

Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells

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Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) niche in a noncycling state and enter the cell cycle at long intervals. However, little is known about inter- and intracellular signaling mechanisms underlying this unique property of HSCs. Here, we show that lipid raft clustering is a key event in the regulation of HSC dormancy. Freshly isolated HSCs from the BM niche lack lipid raft clustering, exhibit repression of the AKT–FOXO signaling pathway, and express abundant p57^{Kip2} cyclin-dependent kinase inhibitor. Lipid raft clustering induced by cytokines is essential for HSC re-entry into the cell cycle. Conversely, inhibition of lipid raft clustering caused sustained nuclear accumulation of FOXO transcription factors and induced HSC hibernation *ex vivo*. These data establish a critical role for lipid rafts in regulating the cell cycle, the survival, and the entry into apoptosis of HSCs and uncover a striking similarity in HSC hibernation and *Caenorhabditis elegans* dauer formation.

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Introduction

Hematopoietic stem cells (HSCs) are clonogenic cells capable of both self-renewal and multilineage differentiation. In adult mouse bone marrow (BM), HSCs reside in the so-called ‘stem-cell niche’ in close contact with supporting cells like osteoblasts and vascular endothelium (Calvi *et al*, 2003; Zhang *et al*, 2003; Kiel *et al*, 2005). The fate of HSCs is determined by signals from their niche through cell-surface or secreted molecules (Fuchs *et al*, 2004; Taichman, 2005). Most of the HSCs are nondividing—in the G₀ phase—in the BM

niche. However, we and others have demonstrated that HSCs are recruited into the cell cycle at long intervals, on average every 1–2 months in analyses using long-term bromodeoxyuridine incorporation (Cheshier *et al*, 1999; Sudo *et al*, 2000). This state of long-term inactivity in HSCs calls to mind hibernation in mammals or dauer formation in *Caenorhabditis elegans*. The capacity to enter and to leave a hibernation-like state is one of the properties necessary for ‘stemness’ and is of critical biological importance in preventing premature HSC exhaustion under conditions of hematopoietic stress (Cheng *et al*, 2001). Several molecular mechanisms that determine HSC quiescence in the niche have been proposed. Molecules that induce or maintain HSC hibernation include the HSC-intrinsic cell cycle regulator p21 and the niche signal agents transforming growth factor- β (TGF- β) (Fortunel *et al*, 2000), N-cadherin (Zhang *et al*, 2003), the Notch ligand jagged-1 (Calvi *et al*, 2003), and the receptor tyrosine kinase Tie-2 ligand Angiopoietin-1 (Ang-1) (Arai *et al*, 2004). However, the precise molecular mechanisms underlying HSC hibernation remain largely elusive.

By contrast, involvement of the DAF-2-DAF-16 signaling pathway in the induction of dauer formation in *C. elegans* is well characterized. Dauer formation is an adaptation for long-term survival and resistance to harsh environmental conditions, such as temperature stress and starvation. DAF-2, an insulin/IGF-1 receptor, activates AGE-1, a phosphatidylinositol-3 kinase (PI3K) that leads to activation of Akt-1 and Akt-2. These phosphorylate DAF-16, a forkhead transcription factor, and negatively regulate its transcriptional activity by excluding DAF-16 from the nucleus (Burgering and Kops, 2002). Under adverse conditions, signaling through the DAF-2 pathway is reduced. DAF-16 then accumulates in the nucleus and activates transcription of a dauer larval gene program. The same pathway is important for hibernation in the 13-lined ground squirrel (Cai *et al*, 2004a, b).

Lipid raft microdomains are cholesterol- and glycosphingolipid-enriched patches in the plasma membrane into which various functional molecules are distributed. Interactive encounters among raft-resident membrane proteins are facilitated by comparison with interactions of raft proteins with nonraft proteins; the latter encounters are limited by segregation of proteins into different membrane phases. Lipid rafts thus act as platforms for cellular functions that include cytokine signaling, membrane trafficking, and cytoskeleton organization. The physiological role of lipid rafts is well characterized in T-cell receptor (TCR) signaling at the ‘immunological synapse’ induced by TCR antigen recognition at the T-cell: antigen-presenting-cell junction. TCR engagement induces clustering of lipid rafts and concentrates activated TCR and downstream signal transducers within lipid rafts. As larger rafts have greater potential for concentration of transducers and for exclusion of negative regulators, lipid raft size

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controls TCR signaling and functional outcomes (Miceli *et al*, 2001). In this regard, lipid raft regulation is a key component of the signal transduction pathway, modulating signal intensity. Nothing is known about lipid rafts in HSCs.

Despite the crucial role of HSCs in regenerative medicine, our knowledge of their physical characteristics and the molecular machineries operating remains limited because HSCs in BM are few. We have established a very efficient method for HSC purification using fluorescence-activated cell sorting (FACS) and have prospectively identified HSCs in adult mouse BM. Mouse BM HSCs are exclusively enriched in CD34⁻ c-Kit⁺ Sca-1⁺ lineage marker-negative (Lin⁻) (CD34⁻KSL) cells, a population representing 0.004% of BM mononuclear cells, whereas CD34⁺KSL cells are progenitors with short-term repopulating capacity (Osawa *et al*, 1996). Using CD34⁻KSL cells, we have succeeded in clonal analyses of HSCs both *in vitro* and *in vivo* (Ema *et al*, 2000, 2005; Takano *et al*, 2004). In the present study, using highly purified HSCs and single-cell immunostaining, we identified the signaling cascade that determines HSC entry into hibernation. As with mammalian hibernation and *C. elegans* dauer formation, the PI3K–Akt–FOXO pathway appeared tightly linked with HSC entry into dormancy. Lipid raft clustering induced by cytokine stimulation activated this pathway and was essential in promoting division by both HSCs and progenitor cells. Inhibition of lipid raft clustering suppressed the PI3K–Akt–FOXO pathway and induced hibernation in HSCs *ex vivo*. It also caused apoptotic death of progenitor cells even in the presence of cytokines.

Results

Lipid raft status in hibernating and cycling HSCs

HSCs in mouse BM are highly enriched in CD34⁻KSL cells. Approximately 40% of freshly isolated single CD34⁻KSL cells exhibit long-term repopulation (LTR) of hematopoiesis in lethally irradiated recipient mice (Takano *et al*, 2004). To evaluate the cell cycle status of BM HSCs, Ki-67 expression was directly analyzed in CD34⁻KSL HSCs and CD34⁺KSL progenitor cells. The Ki-67 antigen is a nuclear protein expressed exclusively in proliferating cells during all active parts of the cell cycle and is not expressed by dormant cells. As expected, most CD34⁻KSL cells (93.6%) did not express Ki-67, while most CD34⁺KSL progenitors did (Figure 1A). Furthermore, most CD34⁻KSL cells (94.5%) scarcely incorporated Pyronin Y, suggesting that they are in the G₀ phase of the cell cycle (Figure 1A). These data confirmed dormancy in HSCs as previously demonstrated (Cheshier *et al*, 1999; Sudo *et al*, 2000; Arai *et al*, 2004).

The signaling pathway that induces HSC quiescence remains elusive. HSC quiescence is reminiscent of mammalian entry into hibernation and *C. elegans* dauer formation. This similarity prompted us to examine the PI3K–Akt–FOXO signaling pathway that regulates these unique states of adaptation to harsh environmental conditions. FOXO1 (FKHR), FOXO3a (FKHRL), and FOXO4 (AFX) are mammalian orthologues of *C. elegans* DAF-16 forkhead transcription factor. In freshly isolated CD34⁻KSL HSCs, phosphorylated Akt, an active form of Akt, was not detected at all, and one of its downstream targets, FOXO3a, accumulated in the nucleus. In contrast, in most CD34⁺KSL progenitor cells, Akt was highly activated, while FOXO3a was largely excluded from

the nucleus and restricted to the cytoplasm (Figure 1B and Supplementary Figure S1). Another orthologue of DAF-16, FOXO1, behaved like FOXO3a (data not shown). Signals mediated by receptor tyrosine kinases and cytokine receptors activate the PI3K–Akt pathway, and thereby negatively regulate FOXO activity (Burgering and Kops, 2002). To evaluate the degree of cytokine signals activated in HSCs, we next examined the localization of c-Kit, an HSC cell-membrane receptor tyrosine kinase, relative to lipid rafts. Lipid raft distribution was assessed by using cholera toxin subunit B (CTxB) to label endogenous GM1 ganglioside, a component of lipid rafts. On most (92%) of the freshly isolated CD34⁻KSL HSCs, both c-Kit and lipid rafts were diffusely distributed. On all CD34⁺KSL progenitor cells, both c-Kit and lipid rafts were polarized and were largely co-localized with one another, indicating that c-Kit was condensed in the lipid raft cluster (Figure 1B and Supplementary Figure S2). Stimulation of HSCs by stem cell factor (SCF) and thrombopoietin (TPO), either independently or in combination, induced clustering within agglomerated lipid rafts of both c-Kit (Figure 2A) and c-Mpl (data not shown), the receptors for SCF and TPO, respectively. These data indicate that cytokine signals concentrate activated receptors together with inactive ones to augment signaling within the lipid raft cluster. As observed in freshly isolated CD34⁺KSL progenitor cells, cytokine stimulation of CD34⁻KSL HSCs induced activation of Akt (Figure 2B and Supplementary Figure S1). It also induced exclusion of FOXO3a from the nucleus to the cytoplasm. Although FOXO3a largely remained in the nucleus at 30 min after cytokine stimulation (Figure 2B), it was completely excluded to the cytoplasm at 24 h after cytokine stimulation (Figure 2B). These data demonstrate a tight correlation of cell cycle status with lipid raft status as well as with activity of the PI3K–Akt–FOXO pathway.

Inhibition of lipid raft clustering modulates cytokine signals in HSCs

The striking contrast in lipid raft and cell cycle status between CD34⁻KSL HSCs and CD34⁺KSL progenitor cells evoked the possibility that lipid rafts regulate the cell cycle status of HSCs by modulating cytokine signals. To address this, we inhibited lipid raft clustering in response to cytokine stimulation by depleting plasma membrane cholesterol with β -cyclodextrin (M β CD). Pretreatment of freshly isolated CD34⁻KSL HSCs with M β CD inhibited lipid raft clustering in 81.2% of the cells tested (Figure 3A). The downstream signals that activate Akt also stayed repressed, leaving FOXO3a in the nucleus (Figure 3A and Supplementary Figure S1). M β CD pretreatment did not inhibit the phosphorylation of tyrosine residues on c-Kit in response to SCF, indicating that the inhibition of lipid raft clustering does not interfere with cytokine receptor activity (data not shown). Treatment of freshly isolated CD34⁺KSL progenitor cells with M β CD showed little effect on preformed lipid raft clustering but it caused inactivation of Akt and subsequent nuclear translocation of Foxo3a (data not shown). Even in hibernating HSCs, D-type G₁ cyclins, *cyclin D1*, *cyclin D2*, and *cyclin D3*, are abundantly expressed (Iwama *et al*, unpublished data). Intriguingly, cyclin D1, D2, and D3, localized exclusively in the cytoplasm in HSCs, were immediately translocated into the nucleus after cytokine stimulation (Figure 3B). Pretreatment of freshly isolated CD34⁻KSL HSCs with M β CD again inhibited cytokine-

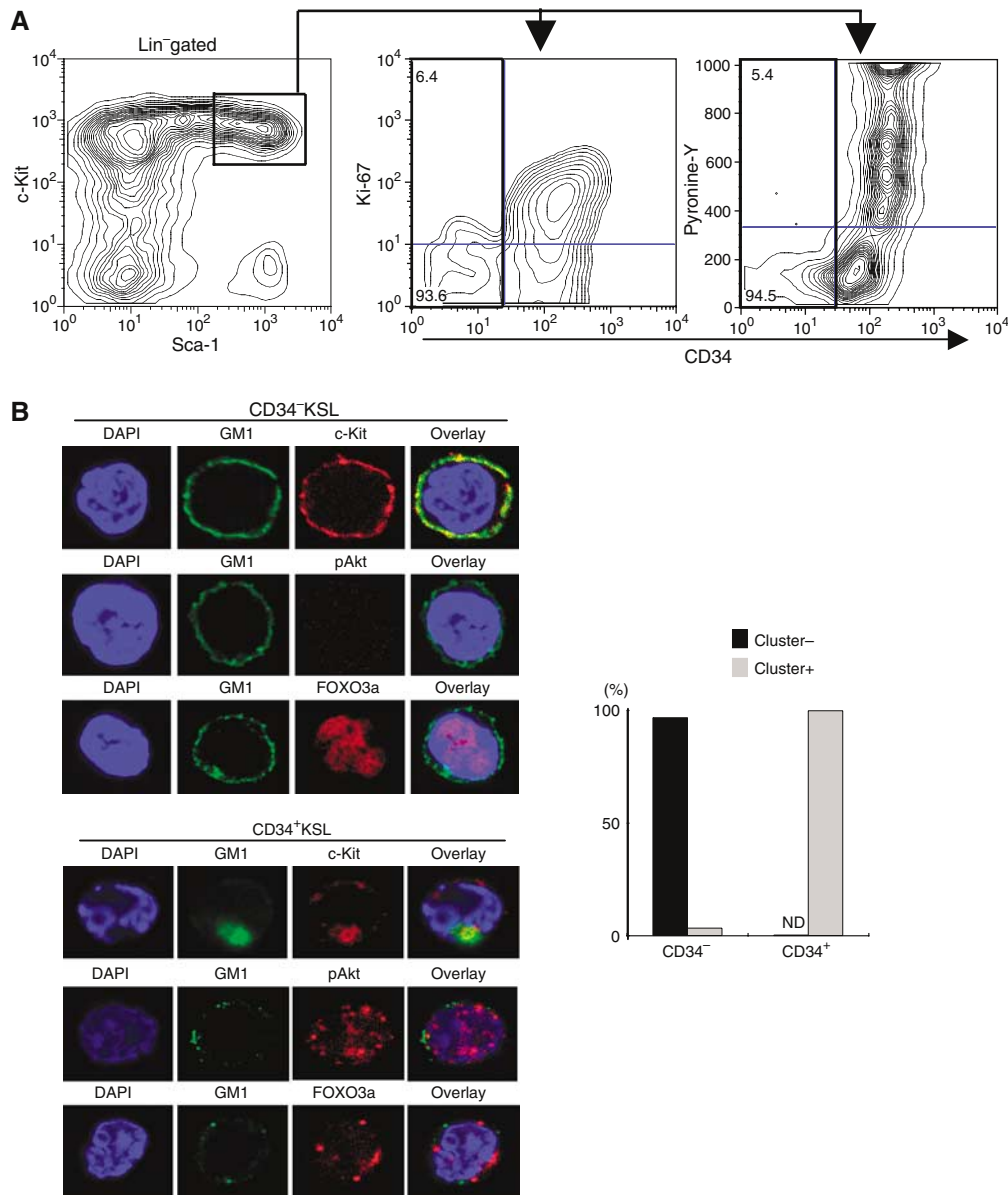


Figure 1 Hibernating HSCs present a striking contrast to cycling progenitors in their lipid raft organization and cytokine signaling. **(A)** Cell cycle status of hematopoietic stem and progenitor cells. Freshly isolated BM KSL cells were analyzed for their expression of CD34 and Ki-67 or CD34 expression and Pyronin Y incorporation on a FACS Vantage. **(B)** Lipid raft organization and cytokine signaling in HSCs. Freshly isolated CD34⁻KSL HSCs and CD34⁺KSL hematopoietic progenitor cells were stained with DAPI (blue) and with an anti-c-Kit, an anti-phospho-Akt, or an anti-FOXO3a antibody (red). Lipid raft distribution was assessed by detecting GM1 ganglioside, a component of lipid rafts, with FITC-conjugated CTB, which marks endogenous GM1 ganglioside (green). Proportions of cells that showed lipid raft clustering or cluster formation are indicated as gray bars (Cluster+) (right panel).

induced nuclear translocation of these cyclins (Figure 3B). On the other hand, cyclin D1, D2, and D3 were present largely in the nucleus in CD34⁺KSL progenitor cells. All these phenomena were reproducible (data not shown) using inhibitors of lipid raft aggregation other than MβCD, such as cytochalasin D, SU6656, and LY294002, which block, respectively, actin polymerization, Src-family kinases, and PI3-K.

HSC hibernation is controlled in part by cyclin-dependent kinase inhibitors (CDKIs). Among *Cip/Kip* gene products (p21, p27, and p57), p21, a late G₁-phase CDKI, is of critical biological importance in preventing premature HSC exhaustion (Cheng *et al*, 2000a), while p27, another late G₁-phase CDKI, regulates proliferation and pool size of hematopoietic progenitor cells rather than of HSCs (Cheng *et al*, 2000b). No

role for p57, another G₁-phase CDKI, has been identified in HSCs. RT-PCR analysis of primitive hematopoietic cell fractions unexpectedly unveiled specific expression of p57 in CD34⁻KSL HSCs (Figure 3C). Immunocytological analysis confirmed abundant expression of p57 in CD34⁻KSL HSCs and drastic downregulation of p57 expression in CD34⁺KSL progenitor cells (Figure 3C). The vast majority of p57 lay in the cytoplasm, like cyclin D1. After cytokine stimulation, p57 immediately disappeared from HSCs. In the presence of MβCD, however, p57 remained in HSC cytoplasm, as did cyclin D1 (Figure 3C). Although p21 and p27 are known direct transcriptional targets for FOXO1, FOXO3a, and FOXO4 (Medema *et al*, 2000; Seoane *et al*, 2004), expression of p27 in CD34⁻KSL HSCs was lower than that in lineage marker-

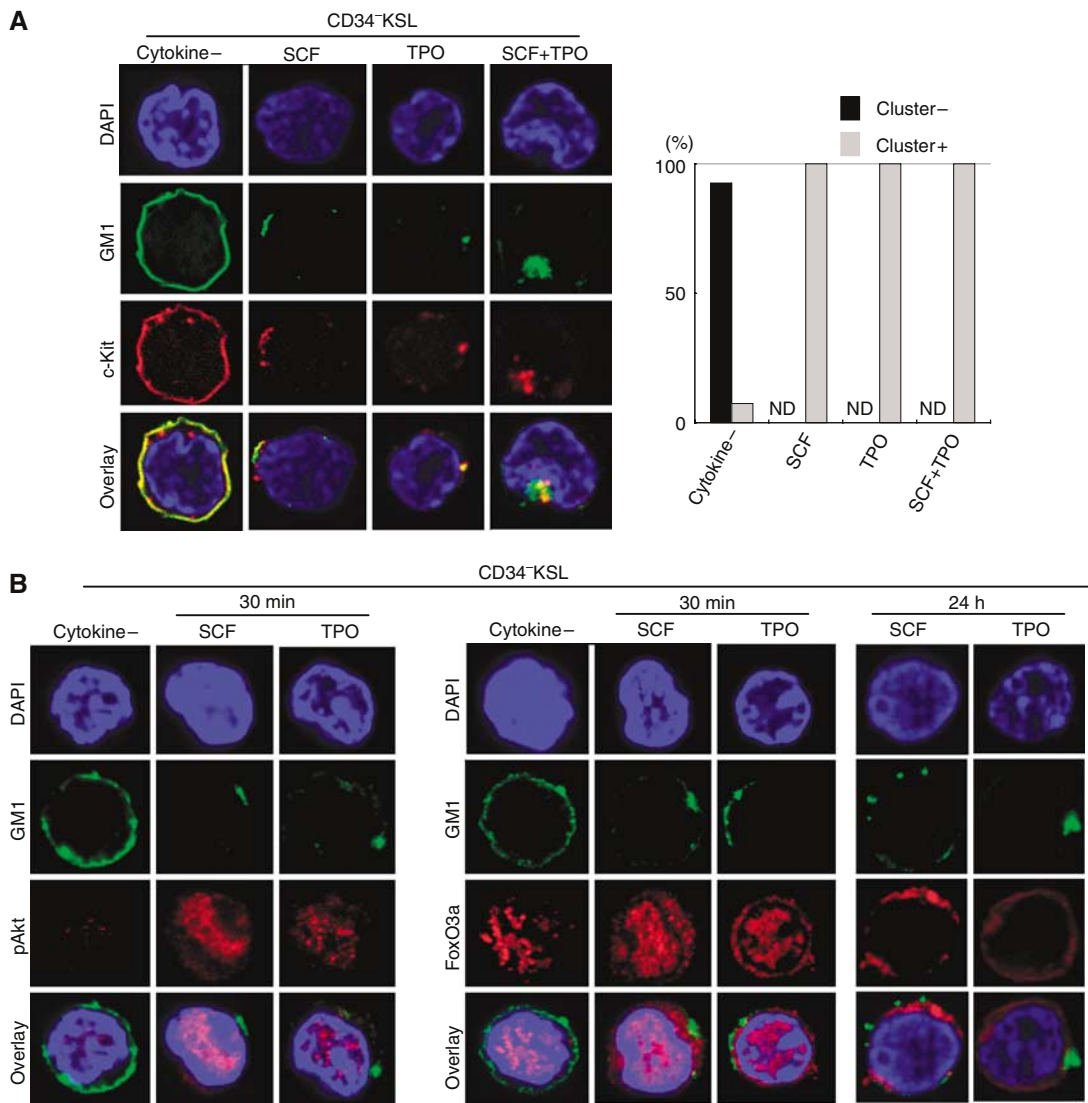


Figure 2 Cytokine stimulation induces lipid raft clustering and activation of the Akt-FOXO signaling pathway in HSCs. Freshly isolated CD34⁺KSL HSCs were incubated for 30 min in the presence or absence of cytokines (SCF and/or TPO) and were stained with DAPI (blue), CTB (green), and an anti-c-Kit (red) (A), an anti-phospho-Akt (red), or an anti-FOXO3a antibody (red) (B). Proportions of CD34⁺KSL HSCs that showed lipid raft cluster formation in response to the indicated cytokines are depicted as gray bars (Cluster+) (A: right panel).

negative (Lin⁻) progenitor fraction cells, and immunocytochemical analyses detected only weak p27 expression in CD34⁺KSL HSC cytoplasm (data not shown). Nor was expression of p21 in CD34⁺KSL HSCs significant. These data indicate a major role of p57 as a specific CDKI that binds to and suppresses the activity of the cyclin D/CDK complex in HSCs.

Modulation of cytokine signals by inhibiting lipid raft clustering induces HSC hibernation *ex vivo*

To understand the biological meaning of lipid raft status for HSC proliferation, M β CD was added to single CD34⁺KSL HSC cultures in the presence of SCF and TPO. High concentrations of M β CD caused cell arrest and apoptotic cell death (data not shown). However, an optimal dose of M β CD (1 mM) efficiently inhibited division of single HSCs with minimal apoptotic death; single HSCs stayed alive for up to 5–10 days (Figure 4A). During this observation period, 93% of single HSCs did not undergo cell division: They remained single cells, without apoptosis (data not shown). By contrast,

CD34⁺KSL progenitor cells underwent apoptotic death by day 2 under the same conditions (Figure 4A). Without cytokines, both CD34⁺KSL HSCs and CD34⁺KSL progenitors died within 24 h (data not shown). These data indicate that lipid raft clustering in response to cytokine stimulation is crucial for the proliferation of both HSCs and progenitors. They also indicate that cytokine signals muted by nonclustering of lipid rafts keep HSCs dormant without inducing apoptosis. Such signals, however, cannot secure survival of progenitor cells.

We next examined whether HSCs treated with both cytokines and a lipid raft aggregation inhibitor are in a hibernating state, *viz.*, whether or not such HSCs can re-enter the cell cycle and retain a full range of HSC functions. We first incubated single HSCs in the presence of SCF, TPO, and 1 mM M β CD. After varying culture periods, surviving single HSCs that had not divided were allowed to form colonies by replacing culture medium with an optimal medium supplemented with SCF, TPO, IL-3, and EPO. Surprisingly, almost

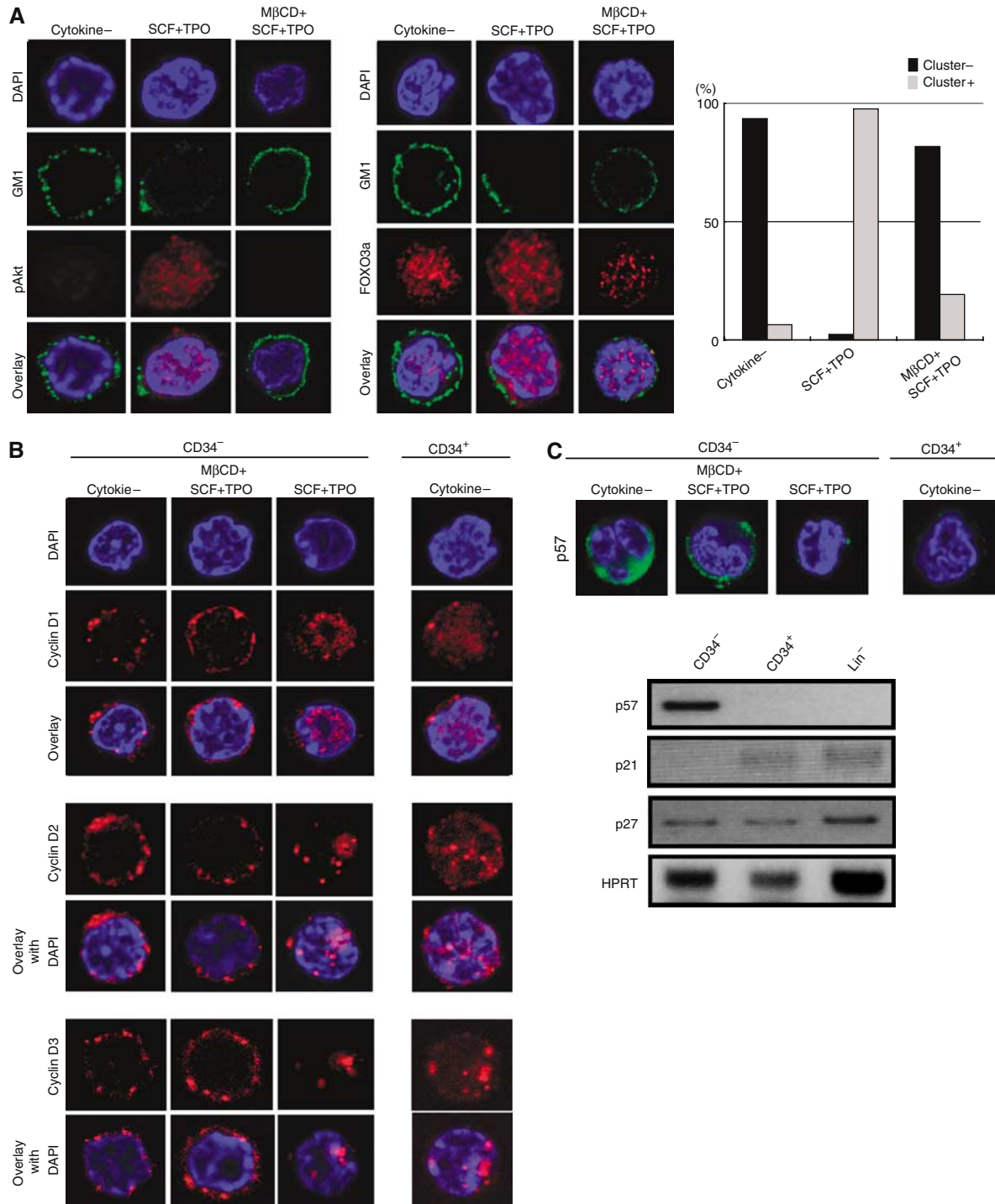


Figure 3 Inhibition of lipid raft clustering attenuates cytokine signals and maintains p57 expression in HSCs. (A) Inhibition of cytokine-induced lipid raft clustering attenuates repression of the Akt-FOXO pathway. Freshly isolated CD34⁻ KSL HSCs were incubated for 30 min in the presence or absence of SCF and TPO (S + T and Cyto-, respectively), or were preincubated with MβCD for 30 min and further incubated in the presence of SCF and TPO for 30 min (MβCD + S + T). The cells were stained with DAPI (blue), CTB (green), and either an anti-phospho-Akt (red) or an anti-FOXO3a antibody (red). The proportions of CD34⁻ KSL HSCs that showed lipid raft cluster formation are indicated as gray bars (Cluster +) (right panel). (B) Subcellular localization of cyclin D1, cyclin D2 and cyclin D3 in HSCs. Freshly isolated CD34⁻ KSL HSCs (CD34⁻) and CD34⁺ KSL hematopoietic progenitor cells (CD34⁺) were incubated for 30 min in the presence or absence of SCF and TPO (S + T and Cyto-, respectively) or were preincubated with MβCD for 30 min and further incubated in the presence of SCF and TPO for another 30 min (MβCD + S + T). The cells were stained with DAPI (blue) and with an anti-cyclin D1 antibody (red). (C) Subcellular localization of p57 in HSCs. Freshly isolated CD34⁻ KSL HSCs (CD34⁻) were preincubated with and without MβCD for 30 min and further incubated in the presence of SCF and TPO for 12 h (MβCD + S + T and S + T, respectively). The incubated cells, together with freshly isolated CD34⁻ KSL HSCs (CD34⁻, Cyto-) and CD34⁺ KSL hematopoietic progenitor cells (CD34⁺, Cyto-), were stained with DAPI (blue) and with an anti-p57 antibody (green) (upper panel). mRNA expression for mouse *Cip/Kip* genes is presented (lower panel). Cells analyzed are BM CD34⁻ KSL HSCs (CD34⁻), CD34⁺ KSL progenitors (CD34⁺), and Lineage marker⁻ cells (Lin⁻).

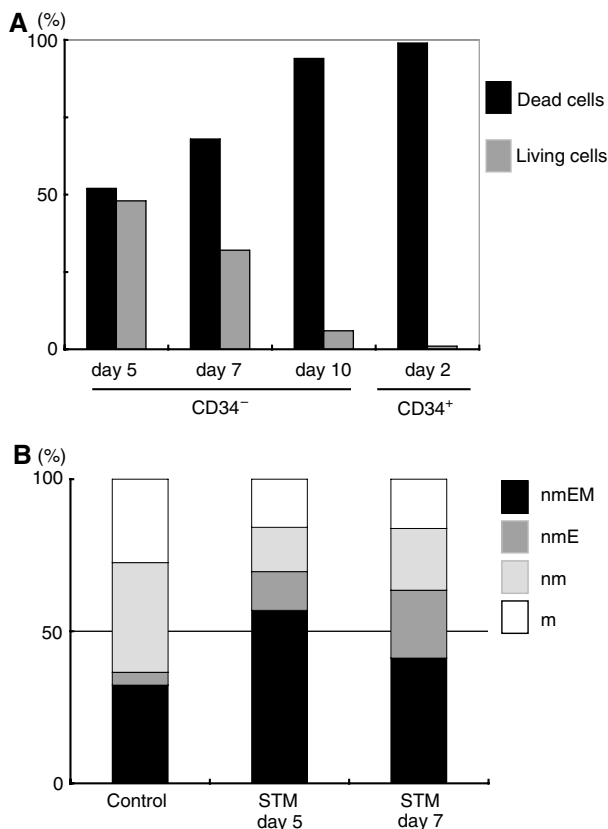


Figure 4 Lipid raft reorganization is essential for hibernating HSCs to re-enter the cell cycle. (A) Freshly isolated CD34⁻ KSL HSCs (CD34⁻) and CD34⁺ KSL hematopoietic progenitor cells (CD34⁺) were sorted clonally into 96-well micro-titer plates and incubated in the presence of SCF, TPO, and 1 mM MβCD (STM). After the indicated culture periods, cell viability and cell numbers were assessed under an inverted microscope. (B) Freshly isolated CD34⁻ KSL HSCs (CD34⁻) were sorted clonally into 96-well micro-titer plates and incubated in the presence of SCF, TPO, and 1 mM MβCD (STM). At the indicated times, surviving single HSCs that had not divided were selected. Culture medium was replaced with one supplemented with SCF, TPO, IL-3, and EPO, permitting colony formation. After 14 subsequent days of culture, the colonies were recovered for morphological examination. As a control, freshly isolated CD34⁻ KSL HSCs were sorted clonally into 96-well micro-titer plates supplemented with SCF, TPO, IL-3, and EPO and cultured for 14 days. Proportions of colony types are depicted: n, neutrophils; m, macrophages; E, erythroblasts; M, megakaryocytes.

half of single HSCs survived up to 5 days in the presence of SCF, TPO, and MβCD (Figure 4A). At day 5 after the change in culture medium, 66% of single HSCs gave rise to colonies (data not shown), and 57% of colonies were neutrophil/macrophage/Erythroblast/Megakaryocyte (nmEM) colonies, derived from colony-forming units-nmEM (CFU-nmEM) with multipotency, that is, a full range of differentiation capacity along myeloid lineages (Figure 4B). Thus, 37.6% of surviving single HSCs could retrospectively be inferred to have been CFU-nmEM. Even after 7 days of culture, most single HSCs retained multipotency (Figure 4A and B). Relative enrichment of the most primitive CFU-nmEM was obtained during CD34⁻ KSL HSC culture in the presence of MβCD compared with the fresh CD34⁻ KSL control. This finding suggests that even the CD34⁻ KSL cells are heterogeneous and less primitive CD34⁻ KSL cells cannot survive in a hibernation-like state and were excluded by apoptosis during MβCD culture like

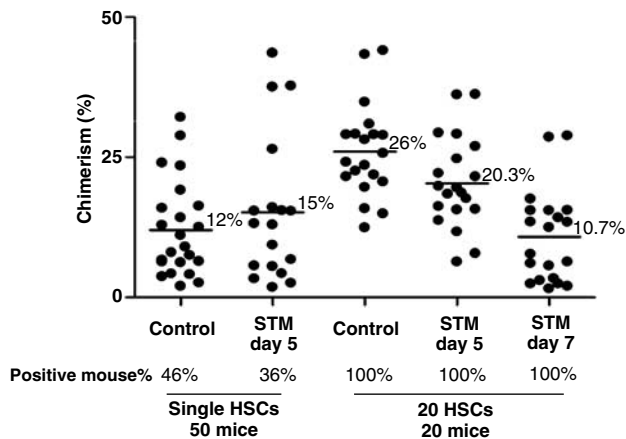


Figure 5 Inhibition of lipid raft clustering attenuates cytokine signals and induces HSC hibernation *ex vivo*. Freshly isolated CD34⁻ KSL HSCs were sorted clonally into 96-well micro-titer plates and were incubated in the presence of SCF, TPO, and 1 mM MβCD (STM). At the indicated times, surviving single HSCs that had not divided during 5- to 7-day culture were selected. Single HSCs or pools of 20 single HSCs (B6-Ly5.1) were mixed with 'competitor cells from B6-Ly5.1 XLy5.2 F1 mice and injected into lethally irradiated B6-Ly5.2 recipient mice. As a control, freshly isolated single HSCs or 20-HSC pools were similarly transplanted into recipient mice. Percentage chimerism of donor cells in recipient mice 12 weeks after transplantation is plotted as dots, with mean values indicated as bars. Recipient mice with donor cell chimerism >1.0% for myeloid and for B- and T-lymphoid lineages were considered multilineage-reconstituted (positive mice).

CD34⁺ KSL progenitor cells. Other lipid raft inhibitors could not be employed in this assay; this was ascribed to cytotoxicity (data not shown). To obtain additional direct evidence of HSC activity, we performed competitive hematopoiesis repopulation assays *in vivo*. We again selected single HSCs that had not divided during 5- to 7-day clonal single-cell culture in the presence of SCF, TPO, and 1 mM MβCD. Single HSCs or pools comprising 20 individual HSCs were transplanted into lethally irradiated recipient mice. As a control, freshly isolated single HSCs or pools comprising 20 individual HSCs were similarly transplanted. Comparable proportions of cultured and freshly isolated hibernating HSCs exhibited LTR activity (freshly isolated controls, 46%; cultured cells, 36%); establishment of chimerism also was comparable (Figure 5). The proportion of LTR HSCs among surviving cultured single HSCs at day 5 (36%) corresponded well with that of CFU-nmEM (37.6%). All recipient mice infused with pools of 20 freshly isolated HSCs showed donor cell repopulation. So did those infused with pools of 20 cultured single HSCs, although established chimerism declined in proportion to the length of HSC culture. All these data strongly support the proposition that inhibition of lipid raft clustering modulates cytokine signals and induces hibernation in HSCs *ex vivo* without affecting HSC capacity to self-renew and to differentiate into a full range of hematopoietic cell lineages.

Discussion

Dormancy or hibernation is indispensable for the maintenance of HSCs. It is established solely in the particular HSC BM microenvironment known as the HSC niche. Cell cycle status of HSCs in the niche is precisely regulated by a specific

combination of niche signals. We report an unexpected role of lipid raft organization in the maintenance of HSC hibernation through regulating the PI3K–Akt–FOXO pathway that lies downstream of cytokine signaling.

Unexpected role of lipid rafts in the maintenance of HSC dormancy

As reported for TCR signaling at the ‘immunological synapse’, regulation of lipid rafts is a key component of signal modulation in the signal transduction pathway (Miceli *et al*, 2001). In this study, we clearly demonstrate that freshly isolated CD34⁻KSL HSCs are devoid of lipid raft clustering or polarization, while in CD34⁺KSL progenitor cells, both c-Kit and lipid rafts are already polarized, with c-Kit condensed in the lipid raft cluster (Figure 1B). Cytokine stimulation induces lipid raft clustering in HSCs and augments downstream signaling pathways, including PI3K–Akt–FOXO. Of interest is that TPO stimulation induced clustering of c-Kit and SCF stimulation induced clustering of c-Mpl in the lipid rafts. These findings represent the recruitment and concentration of signal transducers for efficient and versatile transduction of signals utilizing lipid rafts. Lipid raft reorganization appeared indispensable for HSC emergence from hibernation and re-entry into the cell cycle. Conversely, inhibition of lipid raft clustering attenuated cytokine signals and induced HSC hibernation *ex vivo*.

In the presence of cytokines as well as M β CD, the vast majority of freshly isolated CD34⁻KSL HSCs stayed in G₀. Some of them survived up to day 10 of culture without cell division, although they gradually underwent apoptotic death (Figure 4A). Surviving cells surprisingly retained HSC characteristics, that is, self-renewal capacity and multipotency, and contributed to long-term hematopoiesis reconstitution *in vivo* (Figure 5). It should be noted that this is the first evidence for successful manipulation of HSC hibernation *ex vivo*. We infer that nonclustered lipid raft microdomains finely tune cytokine signals and mediate them toward suitability for HSC survival in the hibernating state. This *ex vivo* observation could also be applied to HSCs in the BM niche. HSCs are exposed to a variety of secreted and membrane-bound growth factors in the niche. Among HSC-active cytokines, SCF, the ligand of c-Kit (KitL), conveys signals that modulate proliferation, survival, and adhesion of HSCs. BM stromal cells express membrane-bound KitL (mKitL). In addition, a number of soluble cytokines, such as TPO, that mediate HSC growth signals should reach and act on HSCs in the niche. Nonetheless, our findings clearly demonstrate that lipid raft reorganization is strictly inhibited in HSCs in the niche, suggesting that some niche signals inhibit lipid raft reorganization to maintain HSC hibernation. These findings support a novel model in which HSC fate, that is, hibernation or cell cycle re-entry, largely depends on lipid raft regulation.

Our findings also may explain in part a critical aspect of HSC mobilization from niche dormancy to reconstitute the HSC and progenitor cell pools (Heissig *et al*, 2002). Soluble KitL, which is cleaved from mKitL under conditions of hematopoietic stress, mediates this process by allowing HSCs to translocate to a permissive vascular niche (Tajima *et al*, 1998; Heissig *et al*, 2002). Perhaps signals inhibiting lipid raft reorganization are lacking in this permissive niche.

The molecular machineries that determine HSC hibernation

The PI3K–Akt–FOXO pathway controls cell cycle, apoptosis, metabolism, and longevity. Its crucial role in cell cycle and death control has been well characterized in *C. elegans* and is now also being tested in mammalian systems. As demonstrated in *C. elegans* dauer formation and in mammalian hibernation, Akt-regulated FOXO family members block cell-cycle progression at phase G₁ (Medema *et al*, 2000), and protect human dormant cells from oxidative stress while antagonizing apoptosis (Kops *et al*, 2002). We have extended these observations to HSCs in the BM niche. Hibernating HSCs isolated from this niche showed repressed Akt activity and nuclear accumulation of FOXO3a (Figure 1B). Cytokine stimulation of freshly harvested HSCs induced lipid raft clustering, drastic activation of Akt, and exclusion of FOXO3a from the nucleus (Figure 2), while M β CD, a lipid raft inhibitor, was highly effective in attenuating cytokine signals that induce Akt activation (Figure 3A). It is intriguing that the PI3K–Akt–FOXO pathway involved in *C. elegans* dauer formation also operates in mammalian hibernation and in dormant HSCs in the BM niche. The tight correlation between lipid raft status and the PI3K–Akt–FOXO pathway indicates that lipid raft reorganization holds the key to precise regulation of the Akt–FOXO pathway in HSCs.

Nuclear accumulation of mammalian orthologues of *C. elegans* DAF-16, FOXO1, FOXO3a, and FOXO4 induces either cell cycle arrest or apoptosis. These proteins upregulate transcription of *p21* and/or *p27* and downregulate protein expression of cyclin D1 and cyclin D2, leading to cell cycle arrest at the G₁ phase in a variety of cells (Medema *et al*, 2000; Seoane *et al*, 2004). Unexpectedly, however, we found that hibernating HSCs predominantly express p57, another member of the Cip/Kip CDKI family, and that p57 presented a striking contrast to p21 and p27 with regard to its specific expression at both mRNA and protein levels in hibernating HSCs (Figure 3C). Moreover, expression of cyclin D1, one of the D-type cyclins that are essential for HSC expansion (Kozar *et al*, 2004), was already high in HSCs (Figure 3B). p57 binds to cyclin D–cdk4/6 complex and inhibits its activity. Interestingly enough, both p57 and cyclin D1 were localized in the cytoplasm, suggesting that they form a complex in HSCs. These findings suggest that in dormant HSCs, cyclin D1 is already available for rapid re-entry into the cell cycle in response to mitogenic signals. However, it is sequestered in the cytoplasm, where it cannot function, with p57 as a fail-safes against cell cycle progression. Although the mechanism that determines cytoplasmic localization of cyclin D1 remains unclear, cytoplasmic localization of D-type G₁ cyclins has been similarly observed in postmitotic nondividing cardiomyocytes and hepatocytes (Jaumot *et al*, 1999; Tamamori-Adachi *et al*, 2003). To test whether p57 is regulated by FOXO transcription factors in HSCs like *p21* and *p27* would be intriguing.

Repressed Akt activity and subsequent nuclear accumulation of FOXO transcription factors can also cause cell death by apoptosis. In the presence of M β CD, most CD34⁻KSL HSCs could survive in a hibernating state, whereas CD34⁺KSL progenitor cells overwhelmingly underwent apoptotic death (Figure 4A). These findings uncover a unique antiapoptotic diathesis of hibernating HSCs. Expression profiling of

pro- and/or antiapoptotic genes (*Bim*, *Bad*, *Noxa*, *Bcl-2*, and *Bcl-xL*) did not detect any significant difference between CD34⁻KSL HSCs and CD34⁺KSL progenitor cells; nor did the absence and presence of MβCD yield any differences. As with *C. elegans* dauer formation and mammalian hibernation, FOXO transcription factors may regulate the genes involved in metabolism and redox control to antagonize apoptosis in dormant HSCs (Kops *et al*, 2002).

Our observations on HSC hibernation and its molecular mechanisms also suggest a novel mechanism for drug resistance in leukemia. In chronic myelogenous leukemia (CML) patients, a specific inhibitor of BCR-ABL, imatinib mesylate, causes apoptotic death of BCR-ABL-transformed primary leukemia cells. Yet, even when imatinib mesylate treatment led to complete cytogenetic remission, the BCR-ABL fusion gene was universally detected in CD34⁺ progenitor cells, indicating persistence of leukemic stem cells (LSC), that is, malignant hematopoietic progenitors (Bhatia *et al*, 2003). Imatinib mesylate treatment of BCR-ABL⁺ leukemic cells represses BCR-ABL kinase activity as well as downstream Akt activity, and induces accumulation of de-repressed FOXO3a in the nucleus (Komatsu *et al*, 2003). De-repressed FOXO3 transcription factors induce apoptosis in progenitor cells, while they induce cell cycle arrest and antagonize apoptotic cell death in HSCs. These observations, viewed in light of our findings, suggest that LSCs with treatment-repressed BCR-ABL activity enter a FOXO-mediated hibernation state in the BM niche and regain BCR-ABL activity after therapy ceases. In effect, antileukemic therapy may activate in LSCs the mechanisms that induce quiescence and maintain hibernation in BM-niche HSCs. To investigate niche signals that inhibit lipid raft reorganization and to manipulate these signals to end LSC hibernation and to induce re-entry into the cell cycle will be important.

Materials and methods

Mice

C57BL/6 (B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) were purchased from Sankyo-Lab Service (Tsukuba, Japan). C57BL/6 Ly5.1 × Ly5.2 F1 mice were bred and maintained in the Animal Research Facility of the Institute of Medical Science, University of Tokyo. Animal care in our laboratory was in accordance with the guidance of Tokyo University for animal and recombinant DNA experiments.

Purification of mouse HSCs and CD34⁺ KSL cells

Mouse CD34⁻KSL HSCs and CD34⁺KSL progenitor cells were purified from BM cells of 2-month-old mice. In brief, low-density cells were isolated on Lymphoprep (1.077 g/ml; Nycomed, Oslo, Norway). The cells were stained with an antibody cocktail consisting of biotinylated anti-Gr-1, -Mac-1, -B220, -CD4, -CD8 and -Ter-119 monoclonal antibodies (PharMingen, San Diego, CA). Lineage-positive cells were depleted with streptavidin (SA)-coupled magnetic beads (M-280; Dinal Biotech, Oslo, Norway). The remaining cells were further stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34, phycoerythrin (PE)-conjugated anti-Sca-1, and allophycocyanin (APC)-conjugated anti-c-Kit antibodies (PharMingen). Biotinylated antibodies were detected with streptavidin-Texas Red (Molecular Probes, Eugene, OR). To detect Ki-67 expression, lineage-depleted cells were stained with FITC-conjugated anti-CD34, PE-Cy5.5-conjugated anti-Sca-1 (PharMingen), and APC-conjugated anti-c-Kit antibodies. The cells were then fixed and permeabilized using a Cell Permeabilization Kit (CALTAG Laboratories, Burlingame, CA) and stained with PE-conjugated anti-Ki-67 antibody (PharMingen). To analyze the cell-cycle status, cells

were incubated with 1 μg/ml Pyronin Y (Sigma-Aldrich, Inc., Saint Louis, Missouri) at 37°C for 45 min. FACS analysis and cell sorting was performed on a FACS Vantage using CELLQuest™ software (Becton Dickinson, Franklin Lakes, NJ) and results were analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunofluorescent staining

Makers and antibodies used are as follows: 4,6-diamidino-2-phenylindole (DAPI), a DNA marker, Alexa-488 or Alexa-647 Cholera toxin B subunit (CTxB), Alexa-647 goat anti-rabbit IgG, goat anti-mouse IgG (Molecular Probes, Inc., Carlsbad, CA), rabbit anti-c-Mpl, rabbit anti-phospho-c-Kit, rabbit anti-phospho-Akt, and rabbit anti-FKHRL1 (FOXO3a), rabbit anti-FKHR (FOXO1) (Upstate Cell Signaling, Charlottesville, VA), mouse anti-cyclin-D1 (Becton Dickinson), mouse anti-cyclin-D2, mouse anti-cyclin-D3 (Sigma-Aldrich, Inc., Saint Louis, Missouri) and rabbit anti-p57 (Santa Cruz Biotechnology, Santa Cruz, CA). CD34⁻ KSL cells were sorted in a serum-free culture-medium drop, supplemented with 20 ng/ml SCF and/or 50 ng/ml TPO, onto slide glasses. The sorted cells were incubated at 37°C for the indicated time periods. After fixation with 2% paraformaldehyde and blocking in 10% goat serum for 1 h at room temperature, cells were incubated with a primary antibody for 12 h at 4°C. The cells were then washed and were incubated with a secondary antibody for 30 min at room temperature. Immunofluorescence was observed with a Leica TCS SP2 AOBs confocal microscope.

Single-cell culture

CD34⁻ KSL cells were clonally deposited into 96-well micro-titer plates containing 200 μl of S-Clone SF-03 (Sanko Junyaku Inc., Tokyo, Japan) supplemented with 5×10^{-5} M 2-β-mercaptoethanol, 10% FCS, and the indicated cytokines (20 ng/ml mouse SCF, 50 ng/ml human TPO, 20 ng/ml mouse IL-3, and 2 U/ml mouse EPO) in the presence or absence of 1 mM MβCD (Junsei, Tokyo, Japan). Survival and division of HSCs were monitored by microscopy. To allow colony formation, single HSCs were cultured in the presence of SCF, TPO, IL-3, and EPO for 11 days. Colonies were recovered, cytopun onto slide glasses, and subjected to May-Grünwald-Giemsa staining for morphological examination.

Competitive repopulation assay

Competitive repopulation assays were performed using the Ly5 system. Single cultured HSCs or pools of single cultured HSCs cells (B6-Ly5.1) were mixed with 2×10^5 bone marrow competitor cells (B6-F1) and were transplanted into B6-Ly5.2 mice irradiated at a dose of 9.5 Gy. After transplantation, peripheral blood cells of the recipients were stained with biotinylated anti-Ly5.1 (A20) and FITC-conjugated anti-Ly5.2. The cells were simultaneously stained with PE-Cy7-conjugated anti-B220 antibody or a mixture of APC-conjugated anti-Mac-1 and -Gr-1 antibodies or a mixture of PE-conjugated anti-CD4 and -CD8 antibodies. Biotinylated antibody was developed with streptavidin Alxa594 (Molecular Probes, Carlsbad, CA). The cells were analyzed on a FACS Vantage. Percentage chimerism was calculated as (% Ly5.1 cells) × 100 / (% Ly5.1 cells + % F1 cells). When percentage chimerism of donor-derived cells was >1.0 for myeloid and for B and T lymphoid lineage reconstitution, recipient mice were considered multilineage reconstituted (positive mice).

RT-PCR

Semiquantitative RT-PCR was carried out using normalized cDNA and quantitative PCR with TaqMan rodent GAPDH control reagent (Perkin-Elmer Applied Biosystems, Foster City, CA) as previously described (Osawa *et al*, 2002). The primer sequences are: *Cip1/p21* sense 5'-TGG AAC TTT GAC TTC GTC ACG-3', antisense 5'-TCT TGC AGA AGA CCA ATC TGC-3'; *Kip1/p27* sense 5'-TCT CAG GCA AAC TCT GAG GAC-3', antisense 5'-TTC GGA GCT GTT TAC GTC TGG-3'; *Kip2/p57* sense 5'-TGA TGA GCT GGG AAC TGA GCC-3', antisense 5'-ACG TCG TTC GAC GCC TTG TTC-3'. Cycling parameters were: Denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s. The amplification proceeded for 38 or 40 cycles. PCR products were separated on an agarose gel and visualized by ethidium bromide staining.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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