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Genetic Models to Study Quiescent Stem Cells and Their Niches

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Abstract

Hematopoietic stem cells (HSC) have been defined by their ability to establish long-term hematopoiesis in myelo-ablated hosts. Prospective isolation using combinations of cell-surface markers and/or dye exclusion can yield highly purified and nearly homogeneous phenotypically defined cells that repopulate irradiated hosts. Although highly informative, these types of analyses may not necessarily reflect ongoing homeostatic hematopoiesis. HSCs are also described as being quiescent. This has been demonstrated by cell cycle analysis of phenotypically defined HSCs. Some studies have challenged the existence of truly quiescent HSCs, suggesting that they continuously cycle, albeit with very slow kinetics. Here we present a pulse–chase system based on the controllable incorporation of H2B-GFP into nucleosomes, which allows the identification, purification, and functional analysis of viable label-retaining cells. Our data complement and extend recent studies using similar strategies. These, together with our present studies, find a rare, quiescent or dormant subset within the population of stringently defined HSC phenotypes. To date, three types of niches, endosteal, vascular, and reticular, have been described; herein we review the cellular and spatial nature of these microenvironments. We propose that HSC label-retention combined with genetically manipulated stem cell niches will allow us to determine their anatomical architecture, to address HSC cell fate proliferation kinetics, and to begin to dissect the molecular cross talk among stem cells and niche cells *in vivo* during both normal and perturbed homeostasis.

Keywords

hematopoietic stem cell; niche; bone marrow; label-retaining cell; quiescence; self-renewal; Tet-On/Off system; pulse–chase; endosteum; osteoblast; vascular; endothelial; reticular cell

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Conflicts of Interest

The authors declare no conflicts of interest.

Introduction

Hematopoietic stem cells (HSCs) were the first stem cells to be prospectively identified and remain the best-characterized adult stem cells. These cells are responsible for the process of blood cell production throughout the life of an individual. As such, they have three basic functional abilities: self-renewal, differentiation, and death. In adults, the manifestation of these abilities and their regulation occurs in their primary residence, the bone marrow (BM) stem cell niche. Phenotypic identification of HSCs has been achieved by a variety of cell-surface markers and dye exclusion, as described in Table 1.^{1–17} Various combinations of these markers have allowed prospective isolation and engraftment of lethally irradiated mice at high frequency with limiting numbers of HSCs. As such, the functional properties of HSCs have largely been defined based on transplantation studies. These assays have been extremely valuable in studies of homing and engraftment by phenotypically defined subsets of HSCs and also for assessing the self-renewal potential of stem cells by serial transplantation. Nevertheless, they shed little light on the behavior and location of HSCs *in situ* during normal physiological homeostatic hematopoiesis. In order to truly investigate interactions of stem cells with their niche, methodologies that allow a spatial, temporal, and kinetic analysis of these cells are necessary.

Quiescence as a Stem Cell Parameter

HSCs are thought to remain mostly quiescent to prevent exhaustion and to protect their genomes from replication errors that may lead to pathological states.¹⁸ In support of this hypothesis is the fact that HSCs appear resistant to antiproliferative chemotherapeutic drugs such as fluorouracil (5FU) or hydroxyurea.^{19–21} During steady-state conditions, about 85–90% of phenotypically identified HSCs are found in the G0/G1 stage of the cell cycle.^{7,22–24} In addition, G0/G1-HSCs are functionally superior to their S/G2/M counterparts in transplantation assays, showing increased long-term (LT)-donor reconstitution potential.^{25,26}

Cell cycle analysis of phenotypically defined HSC populations renders a snapshot view of that particular moment; it does not allow for a dynamic interpretation of cellular mitotic activity over time. Indeed, if stem cells were truly quiescent over an extended period, only a pulse–chase strategy would reveal these cells as label-retaining cells (LRCs). Previous attempts to study these populations have been undertaken using lipophilic dyes or biotin labeling of cell-membrane proteins as well as 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA.^{23,27,28} Lipophilic dye labeling requires *ex vivo* manipulation of the cells followed by transplantation. Although informative as to homing and engraftment kinetics, true LRC study is precluded by the short-term nature of the dye label retention in irradiated hosts. Efficient biotin labeling can be achieved *in vivo*, but analysis requires removal of the labeled cells for study with additional markers and does not allow for *in situ* imaging. The limited detection interval and possible untoward effects of the biotin compound also hamper this methodology. Studies using BrdU incorporation suggest that HSCs cycle slowly but continuously and therefore challenge the existence of long-term, label-retaining cells (LT-LRCs).^{22,23,29} However, there are inherent problems with BrdU labeling. The foremost being that cells must be dividing to incorporate BrdU, so truly quiescent cells should not

label. In addition, to resolve BrdU incorporation, fixation is required, thus negating functional analysis of LRCs prospectively isolated according to their BrdU content. Of concern also are numerous reports that BrdU is cytotoxic.^{30–34} Indeed, a recent report proposed that BrdU triggers an injury response, similar to, although weaker than, 5FU, leading to HSC activation and proliferation and thus allowing labeling of normally quiescent HSCs.³⁴

Alternative methodology that permits temporal, spatial, and functional study of LRCs during both normal and perturbed homeostasis would be ideal. Such a tool would allow study of HSC fate decisions, proliferation kinetics, and their link to the cell cycle in ways heretofore not possible. Toward this goal, pulse–chase systems allowing the identification, isolation, and functional analysis of viable LRCs, have been developed. The basic technique relies on the controllable incorporation of histone H2B-green fluorescent protein (GFP) fusion proteins into nucleosomes.³⁵ Expression of inducible or repressible H2B-GFP, driven by a tet-response element (TRE), is controlled by a tetracycline (Tet)-transactivator (rtTA in Tet-On or tTA in Tet-Off) driven by a promoter of choice (Fig. 1A). Once cells are pulsed with GFP, further labeling is then prohibited during the chase period. As such, with each successive cell division GFP is diluted by one-half due to the continued synthesis of endogenous (unlabeled) histone H2B and the random interchange of histone dimers along the nucleosomes destined for the two daughter cells (Fig. 1B).³⁵ The first *in vivo* use of this double transgenic strategy was to characterize hair follicle stem cells in a Dox-repressible system.⁶⁴ This same pulse–chase system was later used to quantify the proliferation dynamics of hair follicle stem cells.³⁷ The first study using this technology in HSCs suggested that promiscuous expression of the H2B-GFP transgene precluded its use in this system.³⁸ Unfortunately, the promoter used to drive the tTA in this study was neither stem-cell specific nor strong enough to enable expression above the background present in the single transgenic TRE-H2B-GFP mouse. The actual utility of this technology and approach for HSCs was confirmed in two recently published studies using improved designs. One group generated a mouse strain that allowed ubiquitous, Dox-inducible expression of H2B-GFP.³⁶ The other group used a similar double transgenic model as Challen and Good-ell but with SCL/TAL-1-dependent, Dox-repressible expression of H2B-GFP.³⁴ Both groups used their systems in combination with highly purified stem cell populations to independently arrive at the conclusion that there is a great diversity in mitotic activity and proliferation kinetics in otherwise phenotypically identical cells. Within purified HSC populations, there is a subset (~15–20%) that divides with extremely slow kinetics (<1% per day). Indeed, mathematical modeling by Wilson and colleagues predicts that homeostatic quiescent or dormant HSCs proliferate only once every 145 days, or five times in the lifetime of a mouse without systemic stress or injury.³⁴ As a stress-less lifetime is highly unlikely, they also demonstrated that HSCs enter cell cycle upon stress and subsequently return to dormancy. In addition, both groups used transplantation assays to demonstrate that HSCs defined using mitotic quiescence as an additional parameter had a far greater repopulation ability than those that entered cell cycle repeatedly. These data more closely resemble BrdU-labeling studies in nonhuman primates, suggesting that, under steady state conditions, primate HSCs are quiescent but do proliferate at very low rates.³⁹ Both studies demonstrate the utility of

using quiescence as a parameter for HSC identification and subsequent functional and molecular studies.

We have developed a double transgenic mouse system, comparable to the one described by Wilson and colleagues that allows the *in vivo* investigation of HSC proliferation kinetics during normal homeostasis and in situations of systemic perturbation. Our system also allows the visualization and *in situ* location of these LRCs within native and perturbed niches. This is possible because, unlike the two recently published models, we have targeted expression of repressible H2B-GFP predominantly to the HSC compartment using the human CD34 (hCD34) promoter to drive the tTA. The hCD34 regulatory element is faithfully expressed in the most primitive mouse HSCs as it is active in mCD34^{-/lo} HSCs, mirroring the fact that LT-repopulating stem cells are CD34^{-/lo} in mouse and CD34⁺ in humans while other more rapidly dividing primitive and progenitor compartments are CD34⁺ in both.⁴⁰ We have verified the predominantly stem cell specificity of this promoter using combinations of cell-surface markers to identify a wide variety of different stem and progenitor phenotypes. We observed that the highest GFP expression is in the most primitive stem cells and that GFP levels decrease as cells lose repopulating potential (Fig. 2). Our data with this model complement and extend the findings of Foudi and colleagues and Wilson and colleagues.^{34,36} We have also observed a similar dormant subset, an enhanced repopulation potential in the mitotically inactive cells, and demonstrated an ability to image HSCs specifically *in situ* in BM (manuscript in preparation).

In summary, the development of technologies that allow the isolation of viable LRCs has given us new tools with which to advance our understanding of the kinetics of stem and progenitor population usage during both normal and perturbed homeostasis. These tools should allow us to address at which point in the divisional history of a stem cell it loses its self-renewal potential in transplantation assays. We also should be able to determine the kinetics of the return to dormancy after systemic stress. Indeed, we should be able to directly assess whether cells that have returned to dormancy have the same functional potential as those that have been defined as dormant in situations without stress. The ability to isolate viable LRCs will advance molecular studies of the signaling networks mediating the dormancy of these cells as well as those signals that direct activation and subsequent self-renewal or differentiation. A potentially relevant question not directly addressable in these models is the nature of stem cell division at any time, whether with or without stress. Is it symmetrical or asymmetrical? The resolution of this question awaits development of systems that have identified a marker of asymmetric cell division. The use of these models in combination with other mouse models of altered hematopoiesis, whether in HSC or microenvironmental compartments, will aid in unraveling the complexity of this system. Finally, when used with stem cell-specific promoters, these models should expand greatly our ability to image stem cells within their native niches whether normal or perturbed.

The Cellular and Spatial Nature of Stem Cell Niches

Adult HSCs reside predominantly in the BM. Classic studies in the early 1970s demonstrated that the hematopoietic microenvironment provided an inductive influence on HSC fate choices.⁴¹ Other studies have indicated that primitive stem cells are localized

HSCs, sitting atop the sinusoidal endothelium of BM blood vessels, might closely monitor the blood for factors reflecting the current hematopoietic status and be able to immediately respond to homeostatic changes.

In the mid-1970s, scanning and transmission electron microscopy of femoral marrow identified a fibroblastic-like cell, named the reticular cell, which was located adventitial to the vascular sinus and branching into the surrounding space.⁴⁹ It was hypothesized that these reticular cells form a physical niche that not only supports hematopoietic cells but also traps and regulates differentiation of HSCs. A detailed look in the trabecular region of the bone found cells similar to reticular cells, which were named barrier cells. These barrier cells were located in distinct loci next to bone-lining cells within the distal medial metaphysis of the femur. They appeared often in syncytia with extending processes that enveloped bone-lining osteoblasts on one side and putative HSCs (by morphology) on the other, thus forming a barrier between them. These barrier cells could also extend into the marrow and surround the blood vessels, possibly preventing the unprovoked release of HSCs into the bloodstream.⁵⁰ Support for the reticular/barrier cell niche came with the discovery that chemokine (C-X-C motif) receptor 4 (Cxcr4)-deficient mice had drastically reduced numbers of phenotypic HSCs in their BM and that the ligand for Cxcr4, chemokine (C-X-C motif) ligand 12 (Cxcl12), was highly expressed by reticular cells scattered throughout the intertrabecular region of BM.⁴⁸ Few bone-lining osteoblasts expressed Cxcl12 and at lower levels than the reticular cells. In this study, HSCs were found near the endosteum or sinusoidal endothelium always in close association with these reticular cells, termed Cxcl12-adventitial reticular cells or CARs.

Interestingly, downregulation of Cxcl12 follows circadian oscillations triggered by the sympathetic nervous system (SNS) and coincides with the physiological release of HSCs into the blood.⁶⁰ The BM cell targeted by the SNS was recently identified as a CD45⁻ Nestin⁺ perivascular stromal cell expressing high levels of the HSC-associated retention signals Cxcl12 and angiopoietin 1.⁶¹ These cells could give rise to osteoblasts and adipocytes in culture and form ectopic bone replete with marrow. About 60% of phenotypically defined Lin⁻CD150⁺CD48⁻ HSCs were in direct contact with Nestin⁺ cells, and 90% of HSCs were located within 5 cell diameters from Nestin⁺ cells in the endosteal or sinusoidal space. A similar human cell has been described using phenotypically defined skeletal stromal cells. These cells are CD146⁺ cells and are restricted to adventitial reticular cells in hematopoietic tissues.⁴⁷ Purified hCD146⁺ cells were capable of transferring and generating, upon heterotopic transplantation, a hematopoietic microenvironment leading to a miniature vascularized bone replete with mouse marrow. Lacking in this study however, was proof that the ectopic bone was capable of sustaining transplantable HSCs. A new study has now prospectively isolated a skeletal progenitor population with a CD45⁻, Tie2⁻, α -v Integrin⁺, CD105⁺, Thy1⁻ phenotype from fetal endochondral bone. These cells were similarly able to generate heterotopic bones with a marrow cavity through a cartilage intermediate.⁶² These authors were able to show that bona fide transplantable LT-HSCs were recruited to the heterotopic bone. Interestingly, the same progenitors isolated from membranous bone that did not undergo endochondral ossification were able to form ectopic bone but did not recruit and support marrow. Nevertheless, it has been shown that transplantable LT-HSCs can be isolated from membranous calvarial bone of adult mice.⁵⁶

Perhaps the difference is due to the fetal nature of the bones studied where endochondral ossification was required. It will be interesting to see if these studies can be repeated with adult bone progenitors of the same phenotype.

Combining Label Retention with Manipulated Niches

In the above discourse we have discussed ways to phenotypically identify HSCs that have various repopulating and progenitor characteristics. We have also described new methodologies that allow the use of quiescence; defined by mitotic inactivity over prolonged periods, that is, label retention, as a stem cell parameter. Finally we have described various cellular components suggested to participate in the anatomical architecture of the stem cell niche. Herein, we would like to briefly discuss possible scenarios wherein these methodologies can be combined to study the spatial, temporal, and functional dynamics of HSC within their niches as well as the molecular cross-talk that occurs between these elements.

Having identified cellular elements that are involved in stem cell niches one can imagine modifying these elements to express fluorescent markers as well as to constitutively or conditionally express molecules that perturb niche signaling. Indeed the same osteoblast-specific col2.3 promoter used to identify the osteoblast as a niche element was initially used to generate mice expressing different variants of GFP.^{45,63} In addition, the Nestin⁺ cell described above expresses a fluorescent reporter molecule, as do the CAR cells. The first report of niche signaling perturbation was the osteoblast-specific expression of a Wnt inhibitor, Dickkopf 1, via the col2.3 promoter. This study demonstrated a deleterious effect on the ability of stem cells to self-renew in this perturbed microenvironment.²⁵ We have also used the col2.3 promoter to express a Wnt inhibitor, Wnt-inhibitory factor 1, and have seen similar effects on self-renewal (manuscript in preparation). Combining models of niche perturbation with the HSC-specific H2B-GFP LRC strategies would provide interesting ways to address proliferation kinetics of HSCs, the *in situ* localization of the cells, and a means to isolate both niche and stem cells for functional and molecular studies. The generation of transgenic models with niche cells tagged with different fluorescent reporters will greatly expand our ability to image stem cells within their native niches. Combining these genetic models with LRCs should allow us to visualize the anatomical architecture of the stem cell niche at levels of resolution not currently possible. Further extending the models to include specific niche-signaling molecules will allow us to dissect the molecular cross-talk and signaling networks that control the cell fate decisions of stem cells within their niche. Eventually it is hoped that these types of studies will provide the knowledge basis necessary to enable manipulation of the niche as a therapeutic entity.

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Figure 1.

A pulse-chase system for viable label-retaining hematopoietic stem cells. (A) Schematic diagram of a double transgenic system in which cells are labeled by controllable incorporation of a histone H2B-GFP fusion protein into chromatin. H2B-GFP is expressed via a tetracycline response element-containing promoter (TRE), which is activated by the tetracycline transactivator (tTA) driven by a tissue- or cell-specific promoter. Administration of the tetracycline derivative doxycycline (Dox) via drinking water shuts off production of the H2B-GFP transgene (Tet-Off system) and allows retention of the GFP label in

nondividing cells. A similarly suitable label-retaining system would be a Tet-On system, where the reverse tetracycline transactivator (rtTA) induces expression of H2B-GFP in the presence of Dox. **(B)** Quiescent nondividing cells retain GFP label, while dividing cells progressively dilute their GFP label by one-half with each cell division.

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Figure 2.

Stem/progenitor cell-specific chromosomal labeling in a Tet-Off pulse-chase label-retention model system. A double transgenic model system was developed using a human CD34 promoter to drive expression of tTA. When combined with a TRE-H2B-GFP transgenic mouse (described in Fig. 1) stem and progenitor cells differentially label with GFP. The highest GFP-labeling is observed in the most primitive HSC, long-term (LT)-HSC, defined as $CD34^{-/lo}Flt3^{-}LSK$ or $CD150^{+}48^{-}LSK$ cells. Slightly less label is observed in short-term (ST)-HSC defined as $CD34^{+}Flt3^{-}LSK$. There is even less label in the lympho-myeloid

multipotent progenitor (LM-MPP) population defined as $CD34^{+}Flt3^{+}LSK$. Negligible labeling was seen in common lymphoid progenitors, defined as $Lin^{-}IL7R^{+}Kit^{+}Sca^{intermediate}$. No GFP label is detectable in common myeloid progenitors (CMP; $Lin^{-}Sca^{-}Kit^{+}FC\gamma R^{lo}CD34^{+}$), granulocyte macrophage progenitors (GMP; $Lin^{-}Sca^{-}Kit^{+}FC\gamma R^{hi}CD34^{+}$), megakaryocyte erythroid progenitor (MEP; $Lin^{-}Sca^{-}Kit^{+}FC\gamma R^{lo}CD34^{+}$), and mature blood cells.

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TABLE 1**Cell Surface Markers and Dyes Used to Define Hematopoietic Stem Cells**

Markers/Dyes	Abbreviations	LT-HSC-phenotype	Reference
Lymphocyte antigen 6 complex, locus A, Ly-6A/E	Ly-6A/E, Sca1	Sca1 ⁺	15
c-Kit proto-oncogene	cKit, CD117	Kit ⁺	8, 12
Thymus cell antigen 1, theta	Thy-1, CD90	Thy-1 ^{lo}	3, 11
CD34 antigen	CD34	CD34 ^{-/lo}	14
FMS-like tyrosine kinase 3	Flt3/Flk2, CD135	Flt3/Flk2 ⁻	1, 6
Endoglin/CD105	Endo, CD105	Endo ⁺	5
Protein C receptor, endothelial	Procr, Eper, CD201	Procr ⁺	2
Endomucin	Emcn	Emcn ⁺	10
Endothelial cell-specific adhesion molecule	Esam	Esam ⁺	13
Myeloproliferative leukemia virus oncogene	Mpl, c-mpl, Tpo-R, CD110	Mpl ⁺	17
Signaling lymphocyte activation molecule	SLAM (CD150, CD48, CD41)	CD41 ⁻ CD48 ⁺ CD150 ⁺	9
Rhodamine-123	Rho	Rhodamine-123 ^{lo}	4
Side population	SP	Hoechst33342 ^{lo}	7, 16

The above markers and dyes are commonly used in combination with lineage negative (CD2⁻, CD3⁻, CD5⁻, CD8⁻, B220⁻, Mac1⁻, Gr1⁻, Ter119⁻) cells.