Two to 6 months after transplantation, the heparinized peripheral blood was obtained from the transplanted mouse tail. The bone marrow cells were obtained by flushing the tibias and femurs, and filtered through a 100 µm nylon mesh to remove debris. Forty microliters of heparinized peripheral blood or bone marrow cells was incubated with the premixed antibody cocktail (CD3-PE-CY5 (eBioscience Inc.) + CD19-PE (BD Pharmingen) or B220-PE (BD Pharmingen) + CD4-APC (BD pharmingen)) at room temperature for 30 min, added 2 ml of 1x BD FACS Lysing Solution (BD Biosciences) per tube and incubated at room temperature for 5 min to lyse erythrocytes. The samples were washed using cold PBS twice and run by FACS.

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