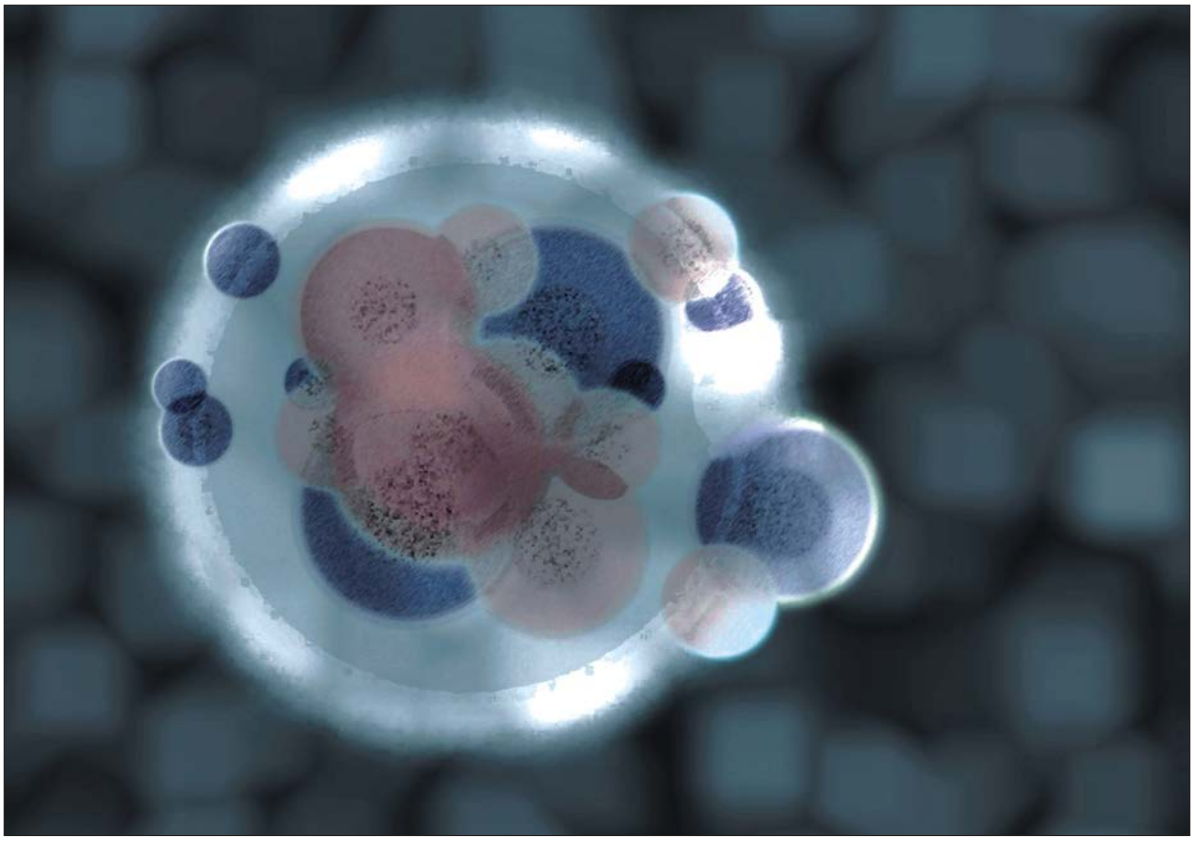

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# Cell Cycle Regulation in Hematopoietic Stem/progenitor Cells

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**Abstract:** Hematopoietic Stem Cells (HSCs) are characterized by two distinct abilities, that is, self-renewal ability and multipotency. To keep the homeostasis of hematopoiesis and protect the exhaustion of HSCs throughout the life, most of HSCs are kept quiescent and only a limited number of HSCs enter cell cycle to supply mature blood cells. Cell cycle state of HSCs is crucially regulated by external factors such as cytokines, Notch ligands and Wnt signals in the Bone Marrow (BM) microenvironment, so called hematopoietic niche. In addition, the intrinsic factors expressed in HSCs such as c-Myb, GATA-2, HOX family proteins and Bmi-1 also control their growth through the gene transcription. Cell cycle regulation in HSCs is not so unique but rather common to other cell types. However, the specific function of each cell cycle regulatory molecule in HSCs has been clarified during the last few years. Especially, $p21^{WAF1}$ and $p18^{INK4C}$ keep the quiescence of HSCs and $p27^{CIP1}$ keeps that of progenitor cells, respectively, thereby governing their pool sizes and/or preventing their exhaustion. On the other hand, the inactivation or deletion of $p16^{INK4A}$ and $p15^{INK4B}$ genes is supposed to contribute to malignant transformation of hematopoietic cells. These results imply that appropriate cell cycle control at the stage of stem/progenitor cells in the BM is required for maintaining normal hematopoiesis.

**Key words:** Hematopoietic stem/progenitor cell, cell cycle

## INTRODUCTION

HSCs are characterized by two distinct abilities; self-renewal ability and multipotency. With these activities, HSCs are capable of maintaining a life-long supply of all lineages of hematopoietic cells according to systemic needs. The durability of the output potential of HSCs is believed to be dependent on their ability to execute self-renewal divisions; that is, an ability to proliferate without activation of a latent readiness to differentiate along restricted lineages. To maintain the homeostasis of hematopoiesis and protect the exhaustion of HSC population, most of HSCs are kept quiescent and only a limited number of cells enter cell cycle to supply mature blood cells. During this cell division, HSCs are obliged to undergo self-renewal, differentiation, or apoptosis. This step is controlled by external stimuli transmitted from the Bone Marrow (BM) microenvironment, including cytokines, Notch ligands, Wnt signals and sonic hedgehog (Shh) signals. Also, intrinsic factors expressed in HSCs, such as transcription regulators and cell cycle regulatory molecules, are crucially involved in this regulation (Fig. 1).

During the last decade, a number of cell cycle regulatory molecules such as cyclins, Cyclin-dependent Kinases (CDKs) and CDK inhibitors (CKIs) have been identified and their roles and regulation have been well characterized in various types of cells[1-3]. Cell cycle is positively regulated by CDKs associated with cyclins and their activities are negatively regulated by CKIs also included in these complexes at the same time. CKIs are classified into two families based on their structures and CDK targets. One class of inhibitors including $p21^{WAF1}$ (hereafter indicated as p21), $p27^{KIP1}$ (p27) and$p57^{KIP2}$ share a CDK2-binding motif in the N-terminus and inhibit the activities of cyclinD-, E- andA-dependent kinases. The other class of inhibitors also known as the INK4 family, including $p16^{INK4A}$ (p16), $p15^{INK4B}$(p15), $p18^{INK4C}$ (p18) and $p19^{INK4D}$, contain fourfold ankyrin repeats and specifically inhibit CDK4 and CDK6. Members of both families are important for executing cell cycle arrest in response to a variety of stimuli such as DNA damage, contact inhibition and transforming growth factor-β1 (TGF-β1) treatment.

Molecular mechanisms governing the stemness of HSCs from a viewpoint of cell cycle regulation are presented in this study.

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**Characteristic of HSCs:** The procedure for the purification of HSCs has made great progress along with the identification of molecular markers that characterize the cells having reconstitution activities in transplanted mice. The most primitive HSCs are considered to be with the $CD34^{low/-}$c-$Kit^{+}Sca\text{-}1^{+}Lin^{-}$ (CD34-KSL) phenotype, since a single cell with this phenotype could reconstitute whole hematopoiesis *in vivo* with high probability[4]. In addition to the specific surface phenotype, HSCs present in steady-state adult mouse BM are functionally characterized by their ability to efflux Rhodamine-123 and Hoechst 33342[5,6]. When adult mouse BM cells are stained with Hoechst 33342, exposed to the UV light and examined at 2 emission wavelengths simultaneously, HSCs are found in the rare Side Population (SP) with the dim fluorescence because of this ability[7]. The low fluorescence of HSCs after staining with Rhodamine-123 and Hoechst 33342 is attributed to their selective expression of different ABC transporters, P-glycoprotein and bcrp-1, respectively[8-10]. In addition, a more recent study proved that the cells having the strongest dye efflux capacity (Tip-SP cells) with the CD34-KSL phenotype are the most primitive HSCs, which can reconstitute long-term hematopoiesis with almost 100% probability after the single cell transplantation[11]. The cells in the SP fraction is considered to be in the G0 phase and this state is supposed to be restrictedly regulated by "hematopoietic niche'' in the BM as described later.

**Cytokines involved in cell cycle regulation in HSCs:** A number of cytokines regulate growth, differentiation and survival of HSCs both positively and negatively. Among these, stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin (TPO), interleukin-3 (IL-3) and IL-6 are known to promote the growth of HSCs *in vitro*[12-14]. In fact, Sl/Sl and W/W mice each having homozygous defect in the SCF gene and its receptor c-kit gene reveal severe anemia[15]. Also, total number of HSCs was reduced in the BM of c-mpl (TPO receptor)-null mice[16]. In addition, HSCs obtained from $c\text{-}mpl^{-/-}$ mice reveled severely decreased activities in reconstitution assays. These lines of evidence indicate that cytokine signals are required for the growth and survival of HSCs *in vivo* as well as *in vitro*[17].

During the last few years, the number of cases with hematologic malignancies receiving cord blood transplantation has increased more and more. However, the insufficient number of HSCs in each cord blood and the delayed recovery of hematopoiesis have limited their applicability to transplantation for adults. So, it has been of particular interest to expand hematopoietic cells *ex vivo*. Regarding the effects of cytokines in the *ex vivo* expansion of HSCs, a number of cytokine combinations were employed and their effects were evaluated by long-term reconstitution assays in transplanted mice. Among these, the combination of SCF, FL, TPO and IL-6/soluble IL-6 receptor seems to induce the *ex vivo* expansion of HSCs most efficiently with 4.2-fold increase of SRC (SCID-repopulating cells)[18].

TGF-β1 is a 25 kd protein produced by the stromal cells and by the hematopoietic progenitors, which induces the growth arrest in HSCs in autocrine and/or paracrine manners[19-23]. Using antisense oligonucleotides, it was demonstrated that the inhibition of TGF-β1 production could release HSCs in the umbilical cord blood or BM from quiescence[22,24,25]. Furthermore, the inhibition of the TGF-β1 signaling pathways in human HSCs using blocking antibodies against TGF-β1 or its receptor allowed quiescent cells to enter the cell cycle[26]. TGF-β1 has been supposed to induce cell-cycle arrest through p21 and p27 in various cell types including HSCs[27-33]. However, a recent paper provided evidence that TGF-β1 induces growth arrest independently of p21 or p27 by demonstrating that TGF-β1 can suppress the growth of HSCs and progenitor cells lacking both p21 and p27[34]. As for the other possible mechanisms of TGF-β1-induced growth arrest, TGF-β1 was reported to transcriptionally induce the expression of p15[35,36] and to down regulate the expression of c-Kit, FLT3 andIL-6 receptor on HSCs, thereby disrupting cytokine-dependent growth signals[37,38].

In contrast, another TGF-β super family protein, bone morphogenetic protein-4 (BMP-4) was recently reported to induce self-renewal of HSCs[39].

**Effects of the BM microenvironment "hematopoietic niche'' on cell cycle regulation in HSCs:** HSCs receive critical signals for proliferation and differentiation from the BM microenvironment consisting of stromal cells and the extracellular matrix (ECM)[40-42]. ECM is composed of a variety of molecules such as fibronectin (FN), collagens, laminin and proteoglycans. ECM in the BM is not merely an inert framework but mediates specialized functions[43-45]. Some components of ECM have been shown to bind to growth factors produced by stromal cells and to immobilize them around cells, resulting in giving spaces where hematopoietic cells and growth factors colocalize. In addition, ECM can bind to glycoproteins expressed on HSCs. FN, collagens and laminin are ligands for integrins that not only control anchorage, spreading and migration of HSCs but also activate signal transduction pathways in these cells[43,44,46,47].

As were the cases with the niches for gut and certain skin stem cells[48-50], it has been supposed that HSCs also receive critical signals for proliferation and differentiation from the BM microenvironment "hematopoietic niche''. However, it has been unknown where the hematopoietic niche is located in the BM or what types of cells

contribute to it. Recently, two groups individually generated mice lacking the BMP receptor type A (BMPRIA) and those engineered to produce osteoblast-specific, activated Parathyroid Hormone (PTH) and PTH-related protein (PTHrP) receptors (PPRs). In these mice, the osteoblast population was found to increase in the specific regions of bone, 'trabecular bone-like areas'. Also, the increase of the osteoblast population caused the parallel increase of the HSC population, particularly long-term repopulating HSCs[51,52]. As for this mechanism, Zhang *et al.*[51] demonstrated that the long-term HSCs were attached to spindle-shaped N-cadherin$^+$CD45$^-$ osteoblastic (SNO) cells. Two adherent junction molecules, N-cadherin and β-catenin, were asymmetrically localized between the SNO cells and the long-term HSCs, suggesting that SNO cells function as a key component of the niche to support HSCs and that BMP signaling through BMPRIA controls the number of HSCs by regulating niche size. Meanwhile, in the latter study, Calvi *et al.*[52] demonstrated that PPR-stimulated osteoblasts produced high levels of the Notch1 ligand, Jagged1 and supported the activity of HSCs through the Notch signaling. Together, these papers indicate that the interaction with osteoblasts contributes to the maintenance of the HSCs.

HSCs expressing the receptor tyrosine kinase Ties were quiescent and Ang-1, the ligand for Tie2 expressed on endothelial cells and HSCs, enhanced the quiescence of HSCs and their adhesion to fibronectin and collagen[53,54]. Therefore, it has been assumed that the Ang-1/Tie2 signaling pathway plays some role in the quiescence of HSCs. In accord with this hypothesis, a recent paper proved that Tie2$^+$ HSCs were in close contact with sub-endosteal osteoblasts expressing Ang-1 and that these Tie2$^+$ cells were included in SP and in the G0 phase of the cell cycle in the pyronin Y staining[55]. These results suggest that HSCs attaching to the specific osteoblasts in the hematopoietic niche are kept quiescent and protected from the myelosuppressive stress such as the treatment with 5-Fluorouracil (5-FU), a cell cycle-specific myelotoxic agent that kills cycling cells. However, it remains unknown which fraction of osteoblasts expresses Ang-1 and how it is regulated. Furthermore, the molecular mechanisms how Tie2/Ang-1 signaling prevents cell cycle progression also remain elusive.

**Effects of the signals from the Notch ligand, Wnt and sonic hedgehog (Shh) on self-renewal of HSCs:** In addition to the cytokines and molecules consisting of the extracellular matrix, various stimuli such as the Notch ligand, Wnt and Shh are transmitted to HSCs in the BM microenvironment. The activation of Notch transmembrane receptors expressed on HSCs by their ligand (Jagged 1 or Jagged 2) expressed on stromal cells promotes self-renewal of HSCs[56-60]. As for the critical target molecule of Notch signals that mediates self-renewal of HSCs, we recently found that c-Myc was transcriptionally induced by Notch[61]. In addition, the ectopic expression of c-Myc induced the growth of HSCs without disrupting their biologic properties in terms of surface phenotypes, colony-forming activities and reconstituting activities. Thus, c-Myc was supposed to play a major role in self-renewal of HSCs as an effector molecule of Notch signals.

Like Jagged1/Notch, a number of Wnt proteins are expressed in the BM and their receptor frizzled was detectable on BM-derived HSCs and progenitor cells[62,63]. Reya *et al.*[64] recently demonstrated that the Wnt signaling is important for the *in vitro* and *in vivo* self-renewal of normal HSCs. Moreover, they demonstrated that the activation of Wnt signaling in HSCs induces the increased expression of HOXB4 and Notch1, thereby inducing proliferation of HSCs. Besides Wnt3a that activates the canonical pathway through Frizzled/β-catenin/TCF/LEF, non-canonical Wnt, Wnt-5a, was also reported to expanded HSCs *in vitro*[65]. However, its mechanisms remain to be clarified.

Shh is a family member of human homologs of *Drosophila* Hedgehog (Hh) and expressed on the cell surface as transmembrane proteins. Hh signals can be mediated through cell-to-cell contact between adjacent cells expressing the Patched (Ptc) receptor. Alternatively, NH2-terminal cleavage of Hh can generate a soluble Hh ligand that can interact with distal cells expressing Ptc[66,67]. In the BM, Shh and their receptors Ptc and Smoothened (Smo) are expressed in highly purified HSCs. Cytokine-induced proliferation of HSCs could be inhibited by the anti-Hh Ab, implying that endogenously produced Hh proteins play a role in the expansion of HSCs. Addition of soluble forms of Shh resulted in an increase in the number of HSCs with pluripotent repopulating capacities. In addition, Noggin, a potent BMP-4 inhibitor, was found to inhibit the mitogenic effects of Shh, indicating that Shh signaling acts upstream of BMP-4 signaling to induce proliferation of HSCs[68].

**Intrinsic factors that regulate the growth of HSCs:** In addition to extrinsic factors, accumulated evidence indicates that cell cycle state of HSCs is regulated by intrinsic transcription regulatory factors, such c-Myb, GATA-2, HOX proteins and Bmi-1 (Fig. 2).

A transcriptional factor, c-Myb promotes the growth of HSCs, probably through the induction of c-myc and upregulated expression of c-kit and Flt3[69,70] and c-Myb-deficient mice die at embryonic day 15.5 (E15.5) due to the defect of definitive hematopoiesis[71]. Similarly, GATA-2$^{-/-}$

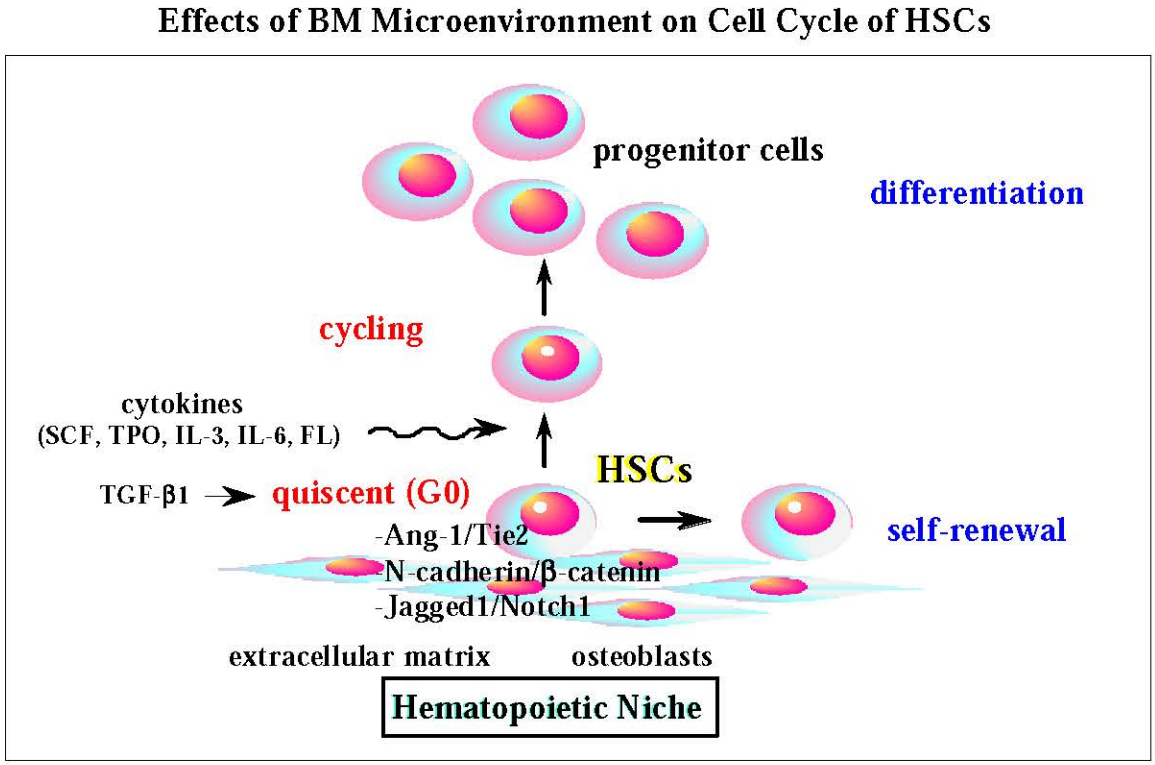


Fig. 1: Effects of BM microenvironment on cell cycle of HSCs

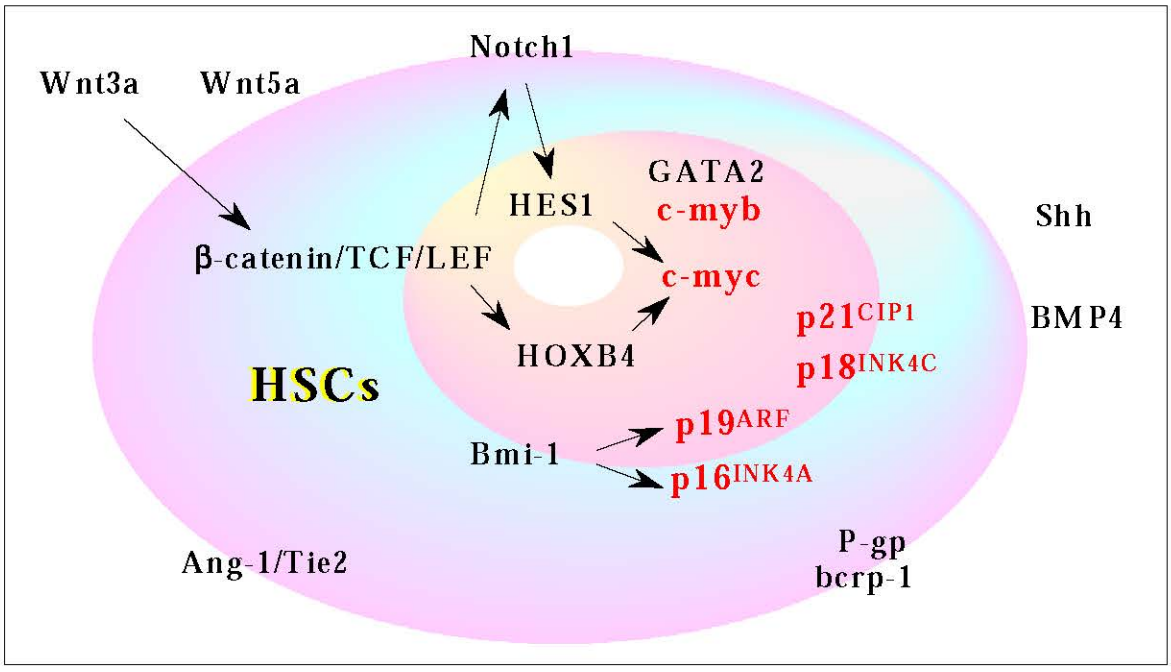


Fig. 2: Regulation of stemness by intrinsic factors in HSCs

mice are embryonic lethal around E11.5 because of the defect in the development and/or maintenance of HSCs[72]. However, functional roles of GATA-2 in the growth of HSCs are still controversial[73-76]. So, it remains unknown whether GATA-2 enhances or suppresses the growth of HSCs.

Among HOX family of transcription factors, HOXB4 is of particular remark as it promotes the growth of HSCs without the induction of leukemias[77-79]. As a result of the HOXB4 gene transfer or the protein delivery, HSCs could be expanded retaining their normal *in vivo* potential of differentiation and long-term repopulation[78,80]. Moreover, a recent study using HOXB4$^{-/-}$HOXB3$^{-/-}$ mice demonstrated that both HOXB4 and HOXB3 are required for the maximal growth potential of HSCs[81].

Bmi-1, a member of the Polycomb Group family of transcriptional repressors[82], was recently shown to be essential for maintenance of adult self-renewing HSCs[83]. Although the number of HSCs in the fetal liver of Bmi-1$^{-/-}$ mice was normal, the number of HSCs was markedly reduced in postnatal Bmi-1$^{-/-}$ mice. Furthermore, transplanted fetal liver and bone marrow cells obtained from Bmi-1$^{-/-}$ mice were able to contribute to hematopoiesis only transiently. Regarding this mechanism, in accord with the previous data obtained from embryonic fibroblasts[84], the micro array analysis on the BM mononuclear cells isolated from wild-type and Bmi-1$^{-/-}$ mice showed that the expression of p16 and p19$^{ARF}$, which is generated from the same INK4A locus by alternative splicing and inhibits MDM-2-mediated p53 degradation, was upregulated in Bmi-1$^{-/-}$ BM cells.

During neural development in mouse embryos, the cell-cycle regulator geminin controls replication by binding to the licensing factor Cdt1[85,86]. Recently, Luo *et al.*[87] reported that murine geminin transiently associated with members of the HOX-repressing polycomb complex, with the chromatin of HOX regulatory DNA elements and with HOX proteins[87]. Through these interactions, geminin displaces HOX proteins from their target genes and/or can interact with polycomb proteins to influence HOX activities. Therefore, the activities of HOX and polycomb protein families might be similarly regulated in HSCs.

**Roles of p21 and p27 in quiescence of HSCs and progenitor cells:** Embryonic fibroblasts obtained from p21$^{-/-}$ mice had a defect in their ability to achieve cell cycle arrest after irradiation[88,89] and antisense oligonucleotides against p21 was shown to release human mesenchymal cells from G0[90]. Therefore, p21 is required for the cell cycle arrest in G0 or G1 in some cell types. As for the roles for p21 in hematopoiesis, the expression level of p21 was reported be low in CD34$^{+}$ cells[91,92] and p21$^{-/-}$ mice did not exhibit an apparent hematologic defect[88,89]. However, in a subsequent analysis, Cheng *et al.*[93] found that p21 was highly expressed in the quiescent stem cell-like fraction of BM cells[93]. They also found that, under normal homeostatic conditions, the proportion of quiescent HSCs in the G0 phase was reduced and that total number of HSCs increased in p21$^{-/-}$ mice. In accord with these findings, when p21$^{-/-}$ mice were treated with 5-FU, the survival percentage was much lower in p21$^{-/-}$ mice than in littermate controls. They also directly assessed stem cell self-renewal capability using a serial transplantation approach. As a result, no mice transplanted with p21$^{-/-}$ BM cells survived after the fifth transplant due to the exhaustion of HSC population, whereas those transplanted with p21$^{+/+}$ BM cells had a 50% survival. Together, these results indicate that p21 is a key molecule that restricts cell cycle entry of HSCs, thereby keeping their pool size and preventing their exhaustion under certain stress.

p27 is molecularly distinct from p21 in its carboxyl terminus; it interacts with similar, though not identical, cyclin-CDK complex and lacks p53-regulated expression. In hematopoietic system, the expression of p27 is observed in more mature progenitors than p21[91,92]. The p27$^{-/-}$ mice have a larger body and hyperplasia of most organs including hematopoietic organs[94-96]. In striking contrast to p21$^{-/-}$ mice, the number, cell cycling and self-renewal of HSCs were normal in p27$^{-/-}$ mice, while these mice had an increase in hematopoietic progenitor cells[97]. In addition, these progenitor cells in p27$^{-/-}$ mice were more proliferative than p27$^{+/+}$ progenitor cells. Furthermore, progenitor cells from p27$^{-/-}$ mice were able to expand and regenerate hematopoiesis after serial transplantation, while p27$^{+/+}$ progenitors were markedly depleted. Thus, p21 and p27 govern the divergent stem and progenitor cell populations, respectively.

**Roles for the INK4 family in self-renewing division of HSCs and as tumor suppressor genes:** Several of INK4 proteins have been supposed to be implicated in the regulation of HSCs numbers and self-renewal. Yuen *et al.*[98] recently clarified a function of p18 in HSCs and the early progenitor cells[98]. Mice deficient for p18 had an increased number of HSCs in the bone marrow. Also, competitive repopulation assays showed that p18$^{-/-}$ HSCs are far more competitive than normal HSCs with 14-fold activities. In contrast to p21$^{-/-}$ HSCs, the exhaustion of p18$^{-/-}$ HSCs was not observed during serial bone marrow transplants, indicating that p18 is a strong inhibitor limiting the potential of stem cell self-renewal *in vivo*.

On the other hands, p16 is highly expressed in CD34$^{+}$ cells and its expression is down regulated during differentiation process towards all lineages[99]. So, p16 was assumed to play some role in cell cycle arrest in HSCs. However, since p16$^{-/-}$ mice did not show an apparent

abnormality in hematopoiesis, p16 was supposed to be dispensable for the quiescence of HSCs[100,101]. In contrast to the expression pattern of p16, the expression of p15 was not detected in $CD34^+$ cells, but increased specifically during myeloid differentiation[99,102]. However, the functional role of p15 in HSCs remained to be clarified. Both p16 and p15 inhibit the function of cyclin D-CDK4/6 complex and suppress the phosphorylation of pRb, thereby inducing cell cycle arrest at G0/G1 phase. Especially, under tumorgenetic stress such as the presence of oncogenic ras gene, p16 and p15 are induced to express and suppress tumor progression through the induction of premature senescence[103,104]. With these activities, both p16 and p15 are supposed to act as tumor suppressor genes. In fact, inactivation and/or deletion of p16 and p15 genes are observed in various human cancers very frequently[105,106]. As for hematologic malignancies, their defects caused by the homozygotic deletion or methylation were observed in a substantial proportion of AML, ALL, ATL, malignant lymphoma and MDS cases[107-111]. These results indicate that appropriate cell cycle control, particularly at the stage of stem/progenitor cells, is required for maintaining normal hematopoiesis.

## CONCLUSIONS

Although a great advance has been made in stem cell biology, particularly in terms of purifying and evaluating the function of HSCs, precise mechanisms of cell cycle regulation that assign self-renewal or differentiation to HSCs remain unknown. So, further studies are required to disclose the whole feature of cell cycle regulation in HSCs. These studies would undoubtfully bring about useful information to establish therapeutic strategies for *ex vivo* stem cell expansion.

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