Life and death in hematopoietic stem cells

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Hematopoietic stem cells (HSCs) are defined as primitive cells that are capable of both self-renewal and differentiation into any of the hematopoietic cell lineages. HSC numbers need to be precisely regulated to maintain hematopoietic homeostasis. HSCs undergo several cell fate decisions, including life-and-death and self-renewal-and-differentiation decisions, which have crucial roles in the regulation of their numbers and lifespan. Defects in these processes have been found to contribute to hematopoietic insufficiencies and the development of hematopoietic malignancies. Recent studies have begun to elucidate how HSCs make life-and-death decisions and the underlying molecular mechanisms involved, highlighting the importance of a balance between survival and death in the regulation of HSCs.

Introduction

Hematopoietic homeostasis is maintained throughout the lifetime of an animal through the self-renewal of HSCs. Self-renewal is a specialized cell division in which one or both of the daughter cells retain the same cellular potential as the parental stem cells. HSCs reside in the bone marrow (BM) in adult animals in a specialized microenvironment, the niche. A subpopulation of osteoblasts and endothelial cells has been implicated as an important component of the HSC niche. In the BM niche, most HSCs remain in a quiescent state (G_0 stage), thereby preserving their capacity to self-renew. However, HSCs usually enter the cell cycle once a month on average, a shorter interval than has been previously considered. The size of the HSC pool is regulated by the balance between self-renewal and differentiation, symmetric (HSC expansion) and asymmetric (HSC maintenance) cell divisions, and survival and apoptosis of HSCs. Recent advances in gene manipulation techniques have led to the identification of cell-intrinsic and cell-extrinsic regulators of HSC fate decisions, including transcription factors, signal transducers, epigenetic regulators, and anti-apoptotic proteins [1].

Programmed cell death or apoptosis is crucial in determining whether HSCs live or die. Accumulating evidence suggests that the cellular senescence of HSCs can be included in this process. Thus, the life-and-death decisions are more complicated than previously thought. This review describes our current knowledge of the molecular mechanisms operating in the choice between life and death in HSCs.

Regulation of apoptosis in HSCs

There exist two distinct but ultimately converging signaling pathways that mediate apoptosis. Signaling through death receptors is activated when death ligands, such as CD95L (FAS ligand), bind their cognate receptors. The other pathway is regulated by the

interplay of pro-survival and pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family, and is triggered by many developmental cues, by cytokine withdrawal, and by various cytotoxic stimuli [2, 3]. The pro-apoptotic BH3-only proteins sense cellular damage, and Bax and Bak act as critical downstream mediators of apoptosis. Activation of Bax and Bak leads to disruption of the outer mitochondrial membrane, resulting in release of the cytochrome *c* and other apoptogenic proteins. Activation of Bax and Bak is opposed by the pro-survival proteins, including Bcl-2, Bcl- x_L , and Mcl-1. BH3-only proteins trigger apoptosis by engaging the multiple pro-survival Bcl-2-like relatives guarding Bax and Bak, allowing the activation of Bax and Bak to proceed (details are in the other reviews in this section).

An important role for apoptosis as a regulator of HSC numbers is supported by studies of constitutive expression of *Bcl-2* [4, 5]. Overexpression of Bcl-2 protected HSCs from many apoptotic stimuli, and resulted in increased numbers of HSCs in BM (2.4x wild type). Also, transgenic HSCs outcompeted wild-type HSCs in competitive repopulation experiments. In contrast, *Bcl-2*-deficient mice do not exhibit overt problems in HSC function [6]. *Bcl-x_L*-deficient chimeras created by complementation of *Rag2*-deficient blastocysts generate lymphoid cells, albeit in reduced numbers, indicating that *Bcl-x_L*^{-/-} HSCs can function *in vivo* [7]. Among these pro-survival Bcl-2 family members, however, Mcl-1 has been demonstrated to be indispensable for HSC survival [8]. Mcl-1 is highly expressed in HSCs and its expression is regulated by growth factors including stem cell factor (SCF). Inducible deletion of Mcl-1 in mice results in ablation of BM due to a preferential loss of early hematopoietic progenitors including HSCs. These findings highlight the major role that the regulation of life-and-death decision-making has in maintaining the size of the HSC pool.

DNA damage activates the tumor-suppressor protein p53, which elicits either apoptosis or cell-cycle arrest and repair. p53 triggers apoptosis by activating the transcription of the BH3-only pro-apoptotic genes, *Puma* and *Noxa*. Interestingly, a transcriptional repressor, Slug, is transcriptionally induced by p53 upon irradiation and then protects the damaged cells from apoptosis by directly repressing p53-mediated transcription of *Puma*. Slug is preferentially expressed in hematopoietic stem and progenitor cells, and shifts the fate of hematopoietic stem and progenitor cells after genotoxic stress toward survival by selectively blocking the apoptotic pathway triggered by p53 [9, 10]. p53 itself is also involved in regulating HSC population, and *p53*-deficient mice have twice as many HSCs and their HSCs exhibit greater repopulating capacity than do wild-type HSCs [11].

The role of the CD95 (Fas) apoptotic pathway in early hematopoiesis is not well understood and controversial. It is reported that CD95 is expressed on primary human CD34⁺ hematopoietic stem and progenitor cells at low, but significant levels. Nonetheless, agonists of the CD95 pathway do not induce apoptosis of primary human CD34⁺ hematopoietic stem and progenitor cells or reduce the potential of these cells to form colonies *in vitro* or engraft in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [12, 13]. The protection against CD95-mediated apoptosis might be of physiological importance ensuring the maintenance of hematopoietic stem and progenitor cell populations. Several molecular mechanisms that support these findings have been reported, including increased expression of the caspase 8 inhibitor FLICE inhibitory protein (FLIP) [12], and the selective expression of an anti-apoptotic caspase-8L isoform, but not pro-apoptotic caspase-8a/b isoforms, in human CD34⁺ hematopoietic stem and progenitor cells [13].

Regulation of resistance to stress in HSCs

Recent findings have revealed differences in sensitivity to physiologic stress between hematopoietic stem and progenitor cells and underscored the importance of the Akt-FoxO signaling pathway as part of the critical regulatory machinery for resistance to stress in HSCs. The FoxO (Forkhead O) subfamily of transcription factors plays an important role in diverse physiologic processes, including the induction of cell cycle arrest, stress resistance, and apoptosis. Upon activation by growth factors or insulin, the serine/threonine kinase Akt directly phosphorylates FoxO1, FoxO3, and FoxO4, resulting in their nuclear exclusion and subsequent degradation. In the absence of growth factors or insulin, or in the presence of stressful stimuli, FoxOs are translocated to the nucleus and upregulate the expression of a series of target genes, thereby promoting cell cycle arrest, stress resistance, or apoptosis [14].

In freshly isolated mouse CD34^{Kit}^{Sca-1}⁺Lineage-marker⁻ (CD34⁺KSL) HSCs, Akt is not activated and FoxOs are accumulated in the nucleus. In contrast, in most CD34⁺KSL progenitor cells, Akt is activated, while FoxOs are largely excluded from the nucleus and reside in the cytoplasm [15]. In a culture with attenuated cytokine signaling, in which FoxOs remained in the nucleus, CD34⁺KSL progenitor cells immediately underwent apoptosis, whereas CD34⁺KSL HSCs survived in a quiescent state as they are in the BM niche, suggesting the involvement of FoxOs in the regulation of the quiescence and survival of HSCs [15]. This finding was supported by an analysis of mice that were conditionally deleted of *FoxO1*, *FoxO3*, and *FoxO4* in the adult hematopoietic system [16]. *FoxO*-deficient BM cells have defective long-term repopulating activity that correlates with increased cell cycling and apoptosis of HSCs (KSL HSC population). Notably, levels of reactive oxygen species (ROS) are increased in *FoxO*-deficient HSCs and a subset of genes involved in the metabolism of ROS, including manganese superoxide dismutase (MnSOD) and catalase, are altered in their expression. Furthermore, *in vivo* treatment with the

antioxidative agent N-acetyl-L-cysteine (NAC) rescues the *FoxO*-deficient phenotype, restoring the size and function of HSCs as well as correcting the abnormalities in cell cycling and apoptosis. Intriguingly, ROS levels increase in an HSC-restricted manner and HSCs are much more sensitive to oxidative stress than the progenitors. These results suggest that FoxOs play essential roles in the establishment of resistance to physiologic oxidative stress that have deleterious effects on survival, quiescence, and function of HSCs (Fig. 1).

The essential need to regulate ROS in HSCs was first demonstrated in a study of *ATM*-deficient mice [17]. The regulation of oxidative stress by ATM is required for the maintenance of HSCs and defective HSC function in *ATM*-deficient mice can be successfully rescued by treatment with NAC. ROS induces phosphorylation of p38 MAPK in an HSC-specific manner and inhibition of p38 MAPK actually rescues ROS-induced HSC defects. Thus, p38 MAPK functions as a downstream mediator of ROS in HSCs [18]. The same group analyzed *FoxO3a*-deficient mice, and also observed an elevation of ROS levels, the activation of p38 MAPK, and a reduction in the expression of FoxO target genes involved in the detoxication of ROS even in mice deficient for a single *FoxO* gene [19]. All these findings inspire our interest in the regulation of intracellular oxidants in the context of the survival and lifespan of HSCs (Fig. 1).

On the other hand, FoxOs could directly regulate apoptosis by activating the transcription of *Bim*, a pro-apoptotic BH3-only gene [14]. It is likely that the Akt-FoxO signaling is involved in Bim-mediated apoptosis triggered in cytokine-depleted hematopoietic progenitor cells [20] as well as imatinib-treated chronic myeloid leukemia cells expressing the Bcr-Abl oncoprotein [21, 22].

Senescence of HSCs

Emerging evidence supports that prevention of senescence holds the key to the prolonged

self-renewal capacity of HSCs necessary to maintain hematopoietic homeostasis during the lifetime of an animal [23-25]. Senescent cells are viable and metabolically active but no longer have the ability to proliferate, presenting a striking contrast to cells in reversible forms of cell cycle arrest such as quiescence. This specialized form of terminal differentiation is induced by a variety of stimuli including alterations of telomere length and structure, some forms of DNA damage such as oxidative stress, and the activation of certain oncogenes (Fig. 2).

Telomere shortening limits the proliferative lifespan of cells by activating DNA damage pathways and causes replicative senescence. Even though HSCs express telomerase, the level of expression is not sufficient to completely prevent telomere erosion during aging or serial transplantation. Telomerase-deficient HSCs can be serially transplanted for only 2 rounds, whereas wild-type HSCs could be serially transplanted for at least 4 rounds. The rate of telomere shortening is increased approximately 2-fold during serial transplantation of telomerase-deficient HSCs, indicating that telomerase is needed to prevent premature loss of telomere function and provide prolonged replicative capacity to HSCs [26]. Deletion of the cell cycle inhibitor p21, a direct target of p53, in telomerase-deficient mice largely improves HSC function and lifespan, but leaves telomere function defective and apoptotic responses intact, suggesting that p21 mediates HSC senescence in the context of telomere dysfunction [27]. In contrast, overexpression of telomerase reverse transcriptase (TERT) in HSCs does not extend their transplantation capacity, even though it prevents telomeres from shortening during serial transplantation of BM [28]. This observation suggests that HSCs undergo a premature senescence, rather than telomere-dependent replicative senescence, that is associated with excessive stress during serial transplantation.

Two major pathways implicated in the induction of senescence are the p53-p21 and p16-Rb pathways and extensive crosstalk exists between the two (Fig. 2). ARF inhibits

MDM2, which mediates ubiquitin-dependent degradation of p53, leading to the accumulation of p53 protein. This results in the activation of p53 target genes involved in cell cycle arrest and apoptosis, including *p21*. By binding directly to CDK4 and CDK6, p16^{INK4A} blocks the assembly of catalytically active cyclin D-CDK complexes and keeps retinoblastoma protein (Rb) hypophosphorylated. Hypophosphorylated Rb represses E2F-dependent transcription by sequestering E2F, ultimately leading to cell cycle arrest and senescence. p21 associates with and inhibits the cyclin E/A-CDK2 complex, linking the p53 and Rb pathways [29].

HSCs have developed strict regulatory mechanisms to prevent premature senescence to prolong their lifespan. *Polycomb group (PcG)* genes are representative of such regulators. *PcG* genes are involved in cellular memory by maintaining gene silencing through chromatin modifications. Polycomb repressive complex (PRC) 1 includes Bmi1, Rnf110/Mel18, Phc1/Mph1/Rae28, Cbx2/M33, Scmh1, and Ring1a/b, while PRC2/3/4 includes Eed, Enx1/Ezh2, and Suz12. The PRC1 and PRC2 complexes possess H2A-K119 ubiquitin ligase activity and H3-K27 methyltransferase activity, respectively. H3-K27 tri-methylation (H3K27me3), catalyzed by PRC2, serves as a binding site for the Cbx2/M33 chromodomain, thereby recruiting the PRC1 complex. Thus, the two PcG complexes could function in a cooperative manner to maintain gene silencing [30]. Recent studies have implicated the *PcG* genes in the maintenance of HSC self-renewal. Among *PcG* genes, *Bmi1* plays a central role in this process. *Bmi1*-deficient mice exhibit a severe defect in HSC self-renewal [31-34]. Conversely, forced expression of *Bmi1* promotes HSC self-renewal [33]. The capacity of Bmi1 to maintain HSC self-renewal largely depends on the silencing of one of its targets, the *ink4a* locus [35]. This locus encodes a cyclin-dependent kinase inhibitor, p16^{INK4A}, and a tumor suppressor, Arf. In *Bmi1*-deficient mice, the expression of *Ink4a* and *Arf* is markedly increased in hematopoietic cells, and the

enforced expression of *Ink4a* and *Arf* in HSCs induces cell cycle arrest and p53-dependent apoptosis, respectively [32]. Conversely, deletion of both *Ink4a* and *Arf* substantially restores the impaired self-renewal capacity of *Bmi1*^{-/-} HSCs, whereas deletion of either of the *Ink4a* and *Arf* genes alone scarcely does [36][Oguro H and Iwama A, unpublished]. Taken together, *Bmi1* regulates HSCs by acting as a critical failsafe against the premature loss of HSCs induced by p16^{Ink4a} and *Arf*-dependent senescence pathways. Although de-repression of *p16*^{Ink4a} and *Arf* has been attributed to defective HSC self-renewal, the cell cycle status of CD34⁻KSL HSCs is not grossly altered or apoptosis is not increased at the level of HSCs in *Bmi1*^{-/-} mice [33]. One promising hypothesis is that de-repression of the *p16*^{Ink4a} and *p19*^{Arf} genes causes premature senescence of HSCs as is observed in *Bmi1*^{-/-} mouse embryonic fibroblasts and osteoblasts [35, 36]. Actually, *Bmi1*^{-/-} HSCs retain a normal telomere length [36]. In line with the depletion of HSCs in aging mice deficient for telomerase, however, no HSCs showing the characteristics of senescence were detected in the *Bmi1*^{-/-} CD34⁻KSL HSC fraction [27, 36]. These findings suggest that the senescent HSCs did not persist *in vivo* but were removed by additional checkpoint mechanisms, or had immunophenotypically changed (or differentiated), and thus no longer could be detected by conventional combinations of marker antigens. Intriguingly, overexpression of *Ezh2* prevents HSC senescence and preserves HSC potential during serial transplantations, further implicating the stabilization of chromatin structure by PcG complexes in the maintenance of HSC stemness [37].

The additional evidence supports the importance of the *Ink4a* locus in HSC function. Genotoxic stimuli, such as exposure to ionizing radiation or busulfan, also induce HSC senescence associated with increased expression of *p16*^{Ink4a} and *p19*^{Arf} [38]. *Ink4a*-*Arf*^{-/-} HSCs exhibit sustained repopulation activity compared to the wild type in serial transplantation [39]. Furthermore, stem-cell-autonomous effects of p16^{Ink4a} that accumulates

in HSCs with age accounts, at least in part, for age-dependent changes in HSC function, such as decreased per-cell repopulating activity, myeloid skewing of differentiation, and increased apoptosis with stress [40].

Conclusions

Decisions regarding the fate of HSCs include not only the choice between survival and death, but also the regulation of senescence. Within the last few years, much has been clarified about the unique regulatory mechanisms that have evolved in HSCs to prevent apoptosis and premature senescence. Leukemic stem cells (LSCs), which are considered to originate from hematopoietic stem or progenitor cells, not only adopt the regulatory machinery operating in normal HSCs, but also establish their own mechanisms against apoptosis and senescence. The regulation of life-and-death decision-making is essential in sustaining LSCs as it is in sustaining HSCs. On the other hand, the p16-Rb and p53-dependent cellular senescence pathways play a critical role in triggering oncogene-induced senescence, which is of substantial importance to eliminate transforming cells that potentially develop into LSCs. Taken together, understanding how HSCs make life-and-death decisions in response to various forms of physiologic stress will help us to manipulate normal HSCs as well as to establish therapeutic approaches that target LSCs.

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Figure legends

Figure 1. Pathways regulating response to oxidative stress in HSCs

Upon its activation by growth factors, Akt directly phosphorylates FoxO1, FoxO3, and FoxO4, resulting in their nuclear exclusion and subsequent degradation. In the absence of growth factors, FoxOs are translocated to the nucleus. In response to stressful stimuli, including oxidative stress, heat shock, and UV radiation, JNK directly phosphorylates FoxOs at different sites from Akt, and causes the nuclear translocation of FoxOs. In the nucleus, FoxOs upregulate a series of target genes, including those involved in the detoxification of ROS. In the absence of FoxOs or ATM, increased levels of ROS activate the p16-Rb pathway by activating p38 MAPK in an HSC-specific manner, and also activate p53, leading to the induction of apoptosis and senescence of HSCs.

Figure 2. Pathways regulating senescence in HSCs

Cellular senescence is triggered by a variety of stimuli including alterations of telomere length and structure, some forms of DNA damage such as those caused by oxidative stress, and the activation of certain oncogenes. The p53-p21 and p16-Rb pathways are the major pathways implicated in this process. Durable repression of promoters associated with growth control genes is essential for the establishment and maintenance of senescence. This repression is enforced by the construction of stable, heterochromatin-like complexes, the formation of which is directed in part by hypophosphorylated Rb. HSCs have developed strict regulatory mechanisms to prevent premature senescence. PcG proteins, particularly Bmi1, play a crucial role in preventing premature loss of HSCs by stably silencing the *ink4a* locus.

Figure 1

Figure 2