

REVIEW

The analysis, roles and regulation of quiescence in hematopoietic stem cells

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ABSTRACT

Tissue homeostasis requires the presence of multipotent adult stem cells that are capable of efficient self-renewal and differentiation; some of these have been shown to exist in a dormant, or quiescent, cell cycle state. Such quiescence has been proposed as a fundamental property of hematopoietic stem cells (HSCs) in the adult bone marrow, acting to protect HSCs from functional exhaustion and cellular insults to enable lifelong hematopoietic cell production. Recent studies have demonstrated that HSC quiescence is regulated by a complex network of cell-intrinsic and -extrinsic factors. In addition, detailed single-cell analyses and novel imaging techniques have identified functional heterogeneity within quiescent HSC populations and have begun to delineate the topological organization of quiescent HSCs. Here, we review the current methods available to measure quiescence in HSCs and discuss the roles of HSC quiescence and the various mechanisms by which HSC quiescence is maintained.

KEY WORDS: Hematopoiesis, Quiescence, Stem cell

Introduction

Somatic cells divide as they progress through the cell cycle, which is chiefly characterized by four phases: G1 (interphase), S (DNA synthesis phase), G2 (interphase) and M (mitosis phase) (Sisken and Morasca, 1965) (Fig. 1). Cells that proceed past the restriction point in the G1 phase enter the S phase, whereas those that do not pass the restriction point remain undivided. These undivided cells can withdraw from the cell cycle and enter the G0 phase: a state in which cells are termed quiescent or dormant (Pardee, 1974). Such non-cycling cells in the G0 phase can either reversibly re-enter the cell cycle and divide (Cheung and Rando, 2013) or remain dormant, losing the potential to cycle and, in some cases, becoming senescent (Campisi, 2005). Quiescence is thus a property that often characterizes tissue-resident stem cells and allows them to act as a dormant reserve that can replenish tissues during homeostasis.

Quiescence is thought to be a fundamental characteristic of hematopoietic stem cells (HSCs), which possess multi-lineage differentiation and self-renewal potential, and are able to give rise to all cell types within the blood lineage (Pietras et al., 2011). HSCs are a heterogeneous population of cells that are found in the bone marrow and, through the process of hematopoiesis, replenish blood cells during embryogenesis and throughout adulthood (Jagannathan-Bogdan and Zon, 2013). Quiescent HSCs reside at the apex of the hematopoietic hierarchy (Weissman, 2000) (Fig. 2), and precise regulation of their cell cycle is required for the effective production of

mature hematopoietic cells with minimal stem cell exhaustion (Orford and Scadden, 2008). However, as proliferating cells are more susceptible to genetic mutations and become senescent once their turnovers reach their maximum, a limit known as the Hayflick limit (Hayflick and Moorhead, 1961), quiescence supposedly protects HSCs from malignant transformation and malfunction (Wang and Dick, 2005). The decision of whether or not to exit quiescence is considered to be both stochastic and deterministic; both cell-intrinsic and -extrinsic signals induced in response to various stresses, such as inflammation or blood loss, permit quiescent HSCs to re-enter the cell cycle, proliferate and differentiate (Morrison and Weissman, 1994; Suda et al., 1983a).

Uncovering the biology of quiescent HSCs may provide insights into the *ex vivo* expansion and manipulation of highly potent HSCs that are applicable to clinical therapeutics. As such, a number of approaches have been developed in order to efficiently measure and analyze quiescence in HSCs. Here, we first provide an overview of the methods that are currently available for measuring quiescence in HSC populations. We next review the relationship between quiescence and repopulation potential, and the links between quiescence and lineage bias. Finally, we discuss the various intrinsic, extrinsic and systemic factors that are involved in maintaining HSC quiescence.

Methods to measure HSC quiescence

In recent years, several methods have been developed and used to detect quiescence in HSCs and to characterize the quiescent properties of HSCs and relate them to their other functional properties. Below, we provide an overview of each of these methods, highlighting those that have provided key insights into the quiescent nature of HSCs. The key features, together with the advantages and disadvantages of each method, are summarized in Table 1.

Snapshot measurements of quiescence based on nucleic acid content

The quiescent state of cells can readily be assessed through measurements of DNA and RNA content and by staining for proliferation markers (Table 1). For example, dual staining using the DNA-binding dye Hoechst 33342 together with an anti-Ki67 antibody, which labels proliferating cells (Wilson et al., 2004), demonstrated that 70% of mouse HSCs exist in the G0 state (Wilson et al., 2008). Similarly, measurement of the ratio of DNA content (based on Hoechst 33342 staining) and RNA content (using the RNA-binding dye Pylonin Y) within a cell (Gothot et al., 1997) identified approximately two-thirds of long-term (LT)-HSCs as quiescent. In line with this, it was recently identified that S/G2/M phase cells obtained from the whole bone marrow (BM) contained long-term engrafting HSCs (Goldberg et al., 2014). This suggests that HSCs may cycle, although the cycling HSCs may be lost through isolation techniques. Moreover, such staining methods reveal the cell cycle status at only a single time point; they do not

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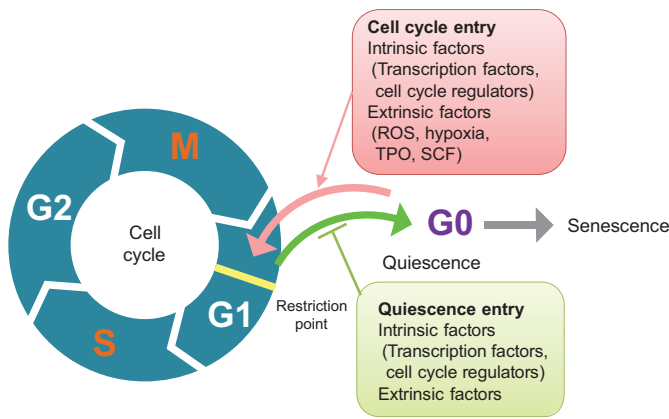


Fig. 1. Regulation of the cell cycle of HSCs. Like all somatic cells, hematopoietic stem cells (HSCs) progress through the four phases of the cell cycle: G1 phase, S phase, G2 phase and M phase. Cells that pass the restriction point in the G1 phase will progress through the cell cycle and divide. By contrast, non-dividing cells can enter the G0 phase and can reversibly re-enter the cell cycle or become quiescent, losing the ability to cycle and sometimes becoming senescent. ROS, reactive oxygen species; SCF, stem cell factor; TPO, thrombopoietin.

take into account the cycling kinetics of HSCs and cannot determine the duration of the G0 phase of HSCs. It should also be noted that live HSCs possess dye efflux activity that can, for example, make their Hoechst 33342 staining appear lower than their actual DNA content.

Label incorporation-based assays

An alternative approach, which allows quiescence to be monitored more dynamically, is based on label incorporation by cells (Table 1). The immortal strand model hypothesizes that adult stem cells divide asymmetrically, passing newly synthesized DNA strands to daughter cells for self-protection from mutations (Cairns, 1975). Based on this theory, cell populations that retain DNA analogs such as BrdU (5'-bromo-2'-deoxyuridine) are thought to consist of quiescent HSCs (Cheshier et al., 1999; Fleming, 1993; Goodell et al., 1996; Zhang et al., 2003). Such BrdU incorporation assays can also be used to characterize the cycling span of cells (Cotsarelis et al., 1990). Short-term BrdU incorporation (24 h), for example, has been used widely to assay the cell cycle state of HSCs and is biased towards marking rapidly proliferating cells (Passegué et al., 2005). Analysis using continuous BrdU incorporation (180 days of BrdU fed to mice) showed that over 99% of HSCs divided once in a 6-month time span (Cheshier et al., 1999). A shorter BrdU incorporation period of 4-10 days followed by a 70-day chase period identified only 0.5% of BrdU-retaining cells as HSCs, suggesting that BrdU retention does not correlate with stem cell phenotype (Kiel et al., 2007). However, in a similar study, using more-refined HSC populations, a BrdU pulse for 10-12 days followed by a 70-day chase revealed that over 30% of HSCs retained BrdU and were thus dormant (Wilson et al., 2008). Furthermore, statistical analysis of the BrdU incorporation kinetics predicted that this subpopulation of dormant HSCs divides once in ~5 months. These inconsistencies in cell cycle state determined by BrdU incorporation may be due to the inefficiency of slow cycling cells to incorporate the dye and to potential effects of the dye itself on cell cycling (Kiel et al., 2007; Takizawa et al., 2011). In addition to these inconsistencies, it should be noted that the detection of BrdU requires cells to be fixed, thus making it impossible to assess their functional properties.

H2B-GFP retention

As an alternative to BrdU, a fusion protein consisting of histone H2B fused to GFP (H2B-GFP), which is incorporated into nucleosome core particles without altering cell division (Kanda et al., 1998), has been used to assess cell cycling. Transgenic mice that can be induced to express H2B-GFP may circumvent the problems of BrdU incorporation. Using induced expression of H2B-GFP specifically in HSCs, it was shown that HSCs exhibited dormancy similar to that measured by BrdU assays (Wilson et al., 2008). Mice that can be induced to express H2B-GFP ubiquitously have also been used to analyze HSC quiescence (Foudi et al., 2009). These studies revealed that HSCs retained the highest level of GFP expression over a 72-week chase, and mathematical analysis of H2B-GFP retention estimated that all HSCs divide and become GFP negative within 300 days. However, some promiscuous H2B-GFP expression has been reported in the absence of the tetracyclin-responsive element that is used to drive the expression of the fusion protein, which suggests some limitations of the H2B-GFP retention model (Challen and Goodell, 2008).

Following cell divisions using dilution assays

Approaches that simply mark cell surface or intracellular proteins, using *in vivo* or *ex vivo* labelling, and subsequently trace cellular divisions by tracking the retention/loss of the labels have also been used (Nygren and Bryder, 2008; Takizawa et al., 2011). For example, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), which is a fluorescent dye that covalently binds to intracellular proteins, was used to label HSCs *ex vivo* and to analyze HSC cell cycle kinetics (Takizawa et al., 2011). In this study, *ex vivo* CFSE-stained HSCs were transplanted into non-irradiated mice and assayed for label retention in order to monitor their divisional history in steady state. Single cell and limiting dilution transplantation combined with mathematical models estimated that HSCs divide every 39 days on average. Compared with BrdU, CFSE label retention is more sensitive and efficient in identifying cell divisions. However, this approach is limited as the HSCs need to be transplanted and to find their way naturally to the bone marrow.

Cell cycle phase reporter mice

The methods discussed above have been used to successfully characterize quiescence and cell cycle status in HSCs, but they each have a number of disadvantages (Table 1) and are all invasive, i.e.

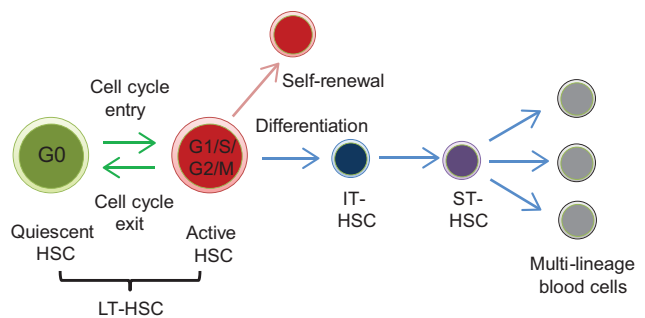


Fig. 2. The stages of HSC differentiation. Quiescent (or dormant) hematopoietic stem cells (HSCs) in the G0 phase can be activated to enter the cell cycle (G1/S/G2/M phases) and either self-renew or differentiate. Active HSCs can also exit the cell cycle and return to quiescence. Upon differentiation, long-term HSCs (LT-HSCs) are thought to give rise to cells with lower repopulation potential – intermediate-term HSCs (IT-HSCs) and short-term HSCs (ST-HSCs) – and subsequently produce multi-lineage blood cells.

Table 1. Advantages and disadvantages of current methods used to determine HSC turnover

Label	Labeling method	Labeling period	Chasing period	References	Label detection	Advantage	Disadvantage
Hoechst/Ki67	<i>Ex vivo</i> staining	n.a.	n.a.	Wilson et al., 2004	Direct fluorescence emission and antibody	<ul style="list-style-type: none"> Simple and efficient labeling Clear separation of cell cycle phase 	<ul style="list-style-type: none"> Snapshot analysis at a single time point No possibility of subsequent functional testing of the labeled cells
Hoechst/Pyronin Y	<i>Ex vivo</i> staining	n.a.	n.a.	Gothot et al., 1997; Passegue et al., 2005	Direct fluorescence emission	<ul style="list-style-type: none"> Simple and efficient labeling Clear separation of cell cycle phase Possibility for functional test of the labeled cells 	<ul style="list-style-type: none"> Snapshot analysis at a single time points
BrdU	<i>In vivo</i> intraperitoneal injection and drinking water with BrdU	180 d 10 d 13 d	180 d 120 d 306 d	Cheshier et al., 1999 Kiel et al., 2007 Wilson et al., 2008	Antibody	<ul style="list-style-type: none"> Simple and efficient labeling <i>In vivo</i> cell cycle kinetics tracking 	<ul style="list-style-type: none"> No possibility of subsequent functional testing of the stained cells Low divisional resolution Mitogenic effect
H2B-GFP (Tet-off)	<i>In vivo</i> transgene induction	Until doxycycline treatment	240 d 84 d	Wilson et al., 2008 Challen and Goodell, 2008	Direct fluorescence emission	<ul style="list-style-type: none"> <i>In vivo</i> cell cycle kinetics tracking Possibility of functional testing of the labeled cells 	<ul style="list-style-type: none"> Inconsistent labeling efficacy reported in different papers Promiscuous and dim H2B-GFP expression
H2B-GFP (Tet-on)	<i>In vivo</i> transgene induction with doxycycline-containing drinking water	42 d	500 d	Foudi et al., 2009	Direct fluorescence emission	<ul style="list-style-type: none"> <i>In vivo</i> cell cycle kinetics tracking Efficient labeling 	<ul style="list-style-type: none"> Unknown divisional resolution although seven to eight divisions assumed
Esterized biotin	<i>In vivo</i> intravenous injection	Single dose	21 d	Nygren and Bryder, 2008	Streptavidin	<ul style="list-style-type: none"> <i>In vivo</i> cell cycle kinetics tracking Simple and efficient labeling Possibility of functional testing of the labeled cells 	<ul style="list-style-type: none"> Low divisional resolution (fewer than four divisions)
CFSE	<i>Ex vivo</i> labeling	7 min	147 d	Takizawa et al., 2011	Direct fluorescence emission	<ul style="list-style-type: none"> <i>In vivo</i> cell cycle kinetics tracking High divisional resolution (seven divisions) Simple and efficient labeling Possibility of functional testing of the labeled cells 	<ul style="list-style-type: none"> Intravenous transfer of <i>ex vivo</i> labeled cells Fluorescence bleaching Limited engraftment of cells in non-conditioned recipients

The methods that can be used to characterize HSC turnover.

CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; d, days; n.a., not applicable; Tet-on, tetracycline-on system; Tet-off, tetracycline-off system.

they require fixing of the cells, thus precluding functional studies. Non-invasive methods that can specifically identify HSCs in G0 and assess the duration of G0 would thus be useful. The fluorescent ubiquitylation-based cell cycle indicator (Fucci) system, which allows visualization of cells in G1 and S/G2/M phases (Sakaue-Sawano et al., 2008), might be a promising option. This system uses the fusion of fluorescent proteins to the cell cycle-specific proteins geminin and Cdt1; owing to the cell cycle-dependent ubiquitylation and degradation of these proteins, nuclei in G1 and S/G2/M phases can be labelled in red and green, respectively. Transgenic mice rendering the Fucci system have been developed (Sakaue-Sawano

et al., 2013) but, to date, they have not been used for characterizing the cell cycle properties of HSCs.

The Fucci system cannot distinguish cells in G0 from cells in G1 but, more recently, a novel cell cycle probe, m-Venus-p27K⁻ (Fig. 3), was developed as a specific indicator of the G0 phase (Oki et al., 2014). Retroviral transduction of this probe into cultured cells was used to identify and isolate quiescent cells, and studies using m-Venus-p27K⁻ transgenic mice also identified quiescent cells in muscle tissues. Improvement of these reporter systems in the future will undoubtedly be a promising approach for identifying and characterizing quiescent HSCs.

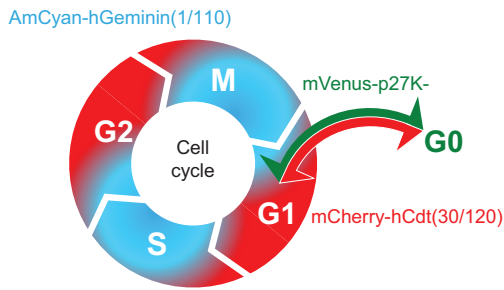


Fig. 3. The Fucci system for analyzing cell cycle status. The fluorescent ubiquitination-based cell cycle indicator (Fucci) system allows visualization of cells in the G1, S/G2/M, and G0 phases of the cell cycle. This system uses the fusion of fluorescent proteins to the cell cycle-specific proteins, geminin, Cdt1 and p27; owing to the cell cycle-dependent ubiquitylation and degradation of these proteins, nuclei in G1, S/G2/M and G0 phases can be labelled in cyan, green and red, respectively.

Cell-surface markers and HSC quiescence

Multiple attempts have been made to identify and isolate purified populations of mouse HSCs using antibodies against cell-surface markers (Table 2). However, the combination of cell-surface markers that specifically identify dormant HSCs is yet to be elucidated. Furthermore, these markers tend not to be involved in the cell cycle; they are simply markers that enable cells with a particular differentiation state to be isolated and, notably, some of these also change with cell cycling and thus should be used with caution (King and Goodell, 2011).

Highly purified HSCs can be isolated using antibodies against signaling lymphocyte activation molecule (SLAM) family members, such as CD150, CD48 and CD41, which are differentially expressed in hematopoietic stem and progenitor cells (Kiel et al., 2005a). Using this approach, it was shown that most (96.2%) CD150⁺CD48⁻CD41⁻ cells are in the G0 state. Recently, it was reported that two other SLAM family markers (CD229 and CD244) could further sub-fractionate CD150⁺CD48⁻LSK cells (Oguro et al., 2013). This study showed that the CD229^{-low}CD244⁻CD150⁺CD48⁻LSK cell fraction contained cells that were deeply quiescent with lower levels of Ki-67 staining, short-term BrdU incorporation and higher histone H2B-GFP label retention. Single cell transplantation of CD229^{-low}CD244⁻CD150⁺CD48⁻LSK cells revealed that 40% of these cells could repopulate donor mice over the long term (>16 weeks). However, the expression of surface markers on HSCs can alter with environmental stimuli, thereby limiting the use of surface markers for the identification of primitive HSCs (King and Goodell, 2011). For example, expression of the stem cell antigen 1 (SCA1) can be highly upregulated by the inflammatory response (Snapper et al., 1991).

Measuring quiescence in human HSCs

Attempts have also been made to directly measure the cycle rate of human HSCs. Using granulocyte telomere length data, human HSCs were estimated to divide once per 45 weeks on average (Shepherd et al., 2004). However, owing to the limitation of assays to study human HSCs, only a limited set of cell-surface markers has been identified for human hematopoietic stem and progenitor cells (Table 3). Delineation of the quiescent properties of human HSCs also relies on transplanting human-derived HSCs into immunocompromised mice. Although clinically manipulated HSCs are enriched according to CD34 positivity, highly purified HSCs with SCID (severe combined immunodeficiency) mice repopulating capacity reside in the Lin⁻CD34⁻CD38⁻CD93^{high} or Lin⁻CD34⁻CD38⁻CD90⁺CD45RA⁻CD49f⁺ populations in the human bone marrow (Danet et al., 2002; Notta et al., 2011). A subsequent study demonstrated that cord blood-derived Lin⁻CD34⁻CD38⁻CD93^{high} cells were more quiescent compared with CD34⁺ fractions (Anjos-Afonso et al., 2013). Further insight into the stem cell potential and the purification of immature human HSCs is necessary for their efficient use in clinical therapeutics.

The link between HSC quiescence and repopulation potential

The stem cell potential, or ‘stemness’ of HSCs relies upon a combination of properties: quiescence, repopulation potential, self-renewal potential and multi-lineage differentiation potential (Rossi et al., 2012). The repopulation potential of HSCs can be assessed through competitive bone marrow transplantation with myeloablative conditioning; this approach is considered to be the golden standard of assessment. However, the correlation between repopulation potential and quiescence is currently unclear (Fig. 4). Generally, it is thought that cell cycle quiescence parallels the self-renewal capacity and repopulation potential of HSCs (Ema et al., 2005; Morrison and Weissman, 1994; Suda et al., 1983b). By contrast, excessive proliferation exhausts HSC populations, resulting in loss of HSC function, which projects to lower repopulation potentials, as exemplified through serially transplanted or aged HSCs (Passegué et al., 2005). Highly dormant HSCs identified by label retention of H2B-GFP exhibit higher repopulation potentials in sublethal irradiation transplantation compared with cycling HSCs (Foudi et al., 2009; Wilson et al., 2008). Consistently, the majority of gene knockout mice with HSC defects suggest that excessive proliferation and loss of quiescence results in defective HSC repopulation (Rossi et al., 2012) implying the existence of cell-autonomous, intrinsic regulatory mechanisms that link quiescence to the repopulating potential of HSCs. However, the knockout of some genes such as

Table 2. Table summarizing the combination of cell markers used to purify murine HSCs

Description	Markers	Adult/fetal	References
Identification using proto-oncogene and lymphocyte antigen	Lin ⁻ KIT ⁺ SCA1 ⁺ (LSK)	Adult	Okada et al., 1992
Identification using a differentiation marker Thy1.1	THY1.1 ^{low} LSK	Adult	Spangrude et al., 1988
Identification using efflux of dye	Side population (SP) LSK	Adult	Goodell et al., 1996
	CD45 ^{mid} Lin ⁻ Rho ^{low} SP	Adult	Dykstra et al., 2006
Identification using a differentiation marker CD34	CD34 ⁻ LSK	Adult	Osawa et al., 1996
Identification using receptor tyrosine kinase Flt-3	FLT3 ⁻ Thy1.1 ^{low} LSK	Adult	Christensen and Weissman, 2001
Identification using lymphocyte markers of the SLAM family	CD150 ⁺ CD48 ⁻ LSK	Adult/fetal	Kim et al., 2006; Kiel et al., 2005a
	CD150 ⁺ CD34 ⁻ LSK	Adult	Morita et al., 2010
	CD229 ^{-low} CD244 ⁻ CD150 ⁺ CD48 ⁻ LSK	Adult	Oguro et al., 2013
Identification using endothelial cell protein	EPCAM ⁺ LSK	Adult	Balazs et al., 2006
	Lin ⁻ CD45 ⁺ EPCAM ⁺ CD48 ⁻ CD150 ⁺	Fetal	Kent et al., 2009

The combination of cell markers used to purify murine HSCs.

Table 3. Table summarizing the combination of cell markers used to purify human HSCs

Marker	Source	Description	Reference
CD34 ⁺ CD45RA ^{lo} CD71 ^{lo} cells	UCB	Multipotent progenitors	Mayani et al., 1993
THY1 ⁺ Lin ⁻ CD34 ⁺ cell	UCB Fetal liver/BM	Multipotent progenitors	Baum et al., 1992 Craig, 1993
CD34 ⁺ CD38 ⁻ cells	UCB BM	Highly quiescent primitive progenitors	Hao et al., 1995
Lin ⁻ CD34 ⁺ CD38 ⁻ CD90 ⁺ CD45RA ⁻	UCB	Multi-potent long-term engrafting cells	Majeti et al., 2007
Lin ⁻ CD34 ⁺ CD38 ⁻ CD45RA ⁻ THY1 ⁺ Rho ^{lo} CD49f ⁺	UCB	Single cell engraftment	Notta et al., 2011
Lin ⁻ CD34 ⁺ CD38 ⁻ CD93 ^{high}	UCB BM	Quiescent cells with multipotent engraftment	Danet et al., 2002 Anjos-Afonso et al., 2013

The combination of cell markers used to purify human HSCs. BM, bone marrow; UCB, umbilical cord blood.

Cdkn2c or *Myb* results in a higher repopulation potential with higher proliferation of HSCs (Yuan et al., 2004; Lieu and Reddy, 2009). In addition, administration of a cell cycle inhibitor can inhibit the engraftment of human HSCs into NOD/SCID mice (Cashman et al., 2002). Furthermore, when HSCs (FLK2⁻ LSK) were fractionated by N-cadherin staining, N-cadherin^{int} HSCs that were more quiescent displayed lower repopulation potentials compared with cycling N-cadherin⁻ HSCs (Haug et al., 2008). The repopulation potential of quiescent N-cadherin^{int} HSCs can be increased upon pre-transplantation culture, raising the possibility that additional stimuli may be needed for deeply quiescent HSCs to exhibit optimal repopulation potential.

It has also been suggested that there is no correlation between the duration of G0 phase and the repopulation potential of HSCs (Takizawa et al., 2011). These contradictory effects may be due to functional heterogeneity within dormant HSCs or to a failure to discriminate between symmetric (producing two HSCs or two progenitors) or asymmetric (producing a HSC and a progenitor cell) cell divisions. In other words, a decrease in self-renewal divisions and an increase in differentiation divisions will likely result in lower repopulation potential and vice versa (Fig. 4). The analysis of cellular behaviors at a single cell level may help to differentiate these cell cycle progression patterns (Suda et al., 1984). Indeed, single-cell competitive bone marrow transplantation of HSCs identified a variation in the length of repopulation by HSCs (Ema et al., 2014; Yamamoto et al., 2013). HSCs can be classified into long- (>12 months), intermediate- (6–12 months) and short- (<6 months) term HSCs, according to the duration of engraftment

after bone marrow transplantation (Fig. 2); these classes likely differ with regards to their quiescent properties. A combination of single cell paired-daughter cell assays along with detailed genetic analysis will be promising for unraveling the relationship between HSC quiescence, self-renewal capacity and repopulation potential (Schroeder, 2013).

HSC quiescence and lineage bias

It is also unclear whether a correlation exists between cell cycle status and the differentiation potential of HSCs. The identification of lineage-biased HSCs within quiescent HSC populations raises the issue of whether multi-lineage repopulation is a factor that determines the ‘stemness’ of HSCs. A recent study showed that HSCs expressing different levels of KIT exhibited variations in the extent to which they could differentiate to megakaryocyte lineages (Shin et al., 2014). It was also identified that stem cell potential correlated with the level of KIT expression on LT-HSCs (Grinenko et al., 2014; Shin et al., 2014); LT-HSCs expressing low levels of KIT were relatively quiescent and exhibited lower repopulation potentials compared with KIT^{high} HSCs. In a similar study, von Willebrand factor-positive HSCs exhibited quiescence along with a strong bias to megakaryocytic differentiation (Sanjuan-Pla et al., 2013). The gene expression profiles of CD229^{-/low}CD244⁻CD150⁺CD48⁻LSK cells also showed higher levels of megakaryocyte/erythrocyte lineage-associated genes than granulocyte/macrophage or lymphoid-associated genes when compared with CD229⁺CD244⁻CD150⁺CD48⁻LSK cells (Oguro et al., 2013). Correspondingly, these CD229^{-/low}CD244⁻CD150⁺CD48⁻LSK cells repopulated with a myeloid lineage bias after transplantation. These studies indicate that quiescent HSCs are a heterogeneous group with varying lineage bias (Quesenberry et al., 2007). In support of this idea, a correlation between cell cycle phase, as detected by the Fucci reporter system, and differentiation potential in human embryonic stem cells was recently proposed (Pauklin and Vallier, 2013). In this study it was shown that the cell cycle regulators cyclin D1 and D3 control differentiation signals, such as transforming growth factor (TGFβ) and SMAD2/3, indicating a strong association between cell fate decisions and the cell cycle machinery in human embryonic stem cells. The delineation of this potential relationship in HSCs will require the further development of methods that can identify variations in G0 length and correlate these with lineage bias.

The possibility of a link between lineage bias and quiescence in HSCs also points towards the existence of a common mechanism that may regulate both of these stem cell properties. One such possible mechanism involves cytokines, which are key extrinsic factors that modulate HSC cell fate. Initially, only a few cytokines, such as stem cell factor (SCF) and thrombopoietin

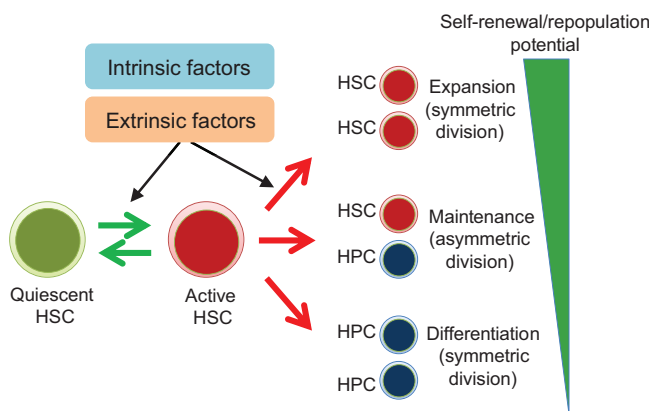


Fig. 4. Symmetric and asymmetric cell divisions in relation to self-renewal and the repopulation potential of HSCs. Quiescent HSCs are activated by cell-intrinsic or -extrinsic factors for symmetric or asymmetric cell divisions. Symmetric cell divisions subsequently result in a stem cell pool with higher repopulation potential.

(TPO), were thought to act directly on HSCs to maintain their quiescence (Gibbs et al., 2011; Kaushansky, 2006). However, recent studies have revealed functions for various cytokines that act directly on HSCs to alter both their cell cycle state and their lineage commitment. Single cell-based analyses of the response to cytokines in HSCs revealed not only a variety of cytokines acting on HSCs but also heterogeneity in HSC responses to cytokines. In one study, flow cytometric analysis in response to various cytokine stimuli revealed heterogeneity in STAT3, STAT5 and ERK phosphorylation patterns in human HSCs (Gibbs et al., 2011). The study was further extended to analyze the effect of granulocyte-stimulating factor (G-CSF) on human HSCs cultured individually in hydrogel microwells, showing a heterogeneous response in cell cycling. When human HSCs were subdivided according to G-CSF receptor (CD114) positivity, it was discovered that CD114^{-/low} HSCs exhibited significantly

higher long-term repopulation potentials in NOD/SCID/IL2 γ ^{null} mice. These results suggest that the exit from quiescence in response to cell extrinsic stimuli is regulated through the cell intrinsic expression of CD114. However, although these data indicate that the cell intrinsic expression of cytokine receptors can determine HSC cell fate, whether or not these lineage-biased stem cells are positioned within the bone marrow niche in accordance with local cytokine production requires further investigation. Indeed, the identification of niche cell-specific production of cytokines has already begun to uncover the physiological availability of cytokines within the BM.

Intrinsic regulation of HSC quiescence

Molecular regulators of cell cycle progression, as well as multiple transcription factors have been identified as intrinsic regulators of HSC quiescence (Table 4). As we discuss below, recent studies have

Table 4. Table summarizing the mouse models used to study the effects of cell-intrinsic regulators of HSCs, primarily focusing on genes that influence the cell cycle status of HSCs

Cell intrinsic regulators	Gene	Mouse model	Adult/fetal	Effect on cell cycle	Effect on repopulation potential	Reference	
Cell cycle regulators	Cyclins D1, D2 and D3	Triple KO	Fetal	Decrease quiescence	↓	Kozar et al., 2004	
	Cyclin A	Conditional KO	Adult	Increase proliferation	↓	Kalaszczynska et al., 2009	
	Rb family (<i>Rb1</i> , <i>Rbl1</i> , <i>Rbl2</i>)	Triple KO (Mx-1-Cre)	Adult	Increase proliferation	↓	Viatour et al., 2008	
	<i>Cdkn2a</i> (p16)	KO (aged mice)	Adult	Increase proliferation	↑	Janzen et al., 2006	
	<i>Cdkn2c</i> (p18)	KO	Adult	Increase proliferation	↑	Yuan and et al., 2004	
	<i>Cdkn1a</i> (p21)	KO	Adult	Decrease or Unchanged quiescence	↓	Cheng et al., 2000a; Van Os et al., 2007; Foudi et al., 2009	
	<i>Cdkn1b</i> (p27)	KO	Adult	No change in cell cycle	↑	Cheng et al., 2000b	
	<i>Cdkn1c</i> (p57)	Conditional KO (Mx1-Cre)	Adult	Decrease quiescence	↓	Matsumoto et al., 2011	
	Nuclear regulators	<i>p53</i> (<i>Trp53</i>)	KO	Adult	Decrease quiescence	↑	Liu et al., 2009
		<i>Mycl/Mycn</i>	Conditional KO (Mx-1-Cre)	Adult	Decrease differentiation	↓	Laurenti et al., 2008
<i>Fbxw7</i>		Conditional KO (Mx-1-Cre)	Adult	Decrease quiescence	↓	Matsuoka et al., 2008	
<i>Runx1</i>		Conditional KO (Mx-1-Cre)	Adult	Increase proliferation	↓	Growney et al., 2005; Motoda et al., 2007	
<i>Gfi1</i>		KO	Adult	Decrease quiescence	↑	Hock et al., 2004	
<i>Necdin</i>		KO	Fetal	Increase proliferation	→	Asai et al., 2012	
<i>Mef</i> (<i>Elf4</i>)		KO	Adult	Increase quiescence and self-renewal	↑	Lacorazza et al., 2006	
<i>Myb</i>		KO	Adult	Decrease proliferation	↓	Lieu and Reddy, 2009	
<i>Gata2</i>		Overexpression	Adult	Increase quiescence	↑	Tipping et al., 2009	
<i>Pbx1</i>		cKO (Mx-1-Cre)	Adult	Decrease quiescence	↓	Ficara, 2008	
<i>Evi1</i> (<i>Mecom</i>)		cKO (Mx-1-Cre)	Adult	Decrease self-renewal	↓	Goyama et al., 2008	
<i>Nurr1</i> (<i>Nr4a2</i>)		KO (heterozygous)	Adult	Decrease quiescence	ND	Sirin et al., 2010	
<i>Foxo1/3/4</i>		KO	Adult	Decrease quiescence	↓	Miyamoto et al., 2007; Tothova et al., 2007	
<i>Nrf2</i> (<i>Nfe2l2</i>)		KO	Adult	Decrease quiescence	↓	Tsai et al., 2013	
<i>Cebpa</i>		Conditional KO	Adult	Decrease quiescence	↓	Ye et al., 2013	
<i>PU.1</i> (<i>Spi1</i>)	Hypomorphs	Adult	Decrease quiescence	↓	Staber et al., 2013		
Epigenetic regulators	<i>Satb1</i>	KO	Fetal	Decrease quiescence	↓	Will et al., 2013	
	<i>Bmi1</i>	KO	Adult and fetal	Decrease self-renewal	↓	Park et al., 2003; Iwama et al., 2004	
	<i>Mysm1</i>	KO	Adult	Decrease proliferation	↓	Wang et al., 2013b	
	<i>Rae28</i> (<i>Phc1</i>)	KO	Fetal	No change in cycling	↓	Ohta et al., 2002	
	<i>Dnmt3a</i>	Conditional KO	Adult	Decrease proliferation	↓	Challen et al., 2012	

The mouse models used to study the effects of cell intrinsic regulators of HSCs, primarily focusing on genes that influence the cell cycle status of HSCs. KO, knockout; ND, not determined.

also started to uncover the relationships between these various factors during HSC regulation.

The transcription factor and chromatin remodeler SATB1 (special AT-rich sequence-binding protein 1) was shown to regulate various cell cycle regulators (including MYC) along with a factor that determines cell polarity (NUMB) in HSCs (Will et al., 2013). HSCs from *Satb1*^{-/-} mice fetal livers display lower quiescence, lower repopulation potentials and decreased symmetric self-renewal cell division frequencies at the single cell level when compared with HSCs from wild-type mice. Similarly, it was identified that the transcription factor PU.1 regulates the transcription of multiple cell cycle regulators in HSCs (Staber et al., 2013). Accordingly, PU.1 hypomorphic mice that express low levels of PU.1 in HSCs exhibited a loss of HSC quiescence and increased proliferation. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) studies of LSK cells revealed that PU.1 binds to the enhancers and promoters of multiple cell cycle inhibitors and activators, including *Gfi1*, *E2f1*, *Cdc25a*, *Cdk1*, *Cdkn1a* and *Cdkn1c*, and regulates their expression. PU.1 was also shown to positively regulate its own expression by binding to a -14 kb upstream regulatory element (URE). However, further studies that unravel the transcriptional and epigenetic networks regulating HSC quiescence are still needed.

HSC entry into quiescence, which occurs in early postnatal development, involves transcriptional regulation by the transcription factor C/EBP α (Ye et al., 2013). During development, murine HSCs are initially proliferative and sustain this cell cycle profile to up to 3 weeks post-birth (Bowie et al., 2006), after which point they start to become quiescent. The conditional deletion of *Cebpa* in HSCs (using a *Mx1-Cre* promoter) in adult mice resulted in Mycn-dependent loss of quiescence in HSCs. By contrast, the deletion of *Cebpa* in fetal liver HSCs did not alter the cell cycle state of these cells (Zhang et al., 2004), suggesting that high C/EBP α expression in adult HSCs serves as a molecular switch that regulates the transition from proliferating primitive HSCs to quiescent definitive HSCs during development.

Extrinsic regulation of HSC quiescence

HSCs reside in a specialized microenvironment termed ‘the niche’, which is composed of cellular and humoral constituents that regulate HSC fate (Schofield, 1978). Components of the HSC niche have been extensively reviewed (Morrison and Scadden, 2014) (Fig. 5). Although various niche cells function to maintain HSCs in a quiescent state, it is not clear whether cycling or quiescent HSCs preferentially occupy a specific niche. Various imaging studies suggest that HSC localization within the bone marrow is not random; HSCs locate in the proximity of bone and are densely associated with the surrounding arterioles and perivascular stromal cells (Arai et al., 2009; Morrison and Scadden, 2014). The identification of HSCs using SLAM markers *in situ* (Kiel et al., 2005b) has allowed detailed visualization of primitive HSCs within the bone marrow in association with various niche cells. It was recently reported that HSCs (CD150⁺CD48⁻CD41⁻Lin⁻) associated significantly with bone marrow arterioles rather than with sinusoidal endothelial cells (Kunisaki et al., 2013). These arterioles are predominantly located near trabecular bone. In another study, novel genetic labeling techniques identified that, in contrast to deeply quiescent HSCs, highly proliferative HSCs (CD150⁺CD48⁻) reside in compartments termed ‘hemospheres’ (Wang et al., 2013a). Clonal proliferation of cells in these hemospheres was identified using *Vav1-Cre:R26R-Confetti* mice, and it was shown that the

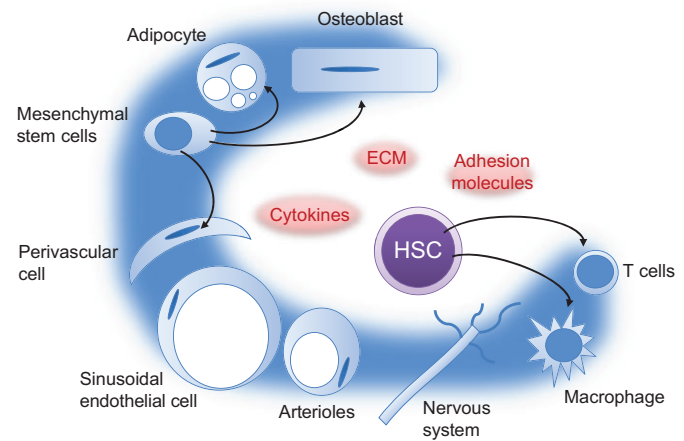


Fig. 5. Scheme showing the major components of the HSC niche in the BM. The hematopoietic stem cell (HSC) niche is composed of various non-hematopoietic cells, including osteoblasts (Arai and Suda, 2007), endothelial cells (ECs; Butler et al., 2010; Ding et al., 2012), perivascular cells (Ding et al., 2012; Sugiyama et al., 2006), mesenchymal stem cells (Méndez-Ferrer et al., 2010), osteoclasts (Kollet et al., 2006), sympathetic nervous system neurons (Katayama et al., 2006), adipocytes (Naveiras et al., 2009) and non-myelinating Schwann cells (Yamazaki et al., 2011). Other studies also indicate that mature hematopoietic cells, such as macrophages/monocytes (Chow et al., 2011) and regulatory T cells (Fujisaki et al., 2011), regulate HSCs. Niche cells regulate HSCs through the production of an array of cytokines and growth factors [e.g. thrombopoietin, stem cell factor, transforming growth factor β and stromal cell factor 1 (SDF1)], extracellular matrix proteins (e.g. tenascin and osteopontin) and adhesion molecules (e.g. N-cadherin) (Arai et al., 2004; Yoshihara et al., 2007; Nilsson et al., 2005; Nakamura-Ishizu et al., 2012; Zhang et al., 2003; Hosokawa et al., 2010; Qian et al., 2007).

maintenance of hemospheres was dependent on VEGFR2-mediated signaling exerted by sinusoidal endothelia located in the center of the structure. These data suggest that HSCs of different proliferative capacities may create compartments that allow for efficient association with their respective niches in the bone marrow, although the visualization of deeply quiescent HSCs in the BM are need to verify this possibility.

Another issue concerning the effects of niche cells on HSC cell cycle is the identification of niche factors that reverse proliferating HSCs to quiescence. Differentiated cells have been proposed to de-differentiate into tissue stem cells in the stomach and lung (Stange et al., 2013; Tata et al., 2013); it is unknown whether differentiated hematopoietic cells can produce HSCs. The mechanisms involved during the reversal of proliferating hematopoietic stem and progenitor cells to quiescence during hematopoietic recovery after myelo-suppression were investigated in a recent study (Brenet et al., 2013). TGF β signaling is known to regulate HSC quiescence during steady state hematopoiesis (Yamazaki et al., 2011; Jacobsen et al., 1991) but, in this study, activation of the TGF β receptor was evident in LSK cells during hematopoietic recovery after 5-FU administration. Antibody-mediated block of TGF β signaling resulted in reduced expansion of hematopoietic stem and progenitor cells, including immature CD150⁺CD48⁻LSK cells, showing that TGF β reverses the proliferative state of HSCs to quiescence during hematopoietic recovery. Furthermore, the study suggested that upregulation of the cyclin-dependent kinase inhibitor (CKI) p57, compared with other CKIs, was responsible for the reversal of the cell cycle. Given that TGF β -mediated block can delay the return of HSCs to quiescence and hence speed the recovery of hematopoiesis after myelo-suppression, similar effects on the cell cycle caused

by other extrinsic niche factors may be of use for clinical applications.

Metabolic regulation of quiescence

HSCs are subjected to a specialized metabolic environment and profile. Owing to their dormancy, the energy demand of quiescent HSCs is lower than that of proliferative progenitor cells. Indeed, HSCs in the BM are located in a predominantly hypoxic environment and their metabolic demands have adapted to chronic hypoxia (Suda et al., 2011). The characteristics of HSC metabolism and how they affect HSC quiescence have been reviewed extensively (Suda et al., 2011; Ito and Suda, 2014). One unsolved issue may be whether the low metabolic profiles of quiescent HSCs are cell intrinsic or a result of the hypoxic microenvironment.

Although evidence confirms that the BM is generally hypoxic (Kubota et al., 2008; Parmar et al., 2007), whether quiescent HSCs distribute towards the region of lowest oxygen tension remains an unresolved issue. Recently, two-photon microscopic analysis with chemical probes displayed that the BM was hypoxic in general (<32 mm Hg) but with a gradient of oxygen tension: the lowest occurring in the peri-sinusoidal regions (Spencer et al., 2014). In this study, the degree of hypoxia did not parallel the presence of Nestin⁺ arterioles, which are known as niches for quiescent HSCs (Kunisaki et al., 2013). High-quality imaging techniques have enabled further assessment of the hypoxic state in relation to the spatial organization of hematopoietic stem and progenitor cells within the BM (Nombela-Arrieta et al., 2013). Pimonidazole-high cells, which are cells exposed to hypoxia, were distributed randomly throughout the BM without a preferential distribution to a specific niche. Pimonidazole incorporation showed no remarkable change in hematopoietic stem and progenitor cells, despite differing cell cycle status. Furthermore, observations of proliferating hematopoietic stem and progenitor cells after 5-FU treatment show that proliferating cells express high levels of the transcription factor hypoxia-inducible factor 1 (HIF1) during hematopoietic reconstitution. It is therefore still not known whether the expression of HIF1A is a consequence of the hypoxic microenvironment within the BM or whether HIF1A expression in HSCs is regulated intrinsically.

Conclusions

Current assessments of the *in vivo* ‘stemness’ of HSCs rely on *ex vivo* purification through a combination of cell-surface markers, assessment of cell cycle status and *in vivo* long-term repopulation potential upon transplantation. To date, many of these characterization methods remain invasive, although advances in *in vivo* imaging and labeling are slowly providing us with useful tools. Nonetheless, it is clear that HSC stem cell potential should be assessed through various phenotypical and functional aspects, not only those that are quiescence related, and both on a population basis and at the single cell level. Indeed, as single cell analyses are improving, the heterogeneity in lineage differentiation in highly purified HSCs is becoming evident, and multi-lineage differentiation potential is no longer an indispensable definition of bona fide HSCs. The further characterization of cell-surface markers that can specifically identify deeply quiescent and highly repopulating HSCs is still needed for enhancing our understanding of HSC biology and for clarifying the relationships between different HSC properties. Improved methods to analyze quiescence and to identify refined populations of HSCs will also deepen our understanding of their intrinsic and extrinsic regulation, thereby providing vital knowledge that can be extrapolated to therapeutics for hematological diseases.

Competing interests

The authors declare no competing financial interests.

Funding

A.N.-I.’s research was supported in part by a fellowship from Japan Society for the Promotion of Science (JSPS) and by a Grants-in-Aid for JSPS Fellows [25-40030 and 05-045-0166/4096]. T.S.’s research is supported by the Seventh Framework Program of the European Union [306240 (SystemAge)], by a Grant-in-Aid from the JSPS, by a National Medical Research Council grant and by a Singapore Translational Research (STaR) Investigator Award.

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