Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 337 (2005) 14–21
BBRC
www.elsevier.com/locate/ybbrc

$p57^{Kip2}$ is expressed in quiescent mouse bone marrow side population cells ☆

Terumasa Umemoto [a], Masayuki Yamato [a], Kohji Nishida [b], Joseph Yang [a], Yasuo Tano [b], Teruo Okano [a,*]

[a] *Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan*
[b] *Department of Ophthalmology, Osaka University Medical School, Osaka, Japan*

Received 31 August 2005
Available online 13 September 2005

Abstract

Hematopoietic stem cells can be accurately identified by the side population (SP) phenotype. It has been previously shown that hematopoietic stem cells are cell cycle arrested, but the mechanisms involved are currently poorly understood. In the present study, results from quantitative real-time RT-PCR show that while SP cells have increased expression of various cyclins and cyclin-dependent kinases, the increased expression of cyclin-dependent kinase inhibitors, in particular $p57^{Kip2}$, is responsible for the observed cell cycle arrest. In addition, gene expression analysis of c-kit^{+}/Sca-1^{+}/$Lineage^{-}$ SP (KSL-SP) cells demonstrates that only $p57^{Kip2}$ shows both higher expression compared to both SP and non-SP cells. Furthermore, immunostaining also demonstrates significantly higher protein expression in KSL-SP cells. These results demonstrate that the maintenance of bone marrow SP cells in G0/G1 may be carefully controlled by $p57^{Kip2}$.

Keywords: Side population; Hematopoietic stem cell; Cell cycle arrest; Cyclin-dependent kinase inhibitor; $p57^{Kip2}$

Hematopoietic stem cells (HSCs) are capable of self-renewal and govern the long-term repopulation of all cell types of the blood forming system [1]. A recently developed technique has employed the differential Hoechst 33342 staining of HSCs to identify a small fraction of bone marrow cells termed "side population" (SP) cells [2]. SP cells have been shown to be highly enriched for HSCs and represent approximately 0.1% of adult nucleated bone marrow cells in mice [3]. This unique ability of SP cells in effluxing Hoechst 33342 is mediated by the ATP-binding cassette transporter G2 (ABCG2), a member of the multiple drug resistance (MDR) family of cell surface transporters [4–6]. SP cells have been found in the hematopoietic compartment of various species [7–10], as well as in other tissues [11–18], demonstrating that the SP phenotype may be a representative characteristic of adult tissue-specific stem cells [19].

A defining feature of adult HSCs is that they are known to reside in the G0/G1 phases of the cell cycle, representing the quiescent state [20], a characteristic which is evidenced by their relatively strong resistance to retroviral infection and cell cycle-specific chemotherapeutic drugs. In somatic cells, the initiation of DNA replication and the associated progression through the cell cycle is known to be controlled by the activity of several classes of cyclin-dependent kinases (Cdks), which are activated by the binding of their respective cyclins. In addition, Cdk activity is carefully regulated by the expression of Cdk inhibitors which can bind to cyclin/Cdk complexes, to prevent cell cycle progression. These Cdk inhibitors are separated into the INK4 family,

☆ *Abbreviations:* HSC, hematopoietic stem cell; SP, side population; ABCG2, ATP binding cassette transporter G2; MDR, multiple drug resistance; Cdk, cyclin-dependent kinase; INK4, inhibitor of cyclin-dependent kinase 4; FACS, fluorescence activated cell sorting; NSP, non-side population; KSL-SP, c-kit^{+}/Sca-1^{+}/$Lineage^{-}$ side population; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* Corresponding author. Fax: +81 3 3359 6046.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.008

which prevents transfer from G1 to S by inhibiting cyclin D/Cdk 4 complex formation; and the Cip/Kip family which inhibits the activities of various cyclin/Cdk complexes.

While the mechanisms governing HSC quiescence are currently not well understood, intuitively it seems that differential expression of proteins involved in mitotic checkpoints may account for the observed cell cycle arrest. It has previously been shown that the induction of the Cdk inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ has an important role in the transforming growth factor (TGF)-β mediated cell cycle arrest in melanoma cells [21]. It was also recently reported that the TGF-β induced cell cycle arrest in hematopoietic progenitor cells was correlated to an increase in $p57^{Kip2}$ expression [22]. $p57^{Kip2}$ has similarly been shown to have an inhibitory effect in the hematopoietic family, by preventing cell cycle progression in human T-lymphocytes [23].

In the present study, we hypothesized that differential expression of various molecules involved in cell cycle checkpoints that regulate progression from G1 to S phase was responsible for the cell cycle arrest observed in bone marrow SP cells. Our findings show that SP cells have increased expression of various cyclins and Cdks, and should therefore have high proliferative potential, but that the increased expression of Cdk inhibitors, in particular $p57^{Kip2}$, blocks the activity of these cyclin/Cdk complexes resulting in the observed quiescent state of HSCs.

Materials and methods

Cell preparation. C57BL/6 mouse (Sankyo Lab Service, Tokyo, Japan) bone marrow cells were obtained by flushing of excised femurs with Hanks' Balanced Salt Solution (Sigma, St. Louis, MO) containing 5% fetal bovine serum (FBS; Moregate Biotech, Queensland, Australia). Cells were collected by centrifugation, followed by disruption of red cells with 0.2% sodium chloride solution. Cells were then washed two times with Dulbecco's phosphate-buffered saline (PBS; Sigma) prior to staining and cell sorting.

Hoechst 33342 exclusion assay using fluorescence activated cell sorting (FACS). Analysis and sorting of SP cells were performed as described previously [17]. Briefly, isolated bone marrow cells were stained with 5 μg/ml Hoechst 33342 (Sigma) at a concentration of 10^6 cells/ml in staining medium (Dulbecco's modified eagle's medium (DMEM) containing 2% FBS and 10 mM Hepes) for 90 min at 37 °C. For inhibition experiments, 50 μM of R (+)-verapamil (Sigma) was added to the staining medium 30 min before the addition of Hoechst 33342. After staining, cells were resuspended in PBS containing 2% FBS and 1 mM Hepes. Prior to analysis and cell sorting, propidium iodide (Sigma) was added at a final concentration of 2 μg/ml, to distinguish between live and non-viable cells. Analysis and cell sorting were then performed using a dual laser fluorescence-activated cell sorter (EPICS ALTRA FACS analysis system, Beckman Coulter, Fullerton, CA).

For the isolation of c-kit$^+$, Sca-1$^+$, Lineage$^-$ SP (KSL-SP) cells, lineage marker-positive cells were first eliminated by magnetic cell sorting (Auto MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany) using the Lineage cell Depletion Kit (Miltenyi Biotec), prior to staining with Hoechst 33342. Lineage$^-$ cells were stained with Hoechst 33342 and then incubated with FITC-conjugated anti-Sca-1 antibody (E13-161.7, BD Biosciences Pharmingen, San Jose, CA) and PE-conjugated anti c-kit antibody (2B8, BD Biosciences Pharmingen) for 30 min on ice. Stained cells were then subjected to sorting by FACS.

Cell cycle analysis. Bone marrow SP cells and total bone marrow cells were pelleted by centrifugation and resuspended in a solution containing 4 mM sodium citrate (pH 7.6), 0.2% Nonidet P-40, and 50 μg/ml propidium iodide. After incubation on ice for 30 min, cell suspensions were treated with 0.25 mg/ml RNase A for 15 min at 37 °C to remove double-stranded RNA. Cells were finally analyzed by flow cytometry at an excitation wavelength of 488 nm.

Gene expression analysis. For gene expression assays, total RNA was obtained from 10,000 SP, non-side population (NSP), or KSL-SP cells using Isogen (Nippongene, Tokyo, Japan) according to the manufacturer's suggested protocol. Single-stranded cDNA was created with the Superscript First-strand System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Invitrogen, Carlsbad, CA), and used as PCR templates. Primer pairs and Taqman MGB probes labeled with 6-carboxyfluorescein (FAM) at the 5′-end and non-fluorescent quencher at the 3′-end were designed with the Taqman gene expression assay (Applied Biosystems). Quantitative PCR was performed with 7300 Real-Time PCR System (Applied Biosystems). Thermocycling programs consisted of an initial cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. All assays were run in duplicate for more than four individual samples. mRNA expression levels were normalized with the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To compare mRNA expression between SP, NSP, and KSL-SP cells, the Mann–Whitney rank sum test was applied. Statistics were calculated using SigmaStat 2.0 (SPSS, Chicago, IL).

Immunofluorescence analysis. Cells isolated by FACS were fixed with Methacarn fixation solution, followed by centrifugation onto glass slides. After incubation with 1% bovine serum albumin (Sigma) to block non-specific reactions, cells were incubated with a 1/50 dilution of anti-$p57^{Kip2}$ antibody (E-17, Santa Cruz Biotechnology, Santa Cruz, CA), for 1 h at room temperature, followed by washing three times with PBS. Cells incubated identically with normal goat IgG were used as negative controls. After incubation with a 1/200 dilution of Alexa Fluor 546-conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature, cells were again washed three times with PBS. Stained cells were finally counter-stained with 10 μg/ml Hoechst 33342 to visualize cell nuclei, and observed using confocal laser scanning microscopy (TCS-SP, Leica Microsystems AG, Wetzlar, Germany).

Results

The SP phenotype is characterized by low blue and red fluorescence intensity on a density plot due to the efflux of Hoechst 33342 via ABCG2. Mouse bone marrow cells show a typical SP staining pattern, comparable to previously reported results [24,25] (Fig. 1A). Additionally, the SP phenotype could not be detected when bone marrow cells were incubated with verapamil, an MDR inhibitor, prior to staining with Hoechst 33342 (Fig. 1B). Quantitative RT-PCR confirmed that SP cells with low Hoechst-derived fluorescence (Fig. 1A, gated cells) had significantly higher expression of ABCG2, the mediator of the SP phenotype (Fig. 1C), as well as Bmi-1, an important factor in the self-renewal of HSCs [26] (Fig. 1D), compared to NSP cells. These results confirmed that cells with low fluorescence intensity were indeed an SP cell population that is enriched for HSCs. To verify that SP cells represented a quiescent HSC population, cell cycle analysis was performed. Although the cell cycle of total bone marrow cells was promoted with active cell division (Fig. 1E), SP cells were nearly all growth arrested in G0/G1, verifying their quiescent state (Fig. 1F).

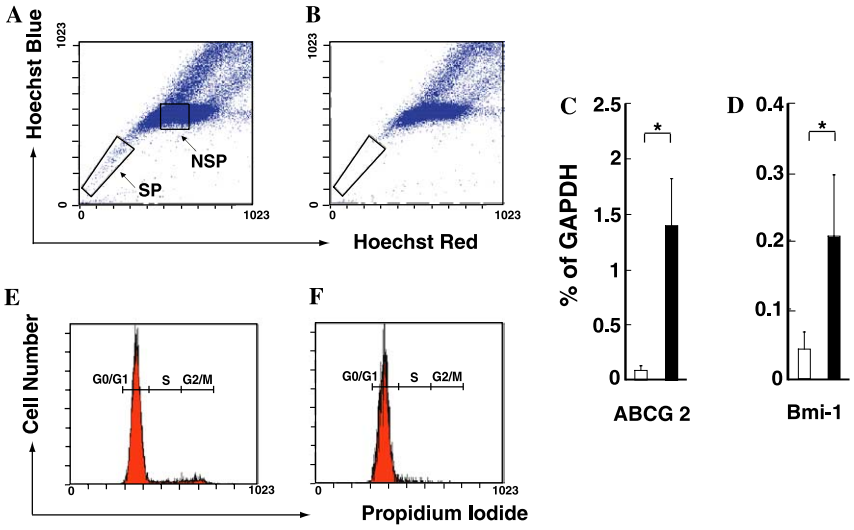

Fig. 1. Mouse bone marrow side population (SP) cells. Bone marrow cells were analyzed for Hoechst 33342 efflux by FACS (A). In the dot plot of (A), cells denoted by each enclosed area were regarded as SP cells or NSP cells for further characterization. When treated with Verapamil, an inhibitor of MDR, Hoechst 33342 efflux was antagonized (B). The expression of the stem cell markers, ABCG2 (C) and Bmi-1 (D), was quantified by real-time RT-PCR. Expression levels were determined from NSP cells: white bar and SP cells: black bar, for each individual mRNA. Data represent mean values from five samples, all performed in duplicate. Error bars indicate the SD (*$p < 0.05$). Additionally, the cell cycle phase in which each individual cell resided from either all viable bone marrow cells (E) or SP cells (F) was detected by flow cytometry.

As SP cells were confirmed to be growth arrested the G1 phase of the cell cycle, we next examined the mRNA expression of cyclin D, cyclin E, Cdk 2, Cdk 4, and Cdk 6, which are required for progression from G1 to S in the cell cycle. Interestingly, all genes examined were more highly expressed in SP cells compared to NSP cells, with significantly greater expression observed for cyclin D1, cyclin D2, and Cdk 4. Therefore, our results indicate that SP cells have high proliferative potential, but that other regulatory mechanisms are likely present to ensure the growth arrest and quiescent state of HSCs (Fig. 2).

As expression of G1- and S-phase Cdk and cyclin mRNAs was higher in SP cells compared to NSP cells, we examined the mRNA expression of the INK4 and Cip/Kip families of Cdk inhibitors. In the INK4 family, both $p15^{INK4b}$ and $p16^{INK4a}$ were barely expressed in either cell fraction (Figs. 3A and B). Additionally, while expression of $p18^{INK4c}$ was higher in SP cells (Fig. 3C), $p19^{INK4d}$ mRNA levels were actually slightly higher in the NSP cell fraction (Fig. 3D). In the Cip/Kip family, both $p21^{Cip1}$ and $p57^{Kip2}$ demonstrated significantly higher expression in SP cells compared to NSP cells, while $p27^{Kip1}$ mRNA expression was higher in NSP cells (Figs. 3E–G).

To investigate the relationship between the observed increase in expression of specific CDK inhibitors and SP cell cycle arrest, bone marrow SP cells were further enriched for

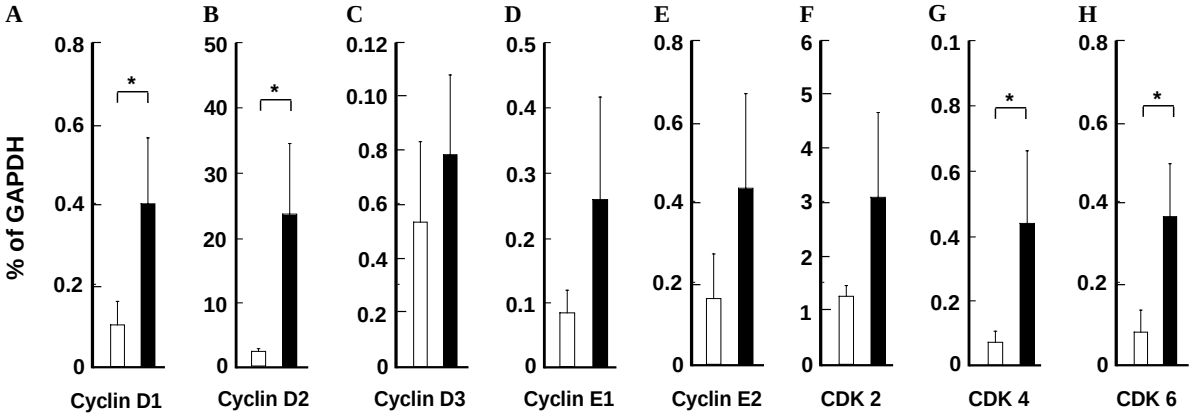

Fig. 2. Expression of cyclin and cyclin-dependent kinase (Cdk) mRNAs in SP and NSP cells. Total RNA was extracted from bone marrow SP cells and NSP cells after FACS, and subjected to real-time quantitative RT-PCR. Relative expression of the selected genes was normalized to that of GAPDH for each sample. mRNA expression of Cyclin D1 (A), Cyclin D2 (B), Cyclin D3 (C), Cyclin E1 (D), Cyclin E2 (E), Cdk 2 (F), Cdk 4 (G), and Cdk 6 (H) is shown. Expression levels were determined from NSP cells: white bar and SP cells: black bar, for each individual mRNA. Data represent mean values from five samples, all performed in duplicate. Error bars indicate the SD (*$p < 0.05$).

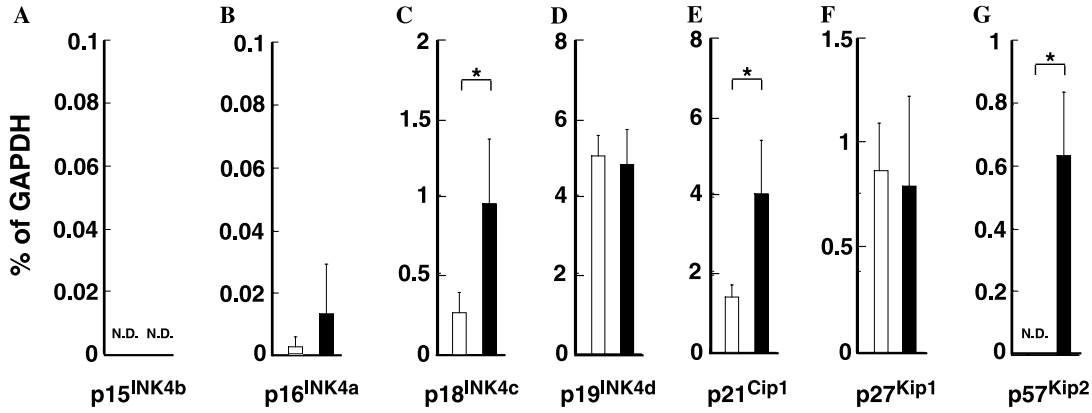

Fig. 3. Expression of cyclin-dependent kinase (Cdk) inhibitor mRNAs in SP and NSP cells. Total RNA was extracted from bone marrow SP cells and NSP cells after FACS, and subjected to real-time quantitative RT-PCR. Relative expression of the selected genes was normalized to that of GAPDH for each sample. mRNA expression of p15 ^{INK4b} (A), p16^{INK4a} (B), p18^{INK4c} (C), p19 ^{INK4d} (D), p21^{Cip1} (E), p27^{Kip1} (F), and p57^{Kip2} (G) is shown. Expression levels were determined from NSP cells: white bar and SP cells: black bar, for each individual mRNA. Data represent the mean value from five samples, all performed in duplicate. Error bars indicate the SD (*$p < 0.05$).

quiescent hematopoietic stem cells by the depletion of lineage-positive cells (Fig. 4A), followed by isolation of c-kit$^+$ and Sca-1$^+$ cells (Figs. 4B and C). mRNA expression of the SP cell marker ABCG2, as well as those CDK inhibitors that showed significantly higher expression in SP cells (p18^{INK4c}, p21^{Cip1}, and p57^{Kip2}), was then examined in the resulting KSL-SP cell population.

Our results demonstrated that KSL-SP cells had much higher expression of ABCG2 and p57^{Kip2} compared to both SP and NSP cells (Figs. 4D and G), but that the expression of both p18^{INK4c} and p21^{Cip1} showed insignificant differences between SP and KSL-SP cells (Figs. 4E and F). To examine p57^{Kip2} protein expression, immunofluorescence was performed with anti-p57^{Kip2} antibodies. Staining visualized with confocal laser scanning microscopy demonstrated that KSL-SP cells had higher expression of p57^{Kip2} protein, compared to NSP cells (Figs. 4H–M). In addition, while KSL-SP cells demonstrated p57^{Kip2} expression that was nearly completely localized to cell nuclei (Figs. 4I, K, and M), the observed low level of p57^{Kip2} in NSP cells showed almost completely cytoplasmic staining (Fig. 4H, J, and L).

These results indicated the possibility that the cell cycle arrest of quiescent bone marrow SP cells was correlated to increased expression of specific Cdk inhibitors, in particular p57^{Kip2}. Therefore, although SP cells may have a strong potential for proliferation due to their increased expression of G1- and S-phase cyclins and Cdks, increased expression of p57^{Kip2} and other Cdk inhibitors was able to induce cell cycle arrest via inhibition of cyclin/Cdk complexes.

Discussion

In the present study, we showed the possibility that the cell cycle arrest observed in bone marrow SP cells, in particular KSL-SP cells, is induced by the Cdk inhibitor p57^{Kip2}. p57^{Kip2} is a well-known member of the Cip/Kip family of Cdk inhibitors that function by binding to cyclin/Cdk complexes to inhibit progression through the cell cycle. Overexpression of p57^{Kip2} has been shown to strongly inhibit somatic cell proliferation [27–29] and p57^{Kip2} has also been shown to be involved in the cell cycle arrest of immature cells in other tissues, such as the retina [30,31] and brain [13]. It was recently reported that TGF-β induced cell cycle arrest of human cord blood derived progenitors required the increased expression of p57^{Kip2} [22]. p57^{Kip2} has also been shown to have genomic imprinting [32,33], which suggests a role in fetal development, as a substantial amount of imprinted genes have critical functions in the control of fetal growth. Therefore, a loss of heterozygosity induces down-regulation and can result in tumor formation [32,34]. It seems likely that p57^{Kip2} is intimately involved in the supervision of cell cycle progression and carefully regulates cell proliferation.

While expression of both p18^{INK4c} and p21^{Cip1} was also significantly higher in SP cells, the difference in the expression of p57^{Kip2} between SP and NSP cells was greatest, with no p57^{Kip2} mRNA detected in NSP cells. Moreover, KSL-SP cells, a cell population more highly enriched for quiescent hematopoietic stem cells, demonstrate a much higher expression of both ABCG2 and p57^{Kip2} than both SP and NSP cells. Hematopoietic stem cells are known to be extremely resistant to various external stresses [35], and the demonstration that the SP cell marker ABCG2 has significantly higher expression in KSL-SP cells compared to both SP and NSP cells further emphasizes that KSL-SP cells are highly enriched for quiescent stem cells.

Previously, Cheng et al. [36] demonstrated that p21^{Cip1} was involved in the maintenance of hematopoietic stem cell quiescence. In the present study, our results also showed that p21^{Cip1} had slightly higher expression in KSL-SP cells. While we cannot exclude the possibility that p21^{Cip1} plays a role in governing the quiescent state of KSL-SP cells, in the previous report by Cheng and colleagues, the authors did

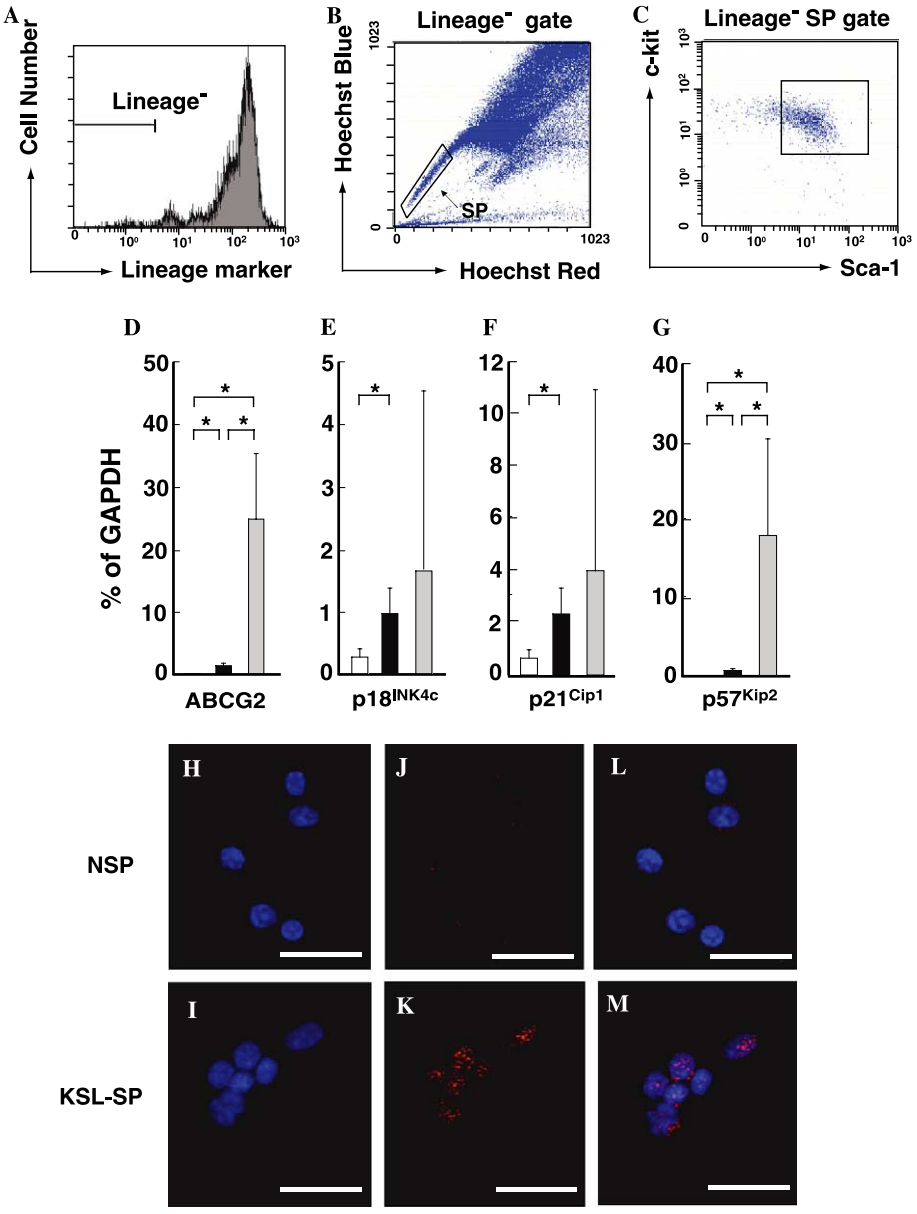

Fig. 4. KSL-SP cells show a high expression of $p57^{Kip2}$. KSL-SP cells were defined by the Lineage$^-$ gate in (A). Lineage$^-$-SP cells (B) were then selected as Sca-1^+ and c-kit^+ (C) for the isolation of KSL-SP cells. mRNA expression of ABCG2 (D), $p18^{INK4c}$ (E), $p21^{Cip1}$ (F), and $p57^{Kip2}$ (G) was then examined using real-time quantitative RT-PCR. Expression levels were determined from NSP cells: white bars, SP cells: black bars, and KSL-SP cells: gray bars, for each individual mRNA. Data represent mean values from four to five samples, all performed in duplicate. Error bars indicate the SD ($*p < 0.05$). Immunofluorescence analysis was performed on NSP cells and KSL-SP cells. Panels represent NSP (H,J,L) and KSL-SP (I,K,M) cells, stained with Hoechst 33342 (H,I), anti-$p57^{Kip2}$ antibody (J,K). (L,M) show merged images. Scale bars represent 20 μm.

not examine the effects of $p57^{Kip2}$. In addition, our findings that $p57^{Kip2}$ expression is specific to SP cells, and in particular KSL-SP cells lead us to believe that $p57^{Kip2}$ is likely the major factor involved in maintaining cell cycle arrest of KSL-SP cells.

Similarly, previous reports have also demonstrated the importance of $p57^{Kip2}$ in the control of the cell cycle. $p57^{Kip2}$ knockout mice often die before birth or else immediately afterwards, due to abnormal apoptosis and morphogenesis [37,38]. In contrast, mice having knockouts for the other Cdk inhibitors examined here, such as $p18^{INK4c}$ [39,40] and $p21^{Cip1}$ [41,42], can survive postnatally. Therefore, $p57^{Kip2}$ seems to be the Cdk inhibitor that is most critically involved in the proper maintenance of cell cycle kinetics.

In many ways, the cancer phenotype resembles a stem cell phenotype, as several cancers are known to express ABCG2, multi-drug resistance protein (MRP), and MDR, and improper regulation of stem cell dynamics is also thought to result in malignant transformation. Loss of function and altered imprinting resulting in altered expression of $p57^{Kip2}$ are known to cause several types of

cancers [43–47] and $p57^{Kip2}$ has been shown to have the strongest tumor suppressor activity in the Cip/Kip family [48]. Therefore, $p57^{Kip2}$ likely has a prominent role in inhibiting cell division.

Interestingly, we also found that SP cells may have a high potential for proliferation due to the increased expression of cyclin D and cyclin E as well as Cdk 2, Cdk 4, and Cdk 6 that are known to promote DNA synthesis and therefore cell cycle progression. Similarly, it has previously been shown that the marker of proliferating cells, Bmi-1, is required for the self-renewal of HSCs, but is also known to decrease the expression of $p16^{INK4a}$ and $p19^{INK4d}$ [26,49,50], and increase telomerase activity [51], which is believed to be indicative of actively proliferating cells. Therefore, it seems that some other regulatory factor, likely $p57^{Kip2}$, is necessary for SP cells to maintain their quiescent state despite their high proliferative potential from increased expression of Bmi-1 as well as, G1- and S-phase cyclins and Cdks.

It has previously been shown that the single characteristic that most accurately identifies a quiescent stem cell population in the bone marrow is the SP phenotype [35]. Bone marrow SP cells have indeed been shown to have the capability for the long-term multi-lineage reconstitution of the hematopoietic system and to reside in a quiescent state in the bone marrow niche [2,35]. It is believed that SP cells from the isolated from the bone marrow by Hoechst 33342 exclusion assays more accurately represent quiescent stem cells than HSCs characterized by other means [35]. Therefore, differences in gene expression between SP and NSP cells isolated from the bone marrow should accurately represent differences between quiescent stem cells and more highly differentiated cells. Because HSCs must govern the long-term maintenance of the entire hematopoietic system, it seems likely that these stem cells require a high proliferative potential to be able to sustain hematopoiesis throughout the host lifetime. However, HSCs must also remain cell cycle arrested in order to carefully regulate this proliferation over time and thus prevent dysfunctions of the hematopoietic system such as various cancers. We therefore hypothesize that the increased expression of the cyclins and Cdks involved in S-phase promotion demonstrates the high proliferative potential of bone marrow SP cells to ensure successful long-term re-population, but that $p57^{Kip2}$ acts to inhibit these cyclin/Cdk complexes and therefore maintain the quiescent state of HSCs and carefully regulate the generation of differentiated progeny.

Previous results have demonstrated that the bone marrow niche in which HSCs reside is critically involved in the maintenance of the quiescent state of the HSCs due to signaling from HSC surface molecules such as Tie-2 [35,52]. It seems likely that the stem cell niche in the bone marrow may be critically involved in inducing $p57^{Kip2}$ expression in order to preserve cell cycle arrest. Further study is needed to identify the system and mechanisms that regulate the expression of $p57^{Kip2}$ such that the proliferation of SP cells, isolated not only from bone marrow, but also other tissues, can be carefully controlled by modifications of gene expression. In the present study, we demonstrate the likely involvement of $p57^{Kip2}$ in inhibiting cell cycle progression of bone marrow SP cells, and thus present a novel mechanism for the maintenance of the quiescent state of HSCs. The careful management of the proliferation of SP cells, which can be found in various tissues, may thus allow for improved applications of cell-based therapies, such as regenerative medicine.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (15390530, 16200036, and 16300161), the High-Tech Research Center Program, the Center of Excellence Program for the 21st Century from the Ministry of Education, Culture, Sports, Science and Technology in Japan, and by the Core Research for Evolution Science and Technology from the Japan Science and Technology Agency.

References

[1] E.A. McCulloch, J.E. Till, The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice, Radiat. Res. 13 (1960) 115–125.
[2] M.A. Goodell, K. Brose, G. Paradis, A.S. Conner, R.C. Mulligan, Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo, J. Exp. Med. 183 (1996) 1797–1806.
[3] M.A. Goodell, M. Rosenzweig, H. Kim, D.F. Marks, M. DeMaria, G. Paradis, S.A. Grupp, C.A. Sieff, R.C. Mulligan, R.P. Johnson, Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species, Nat. Med. 3 (1997) 1337–1345.
[4] S. Zhou, J.D. Schuetz, K.D. Bunting, A.M. Colapietro, J. Sampath, J.J. Morris, I. Lagutina, G.C. Grosveld, M. Osawa, H. Nakauchi, B.P. Sorrentino, The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype, Nat. Med. 7 (2001) 1028–1034.
[5] M. Kim, H. Turnquist, J. Jackson, M. Sgagias, Y. Yan, M. Gong, M. Dean, J.G. Sharp, K. Cowan, The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells, Clin. Cancer Res. 8 (2002) 22–28.
[6] C.W. Scharenberg, M.A. Harkey, B. Torok-Storb, The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors, Blood 99 (2002) 507–512.
[7] T. Leemhuis, M.C. Yoder, S. Grigsby, B. Aguero, P. Eder, E.F. Srour, Isolation of primitive human bone marrow hematopoietic progenitor cells using Hoechst 33342 and Rhodamine 123, Exp. Hematol. 24 (1996) 1215–1224.
[8] F.I. Preffer, D. Dombkowski, M. Sykes, D. Scadden, Y.G. Yang, Lineage-negative side-population (SP) cells with restricted hematopoietic capacity circulate in normal human adult blood: immunophenotypic and functional characterization, Stem Cells 20 (2002) 417–427.
[9] M. Feuring-Buske, D.E. Hogge, Hoechst 33342 efflux identifies a subpopulation of cytogenetically normal CD34(+)CD38(−) progenitor cells from patients with acute myeloid leukemia, Blood 97 (2001) 3882–3889.
[10] H. Iwatani, T. Ito, E. Imai, Y. Matsuzaki, A. Suzuki, M. Yamato, M. Okabe, M. Hori, Hematopoietic and nonhematopoietic potentials of Hoechst(low)/side population cells isolated from adult rat kidney, Kidney Int. 65 (2004) 1604–1614.

[11] A. Asakura, Stem cells in adult skeletal muscle, Trends Cardiovasc. Med. 13 (2003) 123–128.
[12] A.J. Alvi, H. Clayton, C. Joshi, T. Enver, A. Ashworth, M.M. Vivanco, T.C. Dale, M.J. Smalley, Functional and molecular characterisation of mammary side population cells, Breast Cancer Res. 5 (2003) R1–R8.
[13] K.A. Jackson, T. Mi, M.A. Goodell, Hematopoietic potential of stem cells isolated from murine skeletal muscle, Proc. Natl. Acad Sci. USA 96 (1999) 14482–14486.
[14] R. Summer, D.N. Kotton, X. Sun, B. Ma, K. Fitzsimmons, A. Fine, Side population cells and Bcrp1 expression in lung, Am. J. Physiol. Lung Cell Mol. Physiol. 285 (2003) L97–L104.
[15] K. Shimano, M. Satake, A. Okaya, J. Kitanaka, N. Kitanaka, M. Takemura, M. Sakagami, N. Terada, T. Tsujimura, Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1, Am. J. Pathol. 163 (2003) 3–9.
[16] J.X. Zhou, L.W. Jia, Y.J. Yang, S. Peng, Y.J. Cao, E.K. Duan, Enrichment and characterization of mouse putative epidermal stem cells, Cell Biol. Int. 28 (2004) 523–529.
[17] K. Watanabe, K. Nishida, M. Yamato, T. Umemoto, T. Sumide, K. Yamamoto, N. Maeda, H. Watanabe, T. Okano, Y. Tano, Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2, FEBS Lett. 565 (2004) 6–10.
[18] B. Lassalle, H. Bastos, J.P. Louis, L. Riou, J. Testart, B. Dutrillaux, P. Fouchet, I. Allemand, 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells, Development 131 (2004) 479–487.
[19] K.D. Bunting, ABC transporters as phenotypic markers and functional regulators of stem cells, Stem Cells 20 (2002) 11–20.
[20] N. Uchida, D. He, A.M. Friera, M. Reitsma, D. Sasaki, B. Chen, A. Tsukamoto, The unexpected G0/G1 cell cycle status of mobilized hematopoietic stem cells from peripheral blood, Blood 89 (1997) 465–472.
[21] V.A. Florenes, N. Bhattacharya, M.R. Bani, Y. Ben-David, R.S. Kerbel, J.M. Slingerland, TGF-beta mediated G1 arrest in a human melanoma cell line lacking p15INK4B: evidence for cooperation between p21Cip1/WAF1 and p27Kip1, Oncogene 13 (1996) 2447–2457.
[22] J.M. Scandura, P. Boccuni, J. Massague, S.D. Nimer, Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation, Proc. Natl. Acad. Sci. USA 101 (2004) 15231–15236.
[23] G. Li, J. Domenico, J.J. Lucas, E.W. Gelfand, Identification of multiple cell cycle regulatory functions of p57Kip2 in human T lymphocytes, J. Immunol. 173 (2004) 2383–2391.
[24] I. Bertoncello, B. Williams, Hematopoietic stem cell characterization by Hoechst 33342 and rhodamine 123 staining, Methods Mol. Biol. 263 (2004) 181–200.
[25] M.A. Goodell, S. McKinney-Freeman, F.D. Camargo, Isolation and characterization of side population cells, Methods Mol. Biol. 290 (2004) 343–352.
[26] I.K. Park, D. Qian, M. Kiel, M.W. Becker, M. Pihalja, I.L. Weissman, S.J. Morrison, M.F. Clarke, Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells, Nature 423 (2003) 302–305.
[27] S.A. Kassem, I. Ariel, P.S. Thornton, K. Hussain, V. Smith, K.J. Lindley, A. Aynsley-Green, B. Glaser, p57(KIP2) expression in normal islet cells and in hyperinsulinism of infancy, Diabetes 50 (2001) 2763–2769.
[28] M. Joaquin, R.J. Watson, The cell cycle-regulated B-Myb transcription factor overcomes cyclin-dependent kinase inhibitory activity of p57(KIP2) by interacting with its cyclin-binding domain, J. Biol. Chem. 278 (2003) 44255–44264.
[29] T. Urano, T. Hosoi, M. Shiraki, H. Toyoshima, Y. Ouchi, S. Inoue, Possible involvement of the p57(Kip2) gene in bone metabolism, Biochem. Biophys. Res. Commun. 269 (2000) 422–426.
[30] M.A. Dyer, C.L. Cepko, p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina, Development 127 (2000) 3593–3605.
[31] M.A. Dyer, C.L. Cepko, p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations, J. Neurosci. 21 (2001) 4259–4271.
[32] W.Y. Chung, L. Yuan, L. Feng, T. Hensle, B. Tycko, Chromosome 11p15.5 regional imprinting: comparative analysis of KIP2 and H19 in human tissues and Wilms' tumors, Hum. Mol. Genet. 5 (1996) 1101–1108.
[33] T. Taniguchi, K. Okamoto, A.E. Reeve, Human p57(KIP2) defines a new imprinted domain on chromosome 11p but is not a tumour suppressor gene in Wilms tumour, Oncogene 14 (1997) 1201–1206.
[34] I. Hatada, H. Ohashi, Y. Fukushima, Y. Kaneko, M. Inoue, Y. Komoto, A. Okada, S. Ohishi, A. Nabetani, H. Morisaki, M. Nakayama, N. Niikawa, T. Mukai, An imprinted gene p57KIP2 is mutated in Beckwith–Wiedemann syndrome, Nat. Genet. 14 (1996) 171–173.
[35] F. Arai, A. Hirao, M. Ohmura, H. Sato, S. Matsuoka, K. Takubo, K. Ito, G.Y. Koh, T. Suda, Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche, Cell 118 (2004) 149–161.
[36] T. Cheng, N. Rodrigues, H. Shen, Y. Yang, D. Dombkowski, M. Sykes, D.T. Scadden, Hematopoietic stem cell quiescence maintained by p21cip1/waf1, Science 287 (2000) 1804–1808.
[37] Y. Yan, J. Frisen, M.H. Lee, J. Massague, M. Barbacid, Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development, Genes Dev. 11 (1997) 973–983.
[38] P. Zhang, N.J. Liegeois, C. Wong, M. Finegold, H. Hou, J.C. Thompson, A. Silverman, J.W. Harper, R.A. DePinho, S.J. Elledge, Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith–Wiedemann syndrome, Nature 387 (1997) 151–158.
[39] E. Latres, M. Malumbres, R. Sotillo, J. Martin, S. Ortega, J. Martin-Caballero, J.M. Flores, C. Cordon-Cardo, M. Barbacid, Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis, Embo J. 19 (2000) 3496–3506.
[40] G.I. Kovalev, D.S. Franklin, V.M. Coffield, Y. Xiong, L. Su, An important role of CDK inhibitor p18(INK4c) in modulating antigen receptor-mediated T cell proliferation, J. Immunol. 167 (2001) 3285–3292.
[41] M.L. Santiago-Raber, B.R. Lawson, W. Dummer, M. Barnhouse, S. Koundouris, C.B. Wilson, D.H. Kono, A.N. Theofilopoulos, Role of cyclin kinase inhibitor p21 in systemic autoimmunity, J. Immunol. 167 (2001) 4067–4074.
[42] D.J. Bearss, R.J. Lee, D.A. Troyer, R.G. Pestell, J.J. Windle, Differential effects of p21(WAF1/CIP1) deficiency on MMTV-ras and MMTV-myc mammary tumor properties, Cancer Res. 62 (2002) 2077–2084.
[43] M. Kondo, S. Matsuoka, K. Uchida, H. Osada, M. Nagatake, K. Takagi, J.W. Harper, T. Takahashi, S.J. Elledge, T. Takahashi, Selective maternal-allele loss in human lung cancers of the maternally expressed p57KIP2 gene at 11p15.5, Oncogene 12 (1996) 1365–1368.
[44] J.S. Thompson, K.J. Reese, M.R. DeBaun, E.J. Perlman, A.P. Feinberg, Reduced expression of the cyclin-dependent kinase inhibitor gene p57KIP2 in Wilms' tumor, Cancer Res. 56 (1996) 5723–5727.
[45] J.Y. Shin, H.S. Kim, K.S. Lee, J. Kim, J.B. Park, M.H. Won, S.W. Chae, Y.H. Choi, K.C. Choi, Y.E. Park, J.Y. Lee, Mutation and expression of the p27KIP1 and p57KIP2 genes in human gastric cancer, Exp. Mol. Med. 32 (2000) 79–83.
[46] M. Oya, W.A. Schulz, Decreased expression of p57(KIP2)mRNA in human bladder cancer, Br. J. Cancer 83 (2000) 626–631.
[47] Y. Ito, T. Takeda, M. Sakon, M. Tsujimoto, M. Monden, N. Matsuura, Expression of p57/Kip2 protein in hepatocellular carcinoma, Oncology 61 (2001) 221–225.

[48] M.H. Lee, I. Reynisdottir, J. Massague, Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution, Genes Dev. 9 (1995) 639–649.
[49] K.S. Smith, S.K. Chanda, M. Lingbeek, D.T. Ross, D. Botstein, M. van Lohuizen, M.L. Cleary, Bmi-1 regulation of INK4A-ARF is a downstream requirement for transformation of hematopoietic progenitors by E2a-Pbx1, Mol. Cell 12 (2003) 393–400.
[50] A.V. Molofsky, R. Pardal, T. Iwashita, I.K. Park, M.F. Clarke, S.J. Morrison, Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation, Nature 425 (2003) 962–967.
[51] M. Milyavsky, I. Shats, N. Erez, X. Tang, S. Senderovich, A. Meerson, Y. Tabach, N. Goldfinger, D. Ginsberg, C.C. Harris, V. Rotter, Prolonged culture of telomerase-immortalized human fibroblasts leads to a premalignant phenotype, Cancer Res. 63 (2003) 7147–7157.
[52] K.A. Moore, I.R. Lemischka, "Tie-ing" down the hematopoietic niche, Cell 118 (2004) 139–140.