Regulation of Hematopoietic Stem Cell and Its Interaction with Stem Cell Niche

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ABSTRACT

Hematopoietic stem cells (HSCs) are responsible for Blood cell production throughout the lifetime of individuals. Interaction of HSCs with their particular microenvironments, known as stem cell niches, is critical for maintaining the stem cell properties, including self-renewal capacity and the ability of differentiation into single or multiple lineages. In the niche, the niche cells produce signaling molecules, extracellular matrix, and cell adhesion molecules, and regulate stem cell fates. Recently, long-term bone marrow (BM) repopulating (LTR) HSCs exist frequently in BM trabecular bone surface, and it was clarified that an osteoblast (OB) is a critical component for sustainment of HSCs. HSCs balance quiescence and cell division in the osteoblastic niche and also maintain the potential for long-term hematopoiesis. Especially, the quiescent state in the cell cycle is thought to be indispensable for the maintenance of hematopoietic stem cells (HSCs). We demonstrate that c-Kit$^+$Sca-1$^+$Lineage$^-$ (KSL) HSCs expressing the receptor tyrosine kinase Tie2 are quiescent and anti-apoptotic, transplantable and comprise a side-population (SP) of HSCs, which contact closely to Angiopoietin-1 (Ang-1), a ligand for Tie2, expressing osteoblasts in the BM niche. Tie2 and Ang-1 are part of a key signaling interaction between HSC and osteoblasts. Tie2 and Ang-1 are expressed in a complementary pattern, and interaction of Tie2 and Ang-1 induced integrin dependent cell adhesion of HSCs to osteoblasts and extracellular matrix. This signaling pathway regulates functional criteria of HSC in the BM niche, including quiescence, anti-cell death and tight adhesion. These observations led us to a novel model in which Ang-1 produced by osteoblasts activates Tie2 on the HSCs and promote tight adhesion of HSCs to the niche, resulting in quiescence and enhanced survival of HSCs.

Key words: Quiescence, Tie2, Angiopoietin-1, N-cadherin, ATM

Tissue stem cells are characterized by their abilities to self-renew and to produce numerous differentiated daughter cells. These two special properties enable stem cells to play a central role in maintaining tissues. The activity of tissue stem cells is crucial for supply the mature cells in normal tissue turnover. Defective functional activity or low turnover of stem cells leads exhaustion of progenitor or mature cells in tissue and is causing in disease. Unregulated and over proliferation of stem cells is the leading cause of cancer. It now clear that the stem cell niche regulates the stem cell specific property including self-renewal, multi-potentiality, and relative quiescence.

The concept of the stem cell niche was first proposed for the human hematopoietic system in the 1970s (Schofield, 1978). A similar concept has also been proposed for the epidermis, intestinal epithelium, nervous system and gonads (Fuchs et al., 2004; Li and Xie, 2005). We hypothesized that cell cycle regulation by the niche is critical for the fate of HSCs. In the niche, signaling molecules, extracellular matrix, and cell adhesion molecules produced by niche cell regulate quiescence, self-renewal, and cell fate decision of the stem cell. Up to the present date, many adult tissue stem cells and their niches, including hematopoietic system, skin epidermis, gastrointestinal epithelium, brain, and lung were identified. There observation led us the understanding of the common property of the tissue stem cells. First, stem cells are relatively quiescent or slow cycling cells in the tissue. Second, stem cells adhere to the supporting cells (niche cells) in the stem cell niche. We found that the Tie2/Ang-1 signaling pathway between HSCs and OBs contributes to quiescence of HSCs in their stem cell niche, resulting in the maintenance of self-renewal ability and protection from stresses (Arai et al., 2004)

Identification of stem cell niche and niche cells in adult BM

A stem cell niche includes three compartments: localized niche cells (supporting cells), the extracellular matrix (ECM), and soluble factors derived from niche cells (Lin, 2002) (Figure 1). A unique feature of HSCs is that they migrate toward the stem cell niche during their ontogeny. During embryogenesis, development of the hematopoietic system occurs at various anatomical sites, including para-aortic splanchnopleural mesoderm (P-Sp)/ aorta-gonad-mesonephros (AGM) region, yolk sac, fetal liver, spleen, and bone marrow. Intraembryonic hematopoietic development may be associated with the major arterial region. Histological studies have demonstrated the presence of clusters of HSCs in close association with, and often adhering to, endothelial cells on the ventral surface (floor) of the aorta (North et al., 2002). It suggests that endothelial cells play as a niche for developing HSCs. In addition, recently two groups reported that the placental labyrinth region is a source of definitive hematopoiesis and plays as a niche for HSCs during midgestation (Gakas et al., 2005; Ottersbach et al., 2005).

OBs, which derived from mesenchymal stem cell, have long been known to play a central role in skeletal

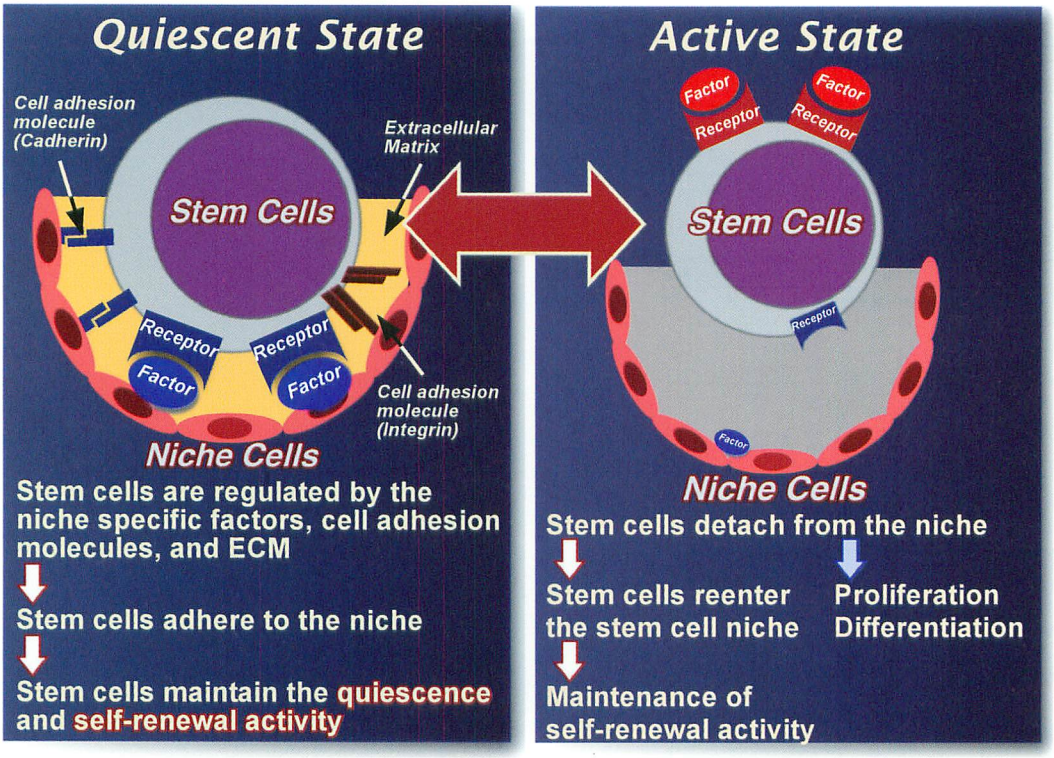

Fig 1. The model of stem cell niche
In the quiescent state, stem cell is regulated by the quiescence inducible factor and receptor complex and adheres to the niche cells, resulting in the maintenance of self-renewal activity and protection from stresses. Once the stem cells are activated by the mitogenic or differentiation factors, stem cell leaves from the niche. Detachment of the stem cell from the niche allows the differentiation and proliferation. This is in turn loss of self-renewal activity. In addition, if activated stem cell reenters in the niche, stem cell comes to quiescent state again and maintains self-renewal activity.

development. OBs also constitute part of the stromal cell support in BM. Taichman and Emerson also reported that OBs are a part of the hematopoietic microenvironment after BM cavity formation (Taichman and Emerson, 1998). Nilsson et al. reported that HSCs were significantly enriched within the endosteal region after BM transplantation (Nilsson et al., 2001).

Recently, two papers reported the identification of a HSC niche (Calvi et al., 2003; Zhang et al., 2003). These reports suggest that OBs are critical regulatory component of adult hematopoiesis. Both groups employed genetic strategies to increase the number of OB population in the trabecular region of the bone. Increasing the number of OBs causes parallel increases in the HSC population, particularly LTR-HSCs, without concomitant increases in other primitive progenitor cells. Such a specific increase in only the LTR-HSC population suggests that a specific niche is functionally enhanced. In addition, Zhang et al. (2003) showed that N-cadherin-positive spindle-shaped OBs (SNO cells) are niche cells, and N-cadherin is asymmetrically localized between HSCs and OBs in the adult BM niche. Other report has addressed the relationship between hematopoiesis and OBs. Visnjic et al. (2004) demonstrated that conditional depletion of OBs using a type I collagen promoter-herpes simplex virus thymidine kinase transgenic mice with ganciclovir treatment led reduction of BM cellularity.

Quiescent HSCs in the adult BM niche

Key features of stem cells in a niche are that they are quiescent and adhere to surrounding niche cells. The quiescent state is thought to be an indispensable property for the long-term maintenance of hematopoietic stem cells (HSCs), and is thought to be an important mechanism for protecting cells from the stress (Cheng et al., 2000). Indeed, it has been reported that HSCs are relatively quiescent when compared to transiently amplifying progenitor cells (Cheshier et al., 1999). We thought that identification of HSCs in quiescent state is viewed as a way to find the stem cell niche. For detection of quiescent HSCs in BM, we used the myelosuppressive model of treatment with 5-FU, a drug that induces apoptosis in cycling cells, and analyzed SP cells in HSCs. SP is a cell fraction weakly or non-labeled with the DNA dye, Hoechst 33342, and is able to enrich the HSCs (Goodell et al., 1996). Moreover, SP cells have been found in several tissue and species, suggesting that SP defines a general property of tissue stem cells (Zhou et al., 2001). We found that the SP cells, but not non-SP cells in HSCs are resistant to BM suppression induced by 5-Fluorouracil (5-FU), suggesting that these cells are quiescent and have anti-apoptotic properties (Figure 2).

To further characterize the cell cycle status of SP cells in the c-Kit$^+$Sca-1$^+$Lineage$^-$ (KSL) fraction, KSL cells were

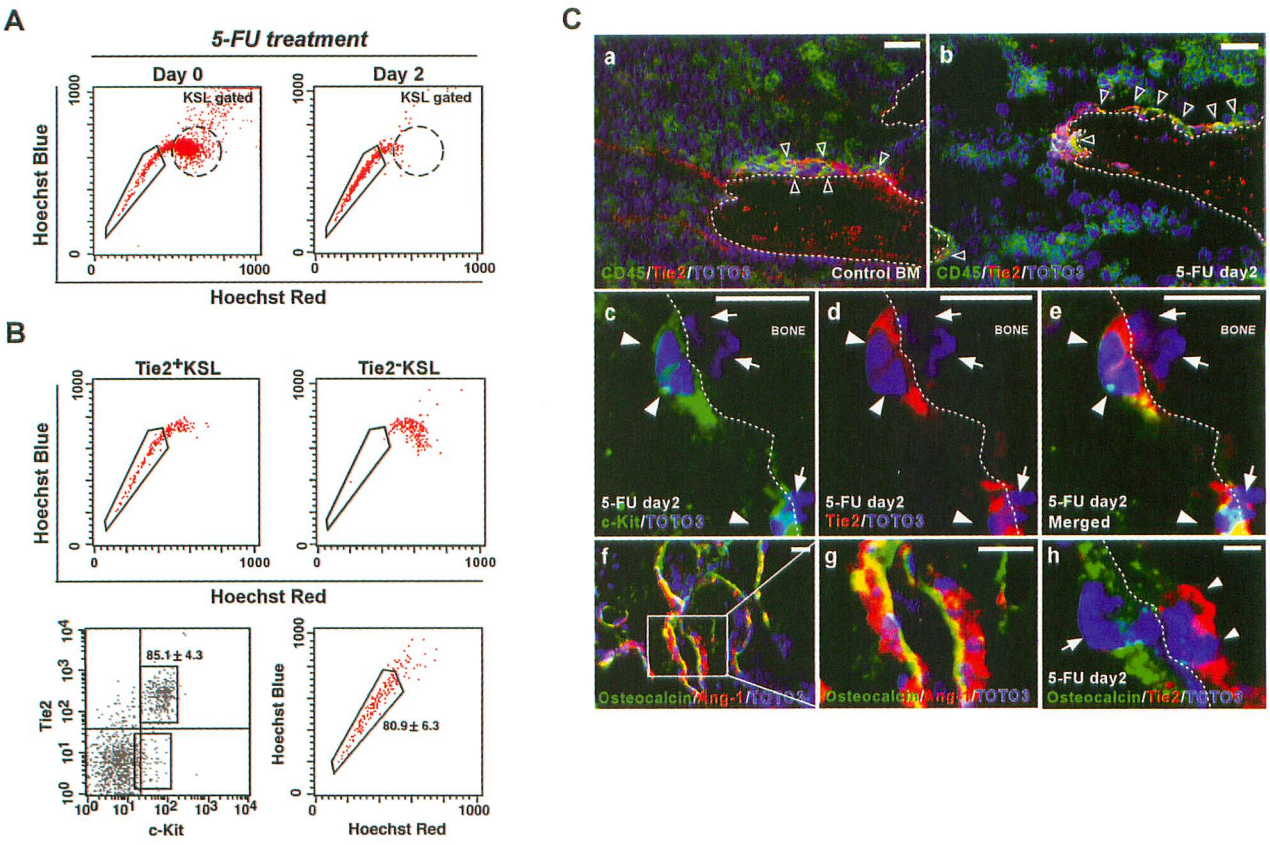

Fig 2. Identification of quiescent HSCs and their niche in adult BM.
(A) Changes in the proportion of SP and non-SP cells in KSL fraction after 5-FU treatment. In control KSL cells, 19.4 ± 0.7 % of cells were in SP, whereas after 2 days of 5-FU treatment the majority of cells in the KSL fraction were SP (70.1 ± 6.2 %), suggesting that KSL-SP cells were in quiescent state. (B) Analysis of the SP cells in $Tie2^+$ and $Tie2^-$ KSL cells. SP cells are specifically enriched in $Tie2^+$ population. (C) Immunohistochemical staining of adult BM. (a) untreated BM. (b-e) are BM section after 2 days of 5-FU treatment. Co-expression of Tie2 and CD45 (a and b). $CD45^+Tie2^+$ cells survived, adhering to the bone surface (open arrowheads). Scale bar, 25 μm. The expression of c-Kit (c) and Tie2 (d). (e) represents the merged image of (c) and (d). $Tie2^+$ cells adhered to bone surface were co-express c-Kit (arrowheads), and adhered to the OBs (arrows). Scale bar, 12.5 μm. The expression of Ang-1 and osteocalcin in BM (f and g). Ang-1 expression was detected in osteocalcin-positive OBs. (g) Higher power view of enclosed boxes in (f). $Tie2^+$ cells (arrowheads) adhered to the $osteocalcin^+$ OBs (arrows) in 5-FU treated BM (h). The dotted lines indicate the margin of the bone surface. Nuclei is labeled with TOTO3.

analyzed by Pyronin Y (PY) staining. It was previously reported that PY^- and PY^+ cells were in G0 and G1 phases of cell cycle, respectively (Huttmann et al., 2001). Expectedly, most KSL-SP cells (92.4 ± 6.7 %) were in G0. Therefore, SP is the most suitable marker for detection of quiescent HSCs. In addition, a proportional increase in the number of cells in G0 among HSCs is observed during mouse development after birth, which correlates closely with the relative increase in SP cells. To identify quiescent HSCs *in situ*, we analyzed specific markers for KSL-SP cells. We found that SP cells were specifically enriched in Tie2 receptor tyrosine kinase expressing KSL cells, and $Tie2^+$KSL cells survived after 5-FU treatment, as well as SP cells (Figure 2). Therefore we analyzed the expression of Tie2 in 5-FU treated BM, and we found that $Tie2^+$ HSCs adhered to OBs at the surface of the trabecular bone (Figure 2), in agreement with previous reports (Calvi et al., 2003; Zhang et al., 2003). Ang-1, a ligand for the Tie2 receptor is mainly produced by OBs, suggesting that Tie2 and Ang-1 are expressed complementarily in the niche (Figure 2). Taken together, our observations support the idea that the niche is a microenvironment reserved for quiescent HSCs. Hence, HSCs may transit between niche and non-niche sites and/or between quiescence and active cell cycling *in vivo*. When this balance is disrupted, such as occurs with $p21^{WAF1/Cip1}$ deficiency, HSCs cannot remain in G0 and long-term repopulating ability is lost (Cheng et al., 2000), indicating that the niche is essential for maintenance of a long-term hematopoietic system.

How does blocking cell division lead to maintenance of stem cell capacity? Every cell division must shorten telomere length in HSCs. Telomeres are composed of the tandem DNA repeats and associated proteins that cap the end of linear chromosomes. Telomeres are maintained by the reverse transcriptase, telomerase. Hematopoietic progenitors have a high replicative potential and telomerase activity to protect the ends of their chromosomes (Greenwood and Landsdrop, 2003). HSCs show telomeric shortening during replicative aging despite expression of telomerase (Greenwood and Landsdrop, 2003). Accelerated telomere erosion reduces the long-term

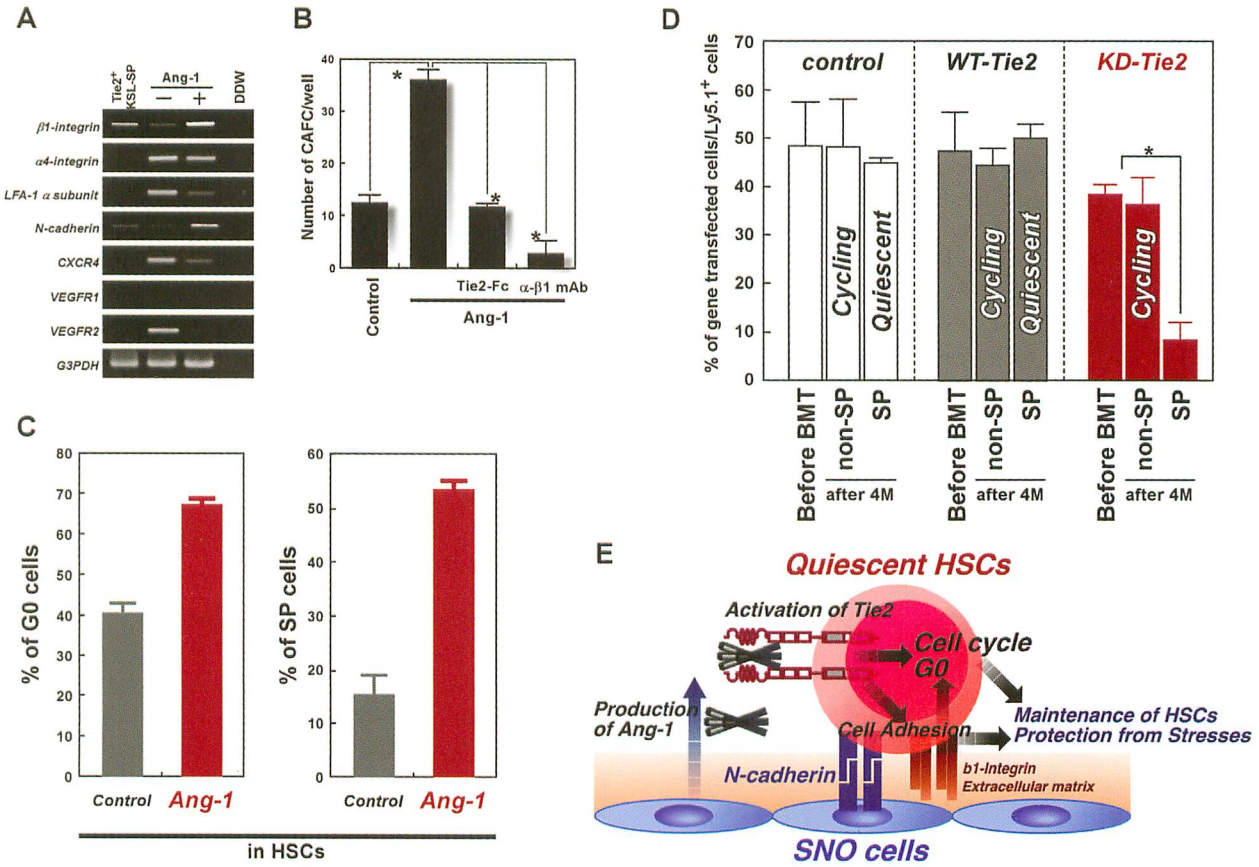

Fig 3. **Role of Tie2/Ang-1 signaling in the regulation of quiescent HSCs**
(A) Tie2+KSL-SP cells were cultured with or without Ang-1. Ang-1 induced the up-regulation of b1-integrin and N-cadherin (B) Cocultivation of Tie2+KSL-SP cells with OP9 stromal cells. Cobblestone formation was induced by the addition of Ang-1. The effect of Ang-1 was inhibited by Tie2-Fc or anti-β1-integrin antibody. (C) Overexpression of Ang-1 in hematopoietic cells. Ang-1 increased the frequency of quiescent cells and SP cells in HSCs. (D) Overexpression of WT and KD-Tie2 in HSCs. HSCs expressed KD-Tie2 failed to express the SP phenotype. (E) Model of the regulation of quiescent HSCs in the osteoblastic niche. Quiescent HSCs adhered to SNO cells in the niche. Ang-1 produced by SNO cells activates Tie2 on the HSCs. Tie2 phospholyration promotes N-cadherin and β1-integrin mediated tight adhesion of HSCs to the niche, and induced quiescence of HSCs. Adhesion and quiescence of HSCs contribute to the maintenance of self-renewal activity of HSCs and enhanced survival of HSCs.

repopulating capacity of HSCs in telomerase mutant mice (Samper et al., 2002; Allsopp et al., 2003). Recently, the important role of telomeres in human disease has been highlighted by the study of dyskeratosis congenta, in which patients exhibit stem cell dysfunction (Ruggero et al., 2003).

Regulation of quiescent hematopoietic stem cells

1) Tie2/Ang-1 signaling

What molecular mechanisms govern the cell cycle, adhesion, and survival of HSCs in the BM niche? We previously reported that Tie2/Ang-1 signaling induced adhesion of HSCs to fibronectin and collagen (Takakura et al., 1998; Sato et al., 1998), and play a critical role in the development of definitive hematopoiesis (Takakura et al., 1998). We showed that Ang-1 activate Tie2 on HSCs resulting induction of HSC adhesion and suppression cell cycle (Figure 3). Tie2/Ang-1 signaling activates β1-integrin and N-cadherin in Tie2+ HSCs. Tie2/Ang-1 signaling also promotes HSC interactions with extracellular matrix and cell components of the niche.

Cell adhesion and quiescence of HSCs act cooperatively in the inhibition of cell division. It was reported that HSCs lose their self-renewal activity after repeated cell divisions under the *in vitro* culture (Ema et al., 2000). Prevention of HSC division by Tie2/Ang-1 signaling associated with maintenance of long-term repopulating activity of Tie2+KSL-SP cells. In addition, exogenous Ang-1 dramatically increased SP cells in the KSL population indicating that increment of quiescent HSCs (Figure 3). Quiescence of cell cycle leads to cell survival from the various stresses. In hematopoietic cell, we showed that Tie2/Ang-1 signaling lead to protection of HSCs from various stresses. For instance, pre-administration of Ang-1 adenovirus or recombinant protein induced adhesion of HSCs to bone surface *in vivo*. Ang-1 treatment followed by a lethal dose of 5-FU injection or X-ray irradiation result in the protection HCS and enhancement of recovery of hematopoietic cell resulting prolonged sur-

vival of mice.

The chimeric mice composed of both normal embryonic cells and Tie receptor Tie1/Tie2-deficient cells showed that these receptors are not required for fetal hematopoiesis, including the emergence of definitive HSCs, or for their relocation to and differentiation in the FL. Although Tie receptor-deficient cells retain the capacity to home to the BM from the FL during ontogeny, they fail to be maintained in the BM microenvironment (Puri and Bernstein, 2003). Tie1-deficient cells, expressing normal levels of Tie2, contribute to hematopoiesis (Partanen et al., 1996; Rodewald et al., 1996), indicating that Tie2 is required for postnatal BM hematopoiesis but not for embryonic hematopoiesis. It is known that Tie2-null mice died at E9.5-10.5 by abnormal interactions between the endothelium and surrounding matrix and pericyte (Suri et al., 1996). We hypothesize that the defect in hematopoiesis in Tie2-null embryos is a consequence of abnormal endothelial cell development in the P-Sp region. The function of Tie2 in endothelial cells might be crucial for the maintenance of the hematopoietic microenvironment. Although Tie2 expression was detected in embryonic HSCs, Tie2 signaling may only function in quiescent HSCs of the adult BM. In addition, in developing mice BM, there were no correlations between Tie2 expression and quiescence or SP phenotype of HSCs.

Analysis of chimeric mice composed of Tie receptor-deficient donors and $Rag2^{-/-}$ hosts, which do not produce mature lymphocytes, show that Tie2/Tie1-deficient cells contribute to lymphopoiesis in the absence of competing host cells (Puri and Bernstein, 2003). We also demonstrated that kinase dead-mutant Tie2 (KD-Tie2) expressing HSCs could not maintain quiescence and failed to express the SP phenotype (Figure 3). These findings strongly suggest that Tie2 is critical for the maintenance and survival of HSCs in the adult BM and that Tie2-deficient or KD-Tie2 cells are unable to occupy the adult BM niche when competing with wild-type cells.

2) N-cadherin mediated cell-cell adhesion

We also found that $Tie2^{+}$KSL-SP cells expressed N- and VE-cadherin, and osteoblasts expressed N-, P-, and OB-cadherin. In addition, we confirmed that Ang-1 treatment upregulated the expression of N-cadherin in $Tie2^{+}$ KSL-SP cells. This suggests that an adherens junction between HSCs and osteoblasts created via N-cadherin may contribute to HSC maintenance. We analyzed the function of N-cadherin in the maintenance of the stem cell specific property, such as cell adhesion, quiescence, and LTR-activity. Homophilic interaction between HSCs and stromal cells via N-cadherin maintained long-term culture-initiating cells (LTC-ICs) and induced slow cell cycling, suggesitng that N-cadherin-mediated cell-cell adhesion between HSCs and stromal cells enhances the quiescence of HSCs and keeps HSCs in immature state in *in vitro*. We found that the overexpression of dominant-negative N-cadherin inhibited long-term reconstitution activity of HSCs. It suggests that the adhesion between HSCs and BM niche cell is indispensable for the LTR-activity. In addition, we also found that WT-N-cadherin overexpressing HSCs were enriched in the SP fraction after 6 month of BM transplantation, indicating that N-cadherin-mediated cell adhesion induced HSCs in the quiescent and kept quiescent HSCs in the niche *in vivo*.

These observations led us the novel model of the maintenance of hematopoiesis. The localization of quiescent HSCs on the bone surface is regulated by stem cell specific adhesion molecules such as N-cadherin. Once the HSCs localize to SNO cells, Ang-1 produced by SNO cells may activate Tie2 on HSCs and Tie2/Ang-1 signaling promote tight adhesion of HSCs to the niche through the up-regulation of N-cadherin. N-cadherin is a critical niche factor for the maintenance of the quiescence and self-renewal activity of HSCs.

Further studies may reveal other molecules or signaling pathways required for cell adhesion and cell cycle regulation by niche factors. Understanding factors underlying cell adhesion and cell cycle regulation in stem cells should lead to development of new strategies for regenerative medicine.

3) Regulation of reactive oxygen species (ROS)

Recently, we demonstrated that a cell cycle checkpoint molecule, ataxia telangiectasia mutated (ATM), regulates the self-renewal activity of the HSCs but not their proliferation or differentiation into progenitors (Ito et al., 2004). The ATM protein maintains genomic stability by activating a key cell cycle checkpoint in response to DNA damage, telomeric instability or oxidative stress. $Atm^{-/-}$ mice over the age of 24 weeks show progressive bone marrow failure due to a defect in HSC function associated with elevated radical oxygen species (ROS), but they do not show telomere dysfunction. Elevated ROS induces upregulation of the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4A}$ and the retinoblastoma (Rb) gene in $Atm^{-/-}$ HSCs. Treatment with anti-oxidative reagents restores the reconstitutive capacity of $Atm^{-/-}$ HSCs (Figure 4). These data demonstrate that prevention of HSC senescence depends on ATM-mediated inhibition of oxidative stress. From these data, it is speculated that

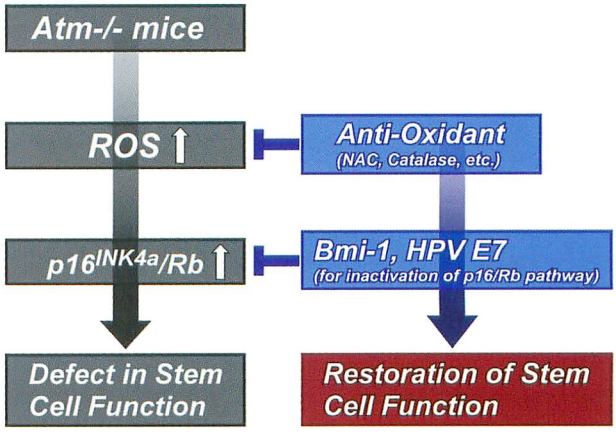

Fig 4. Role of ATM gene in the HSC functions.
Loss of ATM upregulates radical oxygen species (ROS) and $p16^{INK4a}$/Rb, resulting in the defect of stem cell function. Anti-oxidant such as N-acetyl cystein (NAC) or over-expression of Bmi-1/human papilloma virus E7 (HPV E7) restore the stem cell function.

niches or niche cells for quiescent stem cells are located in hypoxic regions of the BM, such as the trabecular zone for HSCs, where they not only keep stem cells quiescent through cell-adhesion but also protect them from ROS.

Recently, a novel concept that cancer cells include self-renewing "cancer stem cells (CSC)" has been proposed (Reya et al., 2001; Passegue et al., 2004). CSCs have the potential for self-renewal to drive tumorigenesis. There is one possibility that the cancer or leukemic stem cells also resided in the niche, and protected from anti-cancer drugs or irradiation therapies. Therefore, regulation of the passage of stem cells in and out of their niche could be a potential strategy for treatment of leukemia.

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