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Dissecting dormancy and quiescence in hematopoietic stem cells

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Quiescence is a fundamental state of adult hematopoietic stem cells (HSCs) characterized by their residence in the G0 phase of the cell cycle. Despite being quiescent, HSCs retain their capacities for self-renewal and multipotency, enabling them to produce all blood lineages. Recent discoveries have shown that HSCs can dive into an even deeper state of quiescence with a very low division rate in steady-state conditions, known as dormancy. Dormant HSCs (dHSCs) have the most superior stem cell properties among HSCs, placing them at the top of the hematopoietic hierarchy. In this review, we argue that quiescence and dormancy are not synonyms in the context of HSCs. Specifically, dHSCs constitute a unique reserve pool of HSCs, mobilized only under stress conditions to protect the HSC compartment throughout life. While HSC quiescence is well-studied, the molecular features of HSC dormancy remain less well-defined. We will discuss the available methods for dHSC isolation and summarize the latest findings on the roles of niche factors, transcription factors, chromatin regulators, and cell cycle-related proteins in maintaining HSC dormancy. Additionally, we will explore whether insights from the quiescent HSC research can be applied to dHSCs. Lastly, we will assess the therapeutic potential of utilizing or targeting dHSCs to improve stem cell transplantation outcomes and treat hematological diseases, opening new avenues for research and clinical applications in regenerative medicine and oncology.

KEYWORDS

hematopoietic stem cells, dormancy, quiescence, bromodeoxyuridine, H2B-GFP, label retention assay, retinoic acid signaling, CD38

1 Introduction

Hematopoietic stem cells (HSCs) are rare cells that continuously regenerate the entire hematopoietic system, producing billions of blood cells. In vertebrates, the primary definitive HSCs originate from the hemogenic endothelium on the ventral wall of the dorsal aorta within the aorta-gonad-mesonephros region via a process termed endothelial-

to-hematopoietic transition. Following their emergence, HSCs migrate to the fetal liver before ultimately colonizing the bone marrow (BM), which then becomes the predominant site of adult hematopoiesis (1). Adult HSCs primarily reside within the distinct environment of the BM, termed the 'BM niche'. This niche is populated by specialized cells that provide HSCs and progenitors with vital factors necessary for their maintenance, survival, and differentiation (2).

Two defining qualities of HSCs are self-renewal and multipotency. Self-renewal refers to the ability of HSCs to replicate themselves to maintain their population, while multipotency denotes their capability to differentiate into all types of mature blood cells (3). According to the hematopoietic tree model, HSCs evolve into mature cells through intermediate stages involving hematopoietic progenitors with varying differentiation and self-renewal potentials (4). These include multipotent progenitors (MPPs), which retain the capacity to produce all blood lineages (5), lineage-committed progenitors, and unipotent progenitors, the latter being capable of producing only a single type of cells. While the classical model of HSC differentiation posits that HSCs undergo distinct step-wise progenitor phases, recent evidence challenges this view, suggesting that HSCs may independently determine their lineage-specific fate (6, 7). For instance, it was demonstrated that HSCs are able to directly differentiate into myeloid progenitors without undergoing cell division upon transplantation into non-irradiated mice (8). Moreover, HSCs could bypass numerous downstream progenitor stages to produce mature erythrocytes (9). Additionally, another study provided evidence that megakaryocytes (Mks) might also originate directly from HSCs (10). Specifically, a single-cell *in vitro* culture of murine HSCs showed that they could produce Mks without undergoing cell division. Therefore, despite significant advances in understanding the differentiation process, the full scope and nature of it is yet to be firmly established. Nevertheless, the immense differentiation capacity of HSCs is crucial for maintaining hemostasis and responding to injury and disease.

Intriguingly, even though blood is a constantly renewing tissue, most of HSCs remain non-cycling or quiescent. In homeostasis, most HSCs reside in the G0 phase of the cell cycle and only a small fraction of HSCs enters the cell cycle to undergo lineage commitment or self-renewal divisions (11). HSCs in the G0 phase of the cell cycle, or quiescent HSCs, can be identified by their DNA content (2N), lower RNA content than in cells in the G1 phase, the absence of expression of proliferation markers, such as Ki67, or by label incorporation assay (12). The simplicity and accessibility of methods for detecting HSCs in the G0 phase of the cell cycle lead to the thorough investigation of HSC quiescence, revealing the key molecular players associated with this state (13–15). HSC quiescence is critical for the long-term sustenance of HSCs, protecting them from the accumulation of replication-associated DNA damage (16). Studies reveal that disruption of HSC quiescence results in excessive replication, leading to impaired capacity for self-renewal and loss of repopulation capacity in transplantation assays (17, 18). Overly proliferation leads to HSC premature exhaustion (19), emphasizing the importance of quiescence for maintaining the "stemness" of HSCs.

For a long time, the terms quiescent and dormant HSCs have been used interchangeably (12, 14, 15, 20). However, the seminal studies by the Trumpp group defined dormant HSCs (dHSCs) as a distinct subpopulation of quiescent HSCs, which are characterized by a deeper state of quiescence and very low overall biosynthetic activity. While quiescent HSCs represent about 70% of the total HSC population (21, 22), the dHSC subset constitutes only 15-30% of the HSC pool (23–25). By definition, dHSCs have extremely low *in vivo* divisional history, reflected by their chromosome label retention in long-term label retention assays (Figure 1A). Importantly, dHSCs demonstrate the highest long-term repopulation capacity in transplantation assay and are less responsive to activating stimuli than quiescent HSCs. Yet, dHSCs can reversibly enter the cell cycle in response to severe stress signals, such as bacterial or viral infection or blood loss (24, 26, 27). Thus, dHSCs are a unique subpopulation of HSCs, which resides at the apex of the hematopoietic hierarchy and serves as a reserve pool of HSCs during the entire life (23, 24, 28). The inability to maintain the dormant state may expose dHSCs to replication stress and promote the accumulation of somatic mutations, increasing the risk of their exhaustion or malignant transformation (29). Conversely, dHSCs failing to enter the cell cycle under stress conditions may limit blood cell production, potentially resulting in bone marrow failure (30). Therefore, tight control of HSC dormancy is vital for effective and healthy hematopoiesis.

While quiescent HSCs are extensively studied (13, 15), the nature of dormancy in HSCs remains less explored. The advent of reporter mice based on retinoic acid-induced gene Gprc5c and the discovery of the surface marker CD38 for the identification of dHSCs shed light on the mechanisms supporting dHSCs' function (24, 25). In this review, we aim to provide the differentiation between the states of quiescence and dormancy in HSCs. We will discuss the latest discoveries that have established the unique features of HSC dormancy and offer insights into the extent to which our understanding of quiescent HSCs can be applied to dHSCs.

2 Identification of dormant HSCs

In the previous paradigm, it was believed that all HSCs periodically enter and exit the cell cycle, with the entire HSC pool undergoing turnover within weeks (31). However, later studies have firmly established the presence of the subpopulation of dHSCs within the quiescent HSC compartment that shows almost no divisional activity under steady-state conditions (23, 28, 32). To identify these dHSCs, long-term label retention assays have been employed. Typically, an initial "pulse" phase includes HSC labeling followed by a "chase" phase, where this label is progressively diluted over an extended period of time due to cell divisions (Figure 1A). One prevalent approach utilizes a thymidine analogue, bromodeoxyuridine (BrdU), which integrates into the DNA during the S-phase of the cell cycle within the labeling period. As cells divide post-labeling, the BrdU is diluted among progeny. Rapidly dividing HSCs will dilute the BrdU quickly (named active HSCs, aHSCs), while dHSCs will retain the BrdU label for more

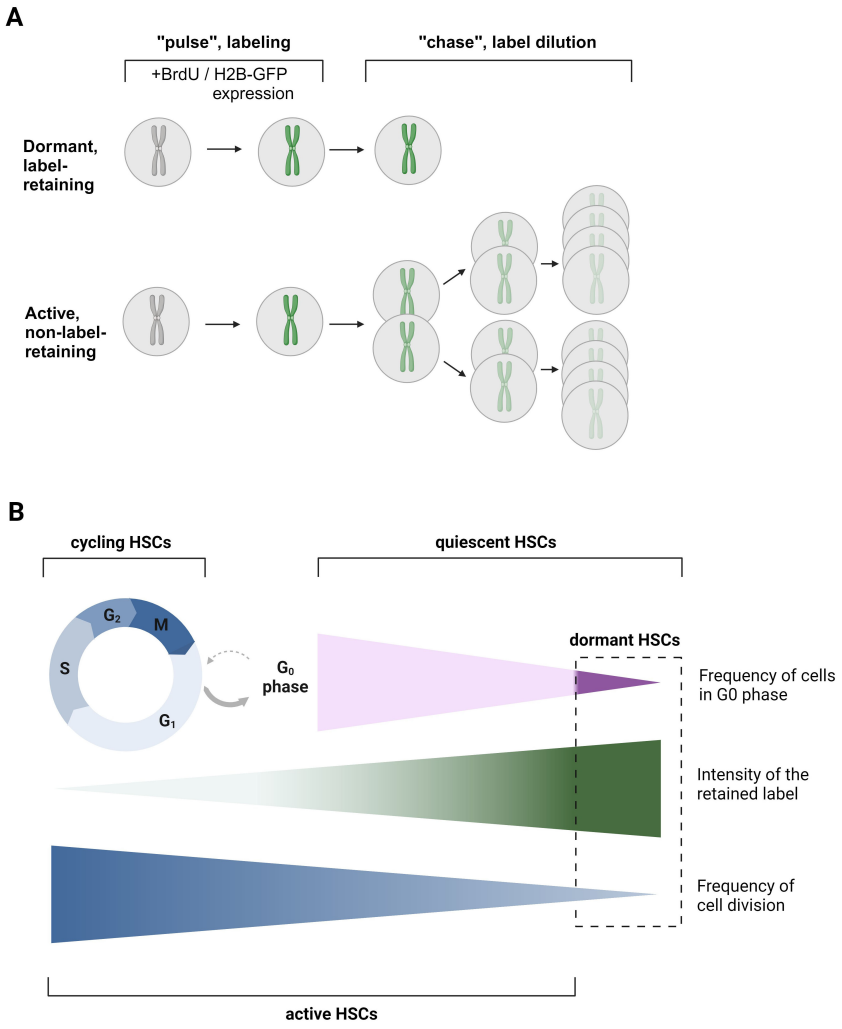


FIGURE 1
Label-retention assay as a classical approach for dHSC detection. **(A)** The principle of label retention assay. During the "pulse" or labeling phase, cells either incorporate BrdU, or the expression of H2B-GFP is induced by the Tet-on system through the administration of doxycycline (dox). In the subsequent 'chase' or label dilution phase, the previously introduced label is diluted out with each cell division after the withdrawal of BrdU/Dox or by turning off H2B-GFP expression in the Tet-off system through dox administration. This dilution allows for the distinction between dormant (non-dividing) and active (dividing) cells. BrdU – bromodeoxyuridine. **(B)** Scheme explaining the differences in terms quiescent HSCs, dHSCs, aHSCs, and cycling HSCs. Quiescent HSCs are those HSCs, which are in the G0 phase of the cell cycle. dHSCs, dormant, HSCs, are the most quiescent, label-retaining HSCs. aHSCs, active HSCs, are those HSCs that have undergone or are undergoing cell division. Cycling HSCs are those HSCs that are in G1-S-G2-M phases of the cell cycle. The figure was created using Biorender.com.

than 70 days. The presence of two subpopulations of HSCs, dHSCs and aHSCs, has been predicted by mathematical modeling based on BrdU dilution kinetics. While the one-population model failed to explain the experimental data, the two-population model describes it with great accuracy, strongly suggesting the presence of dHSCs (23, 32). According to Wilson et al., dHSCs are predicted to divide approximately every 145 days in mice, resulting in only about 5 divisions during a murine lifetime, whereas aHSCs likely divide every 36 days. Importantly, Ki67-Hoechst 33342 staining revealed not only that nearly all dHSCs reside in the G0 phase but also that 83% of aHSCs are in the G0 phase too (Figure 1B) (23). It is worth noting that the percentage of total HSCs in the G0 phase in this study is higher than in previously cited work (21) due to the use of the HSC phenotype (Lineage$^-$ c-Kit$^+$ Sca-1$^+$ CD48$^-$ CD150$^+$ CD34$^-$), enriching for quiescent HSCs. Therefore, those aHSCs are highly quiescent, although at least a part of them are transcriptionally cell-cycle primed (24).

Nevertheless, the BrdU assay has limitations: antibody-mediated BrdU recognition requires cell fixation, and therefore, it cannot be used to study viable dHSCs. Additionally, BrdU has been suggested to cause an injury response in the periphery, which induces a positive feedback loop leading to dHSC activation (23). This can explain why dHSCs are successfully labeled in only a two-week period. Therefore, an advanced system involving transgenic mouse strain R26^{rtTA}/*Col1A1*$^{H2B\text{-}GFP}$ has been established (23, 28). It allows ubiquitous expression of chromatin structural protein histone 2B fused with a green fluorescent protein (H2B-GFP) under the control of an inducible tetracycline-mediated system (using either Tet-on or Tet-off systems), resulting in the labeling of chromosomes. Mirroring the BrdU approach, labeled cells dilute

the H2B-GFP with each division, allowing the identification of dHSCs as those HSCs that retain this label longer than 70 days (Figure 1A).

In contrast to a BrdU assay-based prediction (23), H2B-GFP dilution data argued that dHSCs count their divisions and divide only 4 times before entering permanent quiescence (33). This conclusion, however, has been challenged by Morcos and colleagues, who demonstrated that the H2B-GFP assay cannot be used for accurately determining the number of HSC divisions due to H2B-GFP short degradation time of 4-6 weeks, inhomogeneous initial labeling, and background fluorescence due to leaky H2B-FP expression. Furthermore, performing two consecutive rounds of pulse and chase revealed that HSCs from older mice, following the second pulse, diluted the label at a rate similar to that of younger mice after their first pulse. Therefore, it has been inferred that dHSCs undergo continuous cell cycle activity without losing their potential after 4 divisions (34). However, the H2B-GFP assay allows the labeling of the most quiescent dHSCs (25). The transplantation of H2B-GFP^{hi} HSCs revealed that dHSCs have significantly higher repopulation potential and self-renewal capacity compared with aHSCs with the same surface marker phenotype, which have significantly diluted the H2B-GFP label. The H2B-GFP^{lo} HSCs also failed to reconstitute lethally irradiated mice in serial transplantation assay (23, 35). This superiority in quiescence, self-renewal, and reconstitution capability positions dHSCs at the top of the hematopoietic hierarchy.

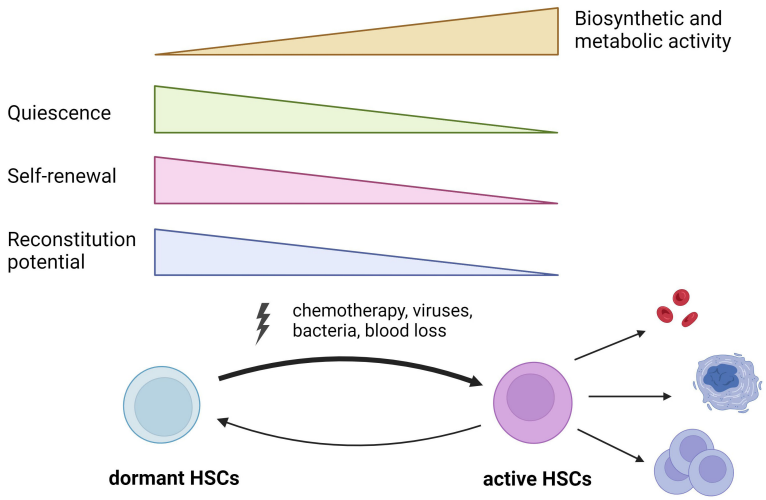
3 dHSCs act as a reserve pool of HSCs

Due to their very infrequent cycling, dHSCs do not significantly contribute to daily blood cell production under steady-state conditions. Instead, dHSCs come into action upon receiving the signals of severe hematopoietic stress. Post *in vivo* treatment with either chemotherapeutic agent 5-fluorouracil (5-FU) or the granulocyte colony-stimulating factor (G-CSF), known for mobilizing HSCs from BM, dHSCs begin cycling as evidenced by the loss of BrdU or H2B-GFP label (23). Another significant factor affecting dHSCs is interferon alpha (IFNα), a cytokine produced by immune cells in response to challenges like viruses, bacteria, and tumors. A single high-dose injection of IFNα or the viral RNA mimetic polyinosinic:polycytidylic acid (polyI:C) - which similarly triggers the IFNα response - can promote dHSC activation *in vivo* (24, 26). A component of the outer wall of Gram-negative bacteria, lipopolysaccharide (LPS), is also capable of inducing dHSC proliferation (24, 27). Importantly, once the stressor is removed and conditions return to normal, dHSCs rapidly revert to their dormant state to preserve the HSC pool (23). Nevertheless, chronic stress conditions, such as repetitive cycles of polyI:C injections can lead to irreversible HSC exhaustion - loss of stem cell function due to reaching the limit of their self-renewal capacity (36). Frequent divisions can also favor the accumulation of somatic DNA mutations, potentially resulting in oncogenic transformation (37). Besides, dormancy protects HSCs from chemotherapeutic agents. When HSC dormancy is disrupted before chemotherapeutic interventions - as observed with repeated polyI:C injections followed by two rounds of 5-FU treatment - it can lead to fatal anemia, likely due to the eradication of all dHSCs (26). Thus, dormancy not only preserves the dHSCs from replication stress, ensuring their functionality, but also positions them as a reserve pool of HSCs throughout an individual's lifetime.

4 Molecular features of dHSCs

Prior research using label retention assays and reporter mice to identify dHSCs has elucidated some of their unique molecular signatures. In this section, we will primarily rely on the studies comparing dHSCs with aHSCs, as the latter represent the best available approximation to quiescent HSCs thus far (Figure 1B). Consistent with their biological function, dHSCs exhibit transcriptional profiles demonstrating markedly reduced biosynthetic activity, including diminished metabolism, downregulated DNA replication machinery, decreased mRNA processing and lower rates of ribosomal biogenesis (23, 24) (Figure 2). Furthermore, dHSCs are characterized by very low levels of *CD34* mRNA (23) and enriched in MolO gene expression signature, which indicates the most functional HSCs (38). Concurrently, tumor suppressor with deubiquitinase activity *Cyld* (cylindromatosis) exhibited high mRNA transcript levels in dHSCs but not in MPPs. Tesio and colleagues demonstrated that CYLD inhibits dHSC cycling by deactivating the pathway involving tumor necrosis factor receptor-associated factor 2 (TRAF2) and p38MAPK. Mechanistically, CYLD removes polyubiquitin chains from TRAF2, thereby decreasing p38MAPK activity and preventing cell proliferation (39).

Notably, Cabezas and colleagues established a critical role of retinoic acid (RA)-induced signaling in the maintenance of HSC dormancy. They found that dHSCs are enriched in retinoic acid-induced gene *Gprc5c* mRNA (orphan G protein-coupled receptor, class C, group 5) compared to aHSCs and MPPs. Subsequently, a transgenic reporter mouse based on EGFP expression under Gprc5c promoter (Gprc5c-EGFP mouse) was developed, enabling the detection of dHSCs. By utilizing this mouse, authors showed that lack of dietary vitamin A in mice results in the disruption of HSC quiescence, whereas administering the active metabolite of vitamin A, all-trans-RA, maintains dHSC quiescence under stress conditions both *in vitro* and *in vivo* (24). In the follow-up study, they discovered the non-classical RA signaling axis, which supports HSC stemness (40). According to their data, all-trans RA is converted by Cyp26b1 enzyme into 4-oxo-RA metabolite, which in turn binds to RA receptor beta (RARβ). The latter is responsible for activating a transcriptional program enhancing HSC identity. Although this study primarily concentrated on HSCs rather than dHSCs, *Cyp26b1* transcript was identified as being enriched in dHSCs in the previous study from this group (24), suggesting that Cyp26b1/4-oxo-RA/RARβ axis might be relevant for dHSC function too. Another research group unveiled the role of another class of RA receptors - RXRs (retinoid X receptor), in the maintenance of HSC quiescence and function. While the individual deletions of either *Rxra* or *Rxrb* do not markedly impact HSC fitness, the double deletion of both *Rxra* and *Rxrb* results in



BrdU^{hi}, H2B-GFP^{hi}	BrdU^{lo}, H2B-GFP^{lo}
Cytosolic Ca^{2+}	
Elevated expression of:	
Gprc5c	*Myc*
Cyld	*CD34*
Gfi-1	Cdk6
Neo-1	
CD38	
c-Fos	
$p57^{Kip2}$	

FIGURE 2
Summary of key characteristics distinguishing dHSCs from aHSCs. BrdU, bromodeoxyuridine, *Cyld*, cylindromatosis; Cdk6, cyclin-dependent kinase 6; Gfi-1, growth factor independent 1; Gprc5c, orphan G protein-coupled receptor, class C group 5; H2B-GFP, histone 2B-green fluorescent protein; Neo-1, neogenin 1. The figure was created using Biorender.com.

HSC activation, subsequently inducing myeloproliferative-like disease (41). Importantly, a study by Zhang and colleagues disclosed that Gprc5c protein is also expressed in human HSCs (hHSCs) (42). Their work also suggests that the Gprc5c^{+} hHSCs can be categorized as dHSCs, resolving a long-standing question regarding the existence of dHSCs in humans.

BM niche has a complex three-dimensional structure involving multiple components: osteoblasts, which form the endosteal niche; endothelial cells that create a sinusoidal and arteriolar vascular niche; mesenchymal stem cells - MSCs, known for their secretory functions; other cells like Mks, immune cells (regulatory T-lymphocytes, macrophages) and both sensory and sympathetic neurons, each contributing uniquely to the niche's complexity (2). For many years, it was widely believed that quiescent HSCs resided in the endosteal niche (20). However, recent insights have revealed that they predominantly occupy perisinusoidal niches in the BM (43, 44). In agreement with this, the interaction of neogenin-1 (Neo-1) expressed on dHSCs with netrin-1, which is most likely produced by arteriolar endothelial and periarteriolar stromal cells in the BM niche, has been suggested (27). Although extracellular Ca^{2+} was once thought to anchor quiescent HSCs near the endosteum (45), the proposed localization of dHSCs in the perivascular niche, alongside the finding emphasizing the significance of intracellular Ca^{2+} for dHSCs, has shifted this perspective. The role of intracellular Ca^{2+} for dHSCs has been discovered using a reporter mouse based on the cell cycle inhibitor $p27^{Kip1}$, which enables the detection of HSCs in the G0 cell cycle phase (mVenus-$p27K^{-}$ mouse). High-throughput small-molecule screening using this reporter mouse revealed that high levels of cytoplasmic Ca^{2+} are associated with HSC dormancy (46). However, the precise function of Ca^{2+} in dHSC maintenance remained unknown. Recently, we revealed that cADPR, synthesized through the conversion of NAD^{+} by CD38, elevates cytoplasmic Ca^{2+} in dHSCs. This elevation induces the expression of the transcription factor (TF) c-Fos, which subsequently promotes dHSC quiescence in $p57^{Kip2}$-dependent manner. Thus, we identified CD38 as an important missing mediator of higher levels of intracellular Ca^{2+} in dHSCs in steady state (25).

Despite significant advancements in the field of dHSCs (Figure 2), progress has been impeded by challenges in identifying and isolating dHSCs. The H2B-GFP label retention assay is time-consuming and necessitates a lengthy chase period,

making the study of dHSCs in young mice unfeasible. Furthermore, Gprc5c-EGFP and mVenus-p27K$^-$ mouse models are not commercially accessible, and to date, there are no known monoclonal antibodies for FACS recognizing dHSC markers Gprc5c or Neo-1, hindering the straightforward isolation of dHSCs. We discovered CD38 as a surface marker for murine dHSCs, which should facilitate the future investigation of HSC dormancy using simple antibody staining for dHSC FACS isolation. Currently, the upstream regulators responsible for very low metabolic and synthetic activities observed in dHSCs, including additional niche factors, signaling proteins, epigenetic regulators, and transcription factors, are not well characterized. Therefore, the next part of the review will focus on determining whether the molecular pathways known to induce quiescence in HSCs also play a role in regulating the dormancy of dHSCs.

5 Known quiescence regulators in the context of dHSCs

Quiescence is a well-studied cellular state in HSCs, with numerous genes shown to play a role in maintaining this state (13–15). As dHSCs represent a subfraction of quiescent HSCs, the molecular features of these two populations most likely overlap significantly. We propose that each known regulator of HSC quiescence should be individually validated in the context of dHSCs. To identify the top candidates of interest, we analyzed the gene lists comparing transcriptional profiles of active HSCs and dHSCs (24), including our own (25). We listed the most promising candidates below, however, in the future, they need to be validated at the protein level using label-retention assay, reporter mice or updated dHSC phenotype (25).

5.1 Niche factors

BM niche produces factors including various cytokines, chemokines, and extracellular matrix components interacting with their specific receptors or adhesion molecules on HSCs to maintain their quiescence in homeostasis or activate them in response to stress ((8), reviewed extensively in (12)). Binding of the quiescence factors to their respective receptors on HSCs (e.g. stem cell factor (SCF) and c-Kit; angiopoietin-1 (Ang-1) and Tie2; thrombopoietin (TPO) and myeloproliferative leukemia protein (MPL)) triggers signaling cascades inducing cell cycle arrest. Such cascades involve signaling tyrosine kinases, transcription factors, epigenetic regulators, and cell cycle-related proteins inhibiting the cell cycle progression, which will be discussed below. Genes related to cell-to-cell and matrix interactions were found to be increased in H2B-GFP^{hi} dHSCs (25, 47), which might indicate ongoing communication between dHSCs and their surrounding microenvironment, keeping them ready to quickly determine their fate. Remarkably, leaving the dormant state was accompanied by an immediate decrease in the expression of genes associated with niche communication, possibly indicating a migration to different niches. Furthermore, dHSCs are enriched in TGF-β signaling signature genes (25, 47). TGF-β is a well-established regulator secreted by Mks and non-myelinating Schwann cells (48–50), which inhibits the cell cycle progression of HSCs via p57^{Kip2} (48). Thus, niche-secreted factors most likely play an important role in the support of HSC dormancy.

The localization of dHSCs in the BM is poorly studied. Confocal microscopy imaging revealed that label-retaining dHSCs were found as individual cells homogenously distributed in the BM, indicating the exceptionally small size of their niches (23, 51). It has been identified earlier that H2B-GFP^{hi} dHSCs were enriched in trabecular bone region and were retained there via non-canonical Wnt signaling (52), in contrast to the later study suggesting that dHSCs reside in perivascular niches guided by Netrin-Neo-1 signaling (27). Additionally, recent studies have demonstrated an important role of extracellular matrix component hyaluronic acid in the maintenance of HSC dormancy in mice and humans (42). Moreover, we have found that CD38 ecto-enzymatic activity on the neighboring cells can promote hHSC quiescence (18). Therefore, BM niche is an essential component of HSC dormancy regulation and requires further investigation.

5.2 Cell cycle regulators

HSCs transit into the G1 cell cycle phase upon receiving the mitogenic stimuli from the niche cells. This transition is tightly controlled by the so-called restriction point, which is mediated through the protein complexes consisting of cyclin-dependent kinases (CDKs) and corresponding cyclins. In the early G1 phase, before the restriction point, cyclin D is produced in response to mitogen stimulation and then forms complexes with either CDK4 or CDK6. Cyclin D-CDK4/6 complex inactivates the tumor suppressor Retinoblastoma protein (Rb) by phosphorylation, which mediates the release of E2F transcription factors from Rb-E2F complex, subsequently leading to the activation of S-phase-related transcriptional program. Rb phosphorylation marks the cell's transition through the restriction point into the late G1 phase. Here, E2F transcription factor activity leads to the expression of cyclin A and cyclin E, which form complexes with CDK2. The resulting cyclin A/E-CDK2 complex hyperphosphorylates Rb, leading to its full inactivation and further transition into S-phase (15, 53). In the absence of mitogenic stimuli, cells enter the reversible state of cell cycle arrest or quiescence. It is further enhanced by cyclin-dependent kinase inhibitors (CKIs) represented by two families, which suppress the activity of CDKs halting the transition through the cell cycle. The members of Cip/Kip CKIs family: p21^{Cip}, p27^{Kip1}, and p57^{Kip2}, suppress the activity of CDK4, CDK6, and CDK2. The members of INK4 family of CKIs: p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D}, have a narrower function inhibiting only CDK4/6 (54).

The roles of cell cycle-related proteins in HSC cell cycle regulation discovered using knock-out mice are extensively discussed in (15, 53, 55). Briefly, cyclins D1/D2/D3 and Cdk2/4/6 likely play redundant roles in HSCs since single deletions of Cyclin D members or a Cdk do not result in significant hematopoietic phenotypes, in contrast to the severe abnormalities observed during embryogenesis with combined deficiencies of either Cyclins D1-3,

Cdk2&4 or Cdk4&6. While full knock-out Cdk6$^{-/-}$ mice do not exhibit any changes in the frequencies of HSCs in the G0 cell cycle phase in a steady state, they fail to start proliferating efficiently upon polyI:C administration (56). In humans, long-term HSCs display lower levels of Cdk6 protein than short-term HSCs, which are primed for activation (57). As for CKIs, p57^{Kip2} is recognized as a primary mediator of HSC quiescence in homeostasis (17, 18). p27^{Kip1} deficiency does not affect HSC and MPP compartments but rather progenitors, causing their increased proliferation (58). p21^{Cip} is not required for HSC quiescence in homeostasis (59) but becomes critical during the stress response, as it is a target gene of p53 (60). INK4 family CKI p16^{INK4A} is dispensable in young mice (61); however, in old mice, it is associated with age-related HSC decline (62). Surprisingly, p18^{INK4C} deficiency leads to increased cycling of HSCs due to enhanced self-renewal capacity, which in turn results in improved functionality (63). The relevance of other INK4 family members for HSC quiescence, p15^{INK4B} and p19^{INK4D}, has to be defined in further research.

Within the context of dHSCs, it has been shown that they are characterized by low protein levels of CDK6 (24) along with high levels of p57^{Kip2} CKI (25). Monitoring p27^{Kip1} activity has been suggested for defining dHSCs using mVenus-p27K^{-} reporter mouse (46). It should be noted, however, that this reporter allows only 50% enrichment for label-retaining dHSCs (46). The deletion p21^{Cip} did not alter the frequency of H2B-GFP^{hi} HSCs (28), indicating its nonessential role for dHSCs. The potential contributions of other CKIs to the deeply quiescent state typical for dHSCs remain to be fully investigated.

5.3 Transcription factors

The expression levels of Myc oncogene, which promotes cell cycle activity via repression of p21^{Cip} and is also necessary for HSC differentiation (64, 65), are low in dHSCs (24). While other TFs have been shown to support HSC quiescence, their role remains unexplored for dHSCs. Gfi-1 (growth factor independent 1) is a zinc-finger transcriptional repressor recruiting histone-modifying enzymes to suppress the transcription of target genes. Gfi-1 is also essential for lymphopoiesis and myelopoiesis (66). Studies using transgenic mice revealed that deletion of Gfi-1 leads to increased proliferation of either LSK (Lin^{-} Sca-1^{+} c-Kit^{+} (67)) compartment and HSCs (LSK Flt3^{-} (68), LSK CD48^{-} CD150^{+} (69)) via decreased *p21^{Cip}* mRNA expression (68), with subsequent loss of their functional capacity in competitive repopulation assay. Additionally, low levels of *Gfi-1* transcripts are associated with an inferior prognosis for acute myeloid leukemia (AML) patients (70) and can lead to early transformation into blast crisis in chronic myeloid leukemia (CML) (71). Gfi1b is a paralogue of Gfi-1 and a transcriptional repressor. The absence of Gfi1b leads to increased HSC proliferation without compromising their repopulation capacity (72). In the bulk RNAseq dataset comparing the transcriptome of dHSCs, aHSCs, and MPPs (24), *Gfi1b* expression is similar in all three populations, whereas *Gfi-1* expression is elevated in dHSCs compared to other populations, suggesting that Gfi-1 can indeed regulate the quiescent state of dHSCs. Indeed, loss of Gfi-1 leads to decreased frequency of H2B-GFP-retaining HSCs, indicating that it is indispensable for dHSCs (28).

Egr1 (Early growth response 1) is an immediate-early response (IER) gene highly expressed in HSCs (LSK Flt3^{-}) in homeostasis, whereas its expression is downregulated upon stimulation with chemotherapy and G-CSF. The lack of Egr1 causes enhanced HSC proliferation along with their egress from BM to PB. Despite this, Egr1$^{-/-}$ HSCs demonstrate comparable regenerative capacity to that of wild-type HSCs in the first two rounds of transplantation into recipient mice, although Egr1$^{-/-}$ HSCs exhibit a more rapid loss of function in subsequent serial transplantation. Mechanistically, it has been suggested that Egr1 supports HSC quiescence via decreased levels of *Bmi1*, an important epigenetic regulator, whose role will be discussed later, and *Cdk4* along with increased levels of *p21^{Cip}* (73). Upon HSC activation, Egr1 is suppressed by Cdk6 (56). As for dHSCs, it has been proposed that signaling mediated by niche-derived netrin-1 via its receptor Neo-1 leads to an increase in *Egr1* levels and a decrease in Cdk6 levels, thereby supporting HSC dormancy (27).

Intriguingly, we found that *Fos*, another member of IER genes, is enriched in dHSCs (25). c-Fos is recognized as a crucial positive regulator of myeloid differentiation (74) and demonstrates transient expression in stimulated cells, leading to cell cycle progression (75, 76). A previous study has reported that the IFNα/β inducible c-Fos overexpression suppresses the *in vitro* proliferation and colony formation of Lin^{-} Sca-1^{+} cells (77). However, this population mainly contains progenitor cells and a very small amount of dHSCs. Therefore, the role of c-Fos in the regulation of dHSC cell cycle remained unexplored. In our work, we have shown for the first time that c-Fos is regulated by the enzymatic activity of CD38 and supports the dormancy of adult HSC under physiological conditions via p57^{Kip2} cell cycle inhibitor (25). In line with our finding, another study reported that in contrast to adult dHSCs, the expression of Fos is downregulated in highly proliferative fetal HSCs (78). While adult HSCs attain quiescence approximately four weeks post-birth (79), the specific timing and factors facilitating the emergence of the dHSC population are yet to be identified and represent an area for future investigation.

Other TFs supporting HSC quiescent state were identified (12). Among these are tumor suppressor p53, which acts via *Gfi1* and *Necdin* (80); FoxO family TF Foxo3a, which suppresses the production of ROS and increases levels of *p27^{Kip1}* and *p57^{Kip2}* (81); homeobox family TF Pbx1, whose loss leads to reduction in *p57^{Kip2}* (82); another homeobox family TF Meis1, which decreases the levels of *Ccnd1* (83, 84); nuclear receptor superfamily TF Nurr1, which likely upregulates *p18^{INK4C}* (85); interferon regulatory factor, IRF2, acting via upregulation of *p27^{Kip1}* and *p57^{Kip2}* (86); and basic leucine zipper family TF HLF (hepatic leukemia factor) exerting its function via upregulation of IRF2 (87). Among these TFs, only *HLF* was suggested as a potential TF that regulates dHSCs, as its expression was exclusively elevated in CD150^{hi} dHSCs (47). Analysis of RNAseq datasets for dHSCs has not revealed any enrichment of other aforementioned TF genes in dHSCs, indicating that their specific roles in the context of dHSCs need to be individually validated.

5.4 Metabolism

The role of metabolism in the maintenance of dHSCs is fundamental to their functionality. By their nature, dHSCs reside in a state of deep quiescence accompanied by markedly low levels of metabolic and synthetic processes. Additionally, most HSCs reside in a hypoxic BM microenvironment with O_2 levels ranging between 1 to 4% (88), subjecting dHSCs to chronic hypoxia. Signaling pathway enrichment analysis has indicated a downregulation of TCA (tricarboxylic acid) cycle-related genes and mitochondrial respiratory chain complex genes in dHSCs (24, 25). Approximately 60% of label-retaining dHSCs exhibited low mitochondrial membrane potential (MMP), as evidenced by TMRE (tetramethylrhodamine ethyl ester) staining, compared to more actively dividing HSCs. The transcriptional profiles of MMP^{lo} HSCs closely resemble those of dHSCs, highlighting low mitochondrial activity as a key determinant of HSC dormancy. This low MMP is responsible for diminished levels of reactive oxygen species (ROS), thereby protecting them from apoptosis and accumulation of DNA damage. Interestingly, dHSCs also demonstrate lower glycolytic rates compared to their cell cycle-primed counterparts, maintaining a minimal level of metabolism essential for their sustenance. Lysosomes have been suggested as principal mediators of this reduced metabolic activity, with quiescent HSCs enriched in large lysosomes, which have a lower degradation capability and engulf mitochondria, thus limiting their metabolic function. Inhibition of lysosomal activity, followed by transplantation assay, revealed that reduced lysosomal function enhances HSC repopulation capacity (89). Therefore, the metabolic profile of dHSCs is not just a passive outcome of their dormancy but actively contributes to the maintenance of their stem cell identity.

The further characterization of dHSC metabolism is challenged by their rarity, with approximately 700-1000 cells present in the entire murine BM (23, 24), making techniques like the Seahorse assay not feasible. However, the development of low-input metabolomics tools should facilitate future research in identifying other metabolic contributors in dHSC biology [reviewed in (90)].

5.5 Epigenetic regulators

Epigenetic regulation modulates gene expression via various mechanisms, including DNA methylation, histone modifications, and non-coding RNAs, such as microRNAs and long non-coding RNAs. The minimal levels of metabolic activity and biosynthesis observed in dHSCs, along with blocked differentiation programs, imply a predominance of closed chromatin states and an abundance of repressive epigenetic marks. The epigenetic state of dHSCs remains largely unexplored primarily due to their scarcity. Nevertheless, few candidates have been suggested as potentially key players in dHSC biology. Bmi1, a component of the Polycomb repressive complex 1 (PRC1) that induces a repressive chromatin state, is critical for the self-renewal capacity of HSCs (91). Despite its consistent expression levels in both dHSCs and active HSCs (24), Bmi1 likely plays an important role in dHSC self-renewal. A study by Qiu and colleagues suggested the potential roles of SmarcA4, a component of SWI-SNF chromatin remodeling complex, and Mllt3/AF9 (myeloid/lymphoid or mixed-lineage leukemia; translocated to, 3), a chromatin reader component of the super elongation complex (SEC), in $CD150^{hi}$ label-retaining dHSCs (47). Later studies have demonstrated that SmarcA4 might be responsible for HSC quiescence upon stress (92), whereas Mllt3 has been shown to be a crucial regulator of hHSC stemness (93). Furthermore, a marked increase in the expression of *Satb1*, a chromatin organizer, was found in dHSCs (24). Previous studies have shown that a lack of Satb1 leads to a loss of HSC quiescence and enhanced differentiation (94), underscoring its potentially important role in dHSC regulation. Finally, advancements in low-input and single-cell epigenomics hold promise for uncovering other players in maintaining the epigenetic landscape of dHSCs (95, 96).

6 Discussion

In this review, we argue that separating dormant and quiescent HSCs is not a question of mere semantics; in fact, distinguishing between them is crucial, highlighting dHSCs as a unique subset. Cabezas-Wallscheid et al. have demonstrated that HSC transition through gradual intermediate states of quiescence before reentering the cell cycle rather than through a binary on/off switch, with dHSCs being the most quiescent subpopulation (24). Another intermediate state, the G_{alert} state, has been first identified for muscle stem cells (MuSCs), implying that such MuSCs are activation-primed but still quiescent. This state, marked by activation of mTORC1 expression, manifests after the distant muscle injury and endows MuSCs with enhanced regeneration potential. Similarly, HSCs exhibit a G_{alert} state in response to muscle injury, indicating at least two distinct quiescent states. G_{alert} stem cells revert to the G0 state after stressor removal and regeneration (97). It is unknown whether some of the HSCs in a steady state are predisposed to switching into G_{alert} upon stress. Nevertheless, this demonstrates a dynamic quiescence landscape.

dHSCs add another level of complexity to this model. While their ability to switch into the G_{alert} state remains unexplored, they are characterized by a deeper quiescence than other HSCs in the G0 cell cycle phase, with markedly reduced biosynthesis and metabolism. These properties are tightly linked with the enhanced functionality of dHSCs, serving as a reservoir for HSCs that are activated upon severe stress. The underlying reason for the difference between the extremely low division rate of dHSCs and more prone to entering the cell cycle quiescent HSCs in homeostasis remains unclear (98). Future studies will reveal whether this difference is mediated by distinct mechanisms keeping quiescent HSCs and dHSCs in the G0 phase or by differing mechanisms controlling the exit from the G0 phase for both populations.

dHSCs hold promise for the development of therapeutic strategies. Identifying specific surface markers for human dHSCs could enable the enrichment of highly potent donor HSCs, enhancing the success of transplantation outcomes. Future studies are essential to explore strategies for mobilizing dHSCs without depleting their reserve. Hematological cancers often lead to healthy HSC dysfunction and resultant pancytopenias (99), as these HSCs become trapped in dormancy by cancer cell byproducts. Developing therapies to activate dHSCs in the context of cancer could unlock further clinical potential. Notably, many hematologic malignancies, such as chronic lymphocytic leukemia, AML, and multiple myeloma, exhibit high

levels of CD38 expression (100). The coculture of hHSCs with $CD38^+$ tumor cells, in the presence of inhibitor targeting CD38 or cADPR antagonist, revealed that inhibition of CD38 enzymatic activity led to the cell cycle entrance of hHSCs. Moreover, human bone marrow imaging showed that quiescent hHSCs colocalized with $CD38^+$ cells in healthy patients (25). We propose that, through a paracrine mechanism, a tumor microenvironment enriched with the products of CD38 ecto-enzymatic activity may suppress the cell cycle of healthy hHSCs, leading to cancer-related pancytopenia. Therefore, inhibiting CD38-mediated cADPR production might support healthy hematopoiesis in patients with hematologic malignancies.

Exploring the regulatory mechanisms behind dHSC quiescence can provide crucial insights into the survival tactics of dormant cancer stem cells, particularly within AML. AML is characterized by a cellular hierarchy dominated by leukemia stem cells (LSCs) that share several stem-like features with dHSCs, such as self-renewal capabilities, the potential to differentiate into blast cells, and notably, a resistance to chemotherapy attributed to their dormant state. This dormancy is a key factor in the persistence of LSCs and a significant cause of AML relapse (101). By comparing the dormancy mechanisms of dHSCs and LSCs, it would be possible to identify novel therapeutic targets to disrupt the persistence of LSCs. Consequently, this could lead to more effective leukemia treatments and a decrease in the rates of relapse in AML.

Author contributions

LI: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. TG: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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