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Quantitative Imaging of Hematopoietic Stem and Progenitor Cell localization and hypoxic status in the Bone Marrow microenvironment

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Abstract

The existence of a hematopoietic stem cell niche as a spatially confined regulatory entity relies on the notion that hematopoietic stem and progenitor cells (HSPCs) are strategically positioned in unique bone marrow (BM) microenvironments with defined anatomical and functional features. Here, we employ a powerful imaging cytometry platform to perform a comprehensive quantitative analysis of HSPC distribution in BM cavities of femoral bones. We find that HSPCs preferentially localize in endosteal zones, where the majority closely interacts with sinusoidal and non-sinusoidal BM microvessels, which form a distinctive circulatory system. *In situ* tissue analysis reveals that HSPCs exhibit a hypoxic profile, defined by strong retention of pimonidazole and expression of HIF-1 α , regardless of localization throughout the BM, adjacency to vascular structures or cell cycle status. These studies argue that the characteristic hypoxic state of HSPCs is not solely the result of a minimally oxygenated niche but may be partially regulated by cell-specific mechanisms.

Introduction

The bone marrow (BM) cavities of long bones are the principal sites of postnatal hematopoiesis, which is sustained by a rare population of hematopoietic stem and progenitor

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Author contributions

C.N-A designed and performed experiments, analyzed data and wrote the manuscript. G.P, B.W, K.J.C, S-Y.P and J.L performed experiments. B.H participated in the design of the study. J.E.M and A.P provided technical help. L.E.S designed the study and wrote the manuscript.

cells (HSPCs) ¹. As for other well-defined adult stem cell types, HSPCs have been hypothesized to reside in defined anatomical locations, where they receive and integrate regulatory cues from neighboring cells, extracellular matrix components and/or soluble factors ²⁻⁴. The precise definition of the physical localization and physiological features of HSPC niches has been greatly hampered by the technical difficulties associated to imaging long bones, the need for complex cell-surface marker combinations to track rare and dispersed HSPC populations, and the lack of tools required for the automated quantitative microscopic analysis of large scale specimens at a single cell level.

Previous attempts to visualize HSPCs in their native context provided relevant information but were limited to the observation of relatively low numbers of events and often lead to controversial views on the compartmentalization of HSPC niches in the BM ⁵. Analysis of the distribution patterns of *ex vivo* purified, transplanted HSPCs or long-term DNA label-retaining cells, suggested that HSPCs preferentially interact with bone-lining osteoblasts ^{4, 6, 7}. An alternative view of HSPC localization was offered by studies visualizing endogenous HSPC-enriched populations in immunostained BM tissue sections, which revealed that the majority of HSPCs reside in bone-distal regions, in direct contact with BM sinusoids and stromal perisinusoidal populations with mesenchymal stem cell and osteoprogenitor potential ⁸⁻¹². Nonetheless, a comprehensive analysis of the global distribution of phenotypically-defined endogenous HSPC-populations in the context of entire BM cavities has not been attempted to date.

A key niche-related feature of HSPCs is their recently reported hypoxic profile ^{2, 13-15}, which has been described on the basis of two lines of experimental evidence. First, HSPCs exhibit enhanced incorporation of pimonidazole (Pimo), the most widely studied hypoxic marker that selectively forms adducts with proteins in cells under low oxygen conditions ¹⁶. Second, HSPCs stably express the α subunit of Hypoxia-inducible transcription factor 1 (HIF-1 α) ¹⁵, which normally undergoes degradation by the proteasome when oxygen levels exceed 5% ^{17, 18}. These experimental observations, together with BM perfusion assays ¹⁹, have inspired a model by which HSPCs localize in areas of the BM with minimal oxygen content, at a certain distance from vascular structures; a condition previously attributed to endosteal regions ^{18, 20}. Adaptation to hypoxia is thought to determine the remodeling of the metabolic profile and induction of quiescence in HPSCs ^{15, 21}. Despite the fundamental physiological implications of this model, evidence demonstrating that defined poorly oxygenated BM domains are enriched in hypoxic HSPCs remains indirect and inconclusive to date.

Here we apply two complementary imaging approaches to perform a comprehensive mapping of the spatial distribution of HSPCs in the BM and analyze their relationship to bone surfaces, as well as to a variety of distinct BM vascular structures, of which we deliver a detailed three-dimensional (3D) characterization. Finally, we exploit these technologies to demonstrate that the hypoxic profile of HSPCs, based on Pimo incorporation and HIF-1 α expression, is unrelated to anatomical positioning in defined BM microenvironments as well as to proximity to vascular structures and cell cycle progression.

Results

Global distribution of c-kit⁺ progenitors in longitudinal BM tissue sections

We adapted the use of Laser Scanning Cytometry (LSC), a technological platform, which enables quantitative imaging cytometry of fluorescently-labeled discrete cell subsets within tissue sections ²², for the analysis of HSPC distribution in the context of whole longitudinal murine BM femoral sections. Cryopreserved, non-decalcified, 5µm-thick sections were systematically scanned using monochromatic laser light excitation, to generate a sequence of high-magnification fluorescent digital images that were assembled into a composite high-resolution image of the entire BM section (Supplementary Fig. 1a). Individual cells were defined and quantified through software-based automatic segmentation of DAPI⁺ nuclei (Supplementary Fig. 1a, lower right panels). Positional information and emitted fluorescent signals in different channels were recorded on a per cell basis, allowing data representation and analysis in the form of tissue maps and scattergrams or histograms (Supplementary Fig. 1b).

We initially employed LSC to determine the spatial distribution of a cellular subset enriched in hematopoietic progenitor cell content and defined by the expression of c-kit, the receptor for stem cell factor (SCF). As explained in the Methods section, autofluorescent cells were excluded from this analysis and isotype control stained sections were systematically scanned to determine base-line levels of fluorescence and define specific gates (Fig. 1a and 1b). C-kit⁺ cells were detected, labeled in high-resolution images throughout the BM cavity (Fig. 1c) and plotted in tissue maps (Fig. 1d and e). To avoid marked differences in cellular content derived from the dissection plane, we restricted our analysis to BM femoral sections in which both proximal and distal metaphysis were exposed and the diameter of the diaphysis was > 800 µm. Quantitative analysis revealed no significant differences in the frequencies of c-kit⁺ progenitor cells in areas of trabecular (metaphysis) and cortical (diaphysis) BM of femurs (Fig. 1d). The presence of c-kit⁺ cells along the width of the diaphyseal compartment was investigated by dividing the cavity in eight longitudinal regions (100–150 µm wide each) spanning the diameter of the diaphysis. This analysis uncovered a gradient in the relative content of hematopoietic progenitors in different regions of the diaphysis; highest percentages of ckit⁺ progenitors were found in bone-proximal endosteal regions (5.59±0.79 %), progressively decreasing towards most central areas (2.68±0.47 %) (Fig. 1e).

Given the crucial role of BM microvasculature in the regulation of early hematopoietic events ^{23, 24}, we investigated the spatial relationship of c-kit⁺ progenitors and Laminin⁺ microvessels. Initial observations revealed a strong tendency of c-kit⁺ progenitors to closely associate with BM vascular structures (Fig. 1f), which was verified in 3D reconstructions obtained by confocal imaging of immunostained thick slices of femoral BM (Supplementary Fig. 2 and Video 1). We next employed software-based segmentation of Laminin⁺ vascular structures to create peripheral contours in which the perimeter of vessel walls was expanded by up to 10µm (Fig. 1g). This enabled us to automatically discriminate perivascular (<10µm from vessel) from non-perivascular populations (Fig. 1h) and determine that perivascular spaces are significantly enriched for c-kit⁺ progenitors (Fig. 1i). Therefore, we uncover a

previously unknown tendency of hematopoietic precursors to spatially arrange according to a gradient throughout the diaphyseal cavity, and concentrate in perivascular areas of BM parenchyma.

HSPCs associate with blood vessels and preferentially localize in endosteal zones

Next we determined the global distribution of three phenotypically defined populations increasingly enriched in HSPC content. First, c-kit⁺ cells lacking lineage marker expression, which encompass a heterogeneous subset of lineage committed progenitor cells with limited self-renewal capacity. Second, Sca-1⁺c-kit⁺ (here on referred to as SK), which are mostly negative for lineage markers, and consist of multipotent progenitors, short-term and long-term repopulating stem cells²⁵. Finally, we tracked very rare Lineage⁻CD48⁻CD41^{lo/-}c-kit⁺ cells, highly enriched for Lin⁻Sca-1⁺c-kit⁺CD48⁻CD150⁺, which are mainly considered as long-term repopulating HSCs and are highly enriched in early HSPC progenitor activity (Supplementary Fig. 3a and 3b). LSC analysis of immunostained sections permitted rapid screening and confirmation of individual cells falling under the gates of interest by automatic relocation and visualization of high-resolution images in every fluorescent channel (Fig. 2a, b, c, d, e and f), which was necessary to exclude possible false positive cells. A total of 1915 Lin⁻c-kit⁺, 1589 SK cells, and 312 Lin⁻CD48⁻CD41^{lo/-}c-kit⁺ cells, were visualized and their distance to vascular structures and endosteal surfaces (detected by bone autofluorescence) was determined.

HSPC populations were equally distributed in metaphysis and diaphysis of femoral cavities (Supplementary Fig. 3c). Within these regions, more primitive subsets were increasingly enriched in endosteal zones, defined as 100 μ m from the closest bone surface (42.72 $\pm$ 2.6 of Lin⁻c-kit⁺, 61.0 $\pm$ 3.3% of SK and 72.64 $\pm$ 4.8% of Lin⁻CD48⁻CD41^{lo/-}c-kit⁺ cells) (Fig. 2h). However, only a minority was found in direct contact or close proximity to endosteal surfaces (Fig. 2h). As expected, the average distance to endosteum of HSPCs residing in metaphysis was significantly lower than those in the diaphysis, where a detectable fraction of these cells resided in central regions, > 200 μ m away from the closest bone surface (Supplementary Fig. 3d and 3e). Notably, the majority of all subsets (64.46 $\pm$ 3.2 of Lin⁻c-kit⁺, 81.99 $\pm$ 2.1% SK and 70.70 $\pm$ 5.3% Lin⁻CD48⁻CD41^{lo/-}c-kit⁺) localized in close adjacency to endothelium (<10 μ m) throughout the entire BM cavity, including densely vascularized periendosteal regions (Fig. 2g). These results offer a comprehensive view of the distribution of primitive HSPCs in BM cavities, highlighting perivascular areas of bone-proximal regions as their preferred microenvironment.

To rule out the possibility that a fraction of HSPCs reside inside blood vessels (as reported for immature B cells) we employed a previously described protocol for the *in vivo* quantification of intravascular populations²⁶. Injection of phycoerythrin-coupled anti-CD45 for very short periods of time prior to euthanasia, results in the specific labeling of intravascular hematopoietic BM populations, which can then be quantified by flow cytometry²⁶. Under steady state conditions, the fraction of intravascular Lin⁻c-kit⁺Sca-1⁺ (LSK) HSPCs and Lin⁻c-kit⁺Sca-1⁻ (LK⁺S⁻) is very low or undetectable (Supplementary Fig. 4a, 4b and 4c and Supplementary Video 2).

Three-dimensional study of BM microvascular compartment

While studying the relationship of HSPCs with blood vessels, we noticed the previously reported, heterogeneous nature of BM microvasculature^{24, 27–29}. The presence of diverse types of vascular structures in thin tissue sections was highly irregular and mostly dependent on the dissection plane. To gain global insight of the 3D structural relationship of diverse types of microvessels, we devised a unique methodology to study whole-mount stained femoral longitudinal slices (300–600µm thick) using confocal or multiphoton microscopy. 3D-reconstructions of optical sections of the diaphysis revealed a highly organized microvascular network in which central longitudinal Sca-1⁺ large arteries gave rise to smaller radial arteries, which in turn branched into arterioles as they migrated towards endosteal regions (Fig. 3a and 3b, Supplementary Video 3). Transition from arterial to venous circulation occurred right along, or in close proximity to the endosteum, where the network of arterial vessels progressively transformed into wider, irregularly shaped Sca-1^{lo/-} sinusoids (Fig. 3b, 3c, and Supplementary Video 4). Sinusoidal vessels extended back into the cavity, forming frequent anastomosis and eventually coalescing into a big collecting central sinus into which the venous circulation drains (Fig. 3c and Supplementary Video 5). The vascular compartment of the metaphysis was less structured. We observed Sca-1⁺ arteries entering the BM through the bone cortex, migrating longitudinally towards the diaphysis (Fig. 3d and Supplementary Video 6) and giving rise to smaller arterioles that frequently ran along the surface of the trabecular bone and drained into sinusoids (white arrow Fig. 3e and Supplementary Video 7). These experiments revealed that densely vascularized endosteal zones contain a unique circulatory system.

Based on morphological criteria and differential expression of Sca-1 and Laminin, we were able to discriminate between sinusoidal and non-sinusoidal endothelium in thin tissue sections and quantify the interactions of HSPCs with different types of BM microvessels (Fig. 4a and 4b). The majority of perivascular Lin⁻CD48⁻CD41^{lo/-}c-kit⁺ directly associated with sinusoidal endothelium (Fig. 4c). However, 12.0 ± 2.6% of this population and 36.72 ± 5.0% of SK cells, were found to lie adjacent to non-sinusoidal endosteal vessels, or central and radial arteries expressing high levels of Sca-1 and Laminin (Fig. 4b and 4c). Thus, HSPCs, in particular SK multipotent progenitors, directly interact with structurally diverse BM microvessels.

Hematopoietic progenitors display a hypoxic state irrespective of their localization in the BM

HSPCs are considered relatively hypoxic compared to other BM populations, as measured by incorporation of the hypoxic probe Pimonidazole (Pimo)^{13, 14}. Interestingly, not only long-term HSCs (LSKCD34⁻), but Lin⁻Sca-1⁺c-kit⁺ (LSK) and LK⁺S⁻ cells, containing mostly committed progenitors, avidly incorporated Pimo as measured by flow cytometry (Fig. 5a and 5b). The hypoxic nature of HSPCs has been proposed to derive from their residency in endosteal regions and relatively distant from blood vessels^{13, 18, 20}, in apparent contradiction with our demonstration of their perivascular localization. We sought to clarify this discrepancy by employing 5-color imaging cytometry to simultaneously analyze the spatial distribution of different BM cell subsets and levels of Pimo incorporation *in situ*. Pimo^{hi} cells were scattered throughout the BM displaying no apparent spatial patterning

(Fig. 5c and 5d). In accordance with our FACS results, the Pimo-specific signal was significantly increased in c-kit⁺ progenitors compared to total BM or B220⁺ cells (Fig. 5e and 5f). Strikingly, throughout the BM we frequently observed Pimo^{hi} perivascular c-kit⁺ progenitors, directly adjacent to Pimo^{lo/neg} B220⁺ cells sharing the same extracellular environment (Fig. 5d). A detailed analysis revealed that Pimo incorporation of c-kit⁺, B220⁺ or total BM cells was not defined by their residency in the metaphysis or defined regions of the diaphysis, nor by their distance to blood vessels (Fig. 5f, 5g, 5h and data not shown). Thus, the hypoxic phenotype of hematopoietic progenitor populations is cell-specific rather than location-dependent.

Pimo incorporation of primitive HSPC populations is unrelated to localization in BM endosteal zones or to cycling status

To further confirm the independence of the hypoxic status of HSPCs from their positioning near endosteal surfaces, we performed an in depth study of SK cell distribution and levels of Pimo incorporation. Again, perivascular SK cells displayed an enhanced Pimo signal compared to that of BM cells (Fig. 6a and 6b). Of note, the mean fluorescence intensity (MFI) in the Pimo channel was consistently increased in SK cells found at distant sites from bone, mainly in the diaphysis, compared to those in the proximity of endosteal surfaces (Fig. 6c). Therefore, contrary to what has been proposed^{18, 20}, endosteal areas of BM do not harbor the most hypoxic HSPCs. Notably, AMD3100-mobilized circulating, as well as splenic HSPCs, incorporated high levels of Pimo, comparable to those of BM HPSCs (Fig. 6d), further reinforcing the notion that the hypoxic status of HSPCs is preserved throughout different environments of the entire organism. Of note, despite the proposed link between hypoxia and quiescence, we found that non-dividing HSPCs in G₀ incorporate similar levels of Pimo to those in G₁ phase of the cell cycle (Fig. 6e and 6f).

HSPCs display stable expression of HIF1- α independently of proximity to blood vessels, localization in BM and cell cycle status

Similar to Pimo incorporation, stable expression of HIF-1 α in HSPCs is thought to result from residency in specific hypoxic regions of the BM. Notably, not only LSKCD34⁻ and LSKCD34⁺, but LK⁺S⁻ cells displayed homogenous expression HIF-1 α , as measured by flow cytometry (Fig. 7a). In accordance, robust HIF-1 α expression of c-kit⁺ progenitors was detected in immunostained BM sections via LSC (Fig. 7b). C-kit⁺ cells localized throughout the different regions of the BM expressed HIF-1 α at comparable levels (Fig. 7c and 7d), regardless of their perivascular localization (Fig. 7e). Similarly, we visualized perivascular SK HSPCs expressing high levels of HIF-1 α , irrespective of their distance to the nearest endosteal surface (Fig. 7f). Moreover, splenic HSPCs conserved displayed similar HIF-1 α expression levels to BM-residing HSPCs (Fig. 8a). Altogether, our results demonstrate that HIF-1 α stabilization in HSPCs occurs independently of the possible differences in oxygenation registered at different anatomical sites.

Finally, we examined HIF-1 α expression levels in cycling and non-cycling cells. Entry into cell cycle did not result in significant changes in HIF-1 α expression of HSPCs (Fig. 8b). Remarkably, highly proliferating HSPCs found in murine BM during the recovery phase post-treatment with the cytotoxic agent 5-fluorouracil (5-FU) (7–9 days), exhibited

increased levels of Pimo incorporation and HIF-1 α expression (Fig. 8c and 8e). As observed during homeostasis, Pimo^{hi} and HIF-1 α ^{hi} cells were found in direct contact with disrupted vascular structures throughout the entire BM cavity after 5-FU treatment (Fig. 8d and 8f). In conjunction with the Pimo incorporation data, these findings clearly demonstrate that the characteristic hypoxic profile of HSPCs is not exclusive of quiescent HPSCs but retained during cycling and upon proliferative stress.

Discussion

In this study, we employ a quantitative imaging approach to systematically map phenotypically-defined HSPC subsets in the context of entire BM femoral sections. Hematopoietic progenitors exhibit a tendency to accumulate in endosteal zones, which becomes increasingly pronounced in primitive HSPC subsets and therefore seems to be hierarchically regulated. Of note, only a minority of HSPCs is observed in direct contact with endosteal surfaces. Contrary to findings of previous studies, which investigated the localization of transplanted HSPCs or long-term DNA-label retaining cells^{4, 6, 7}, our results indicate that direct adjacency to bone-lining osteoblasts is not required. Regardless of their spatial relationship to bone surfaces, the majority of HSPCs, as well as hematopoietic progenitors with restricted self-renewal capacity, lie adjacent (<10 μ m) to BM microvessels. Our findings further extend previous observations^{8–10}, by demonstrating that vascular adjacency is not only a feature of primitive HSCs but also of their immediate progeny, including SK cells, as well as Lin[–]c-kit⁺ multipotent progenitors. In conjunction with previous work³⁰, these results may indicate a functional role of the interaction of c-kit with its ligand SCF, expressed mostly by perivascular stromal cells¹², in the functional positioning of progenitors in the vicinity of BM microvessels.

The widespread distribution of HSPCs throughout substantially large areas of the BM raises the possibility that, unlike what happens in other model systems such as intestinal crypt stem cells, no rigorous spatial constraints apply for HSPC localization within BM. In this scenario, HSPC maintenance capacity would not be restricted to a limited number of spatially confined physical entities, but rather correspond to an extended property of BM parenchyma, which is pervasively infiltrated by dense vascular and stromal cell networks with HSPC supportive capacity^{9, 12}. Alternatively, HSPCs could be strictly required to physically interact with rare niche cell subsets, not investigated in this report, which would provide specific cues to instruct maintenance of HSPCs. Supporting this hypothesis is the recent description of BM GFAP⁺ non-myelinating Schwann cells, which lie adjacent to a fraction of HSPCs and promote TGF- β -dependent HSPC quiescence³¹. Novel imaging methods enabling *in situ* multidimensional analysis will be instrumental to elucidate whether HSPC specific properties vary as a result of anatomical localization.

Of particular relevance is our finding that hypoxic features seem to be physiologically inherent to HSPCs and independent of their confinement in defined tissue regions and organs or their spatial relationship to blood supply. In line with previous reports^{14, 32}, our results challenge the pervasive view that the hypoxic status of HSPCs directly results from their residence at sites of minimal oxygen availability of the BM, traditionally hypothesized to correspond to endosteal surfaces as opposed to well-perfused and oxygenated perivascular

niches^{18, 20}. Pimo is the most widely used and best characterized hypoxic marker in terms of molecular mechanism¹⁶. Pimo competes with intracellular oxygen to bind electrons released from the electron transport chain, converting into a reduced derivative that in turn incorporates into proteins. Thus, Pimo incorporation levels indirectly reflect an intracellular state in which oxygen influx is insufficient to absorb the production of electron release¹⁶. This status is prominently induced in most cells subjected to extremely low oxygen supply, such as those present in a vascularized areas of solid tumors. However, we find that within the range of homeostatic oxygen tensions estimated to exist in BM tissues (1 to 6%)^{18, 20}, different cell types display significant disparities in Pimo binding, despite being physically adjacent and sharing similar extracellular oxygen availability. Thus, it is conceivable that the relatively increased Pimo incorporation capacity of HSPCs compared to other cell subsets derives not directly from different oxygenation, but from their distinctive metabolic dynamics, which result in a relative deficiency of intracellular electron acceptors.

In this regard, it is important to note that HIF-1 α is a master regulator driving the remodeling of the metabolic machinery of HSPCs towards anaerobic glycolysis¹⁵. Our study shows that HIF-1 α expression in HSPCs is again not determined by their spatial localization in BM. This finding can be explained by the fact that oxygen-dependent molecular mechanisms targeting HIF-1 α for rapid degradation are gradually activated at oxygen concentrations above 5%^{18, 33}, and are therefore probably not the limiting factor regulating HIF-1 α expression levels in the context of BM microenvironmental conditions. Furthermore, oxygen-independent mechanisms are known to promote stabilization of HIF-1 α in HSPCs even under saturating oxygen conditions^{34, 35}. Our findings are further supported by the fact that human circulating HSPCs conserve HIF-1 α expression³⁶ and that HSPCs maintain a typical hypoxic transcriptional program several hours after incubation at ambient oxygen pressures¹⁵. In light of our results, it seems reasonable to conclude that the so-called hypoxic status of HSPCs is related to cell-specific mechanisms derived from their glycolytic metabolic profile, rather than measurable differences in intracellular oxygen levels or environmental oxygen availability. Thus, instead of hypoxic, HSPCs would be best described as low oxidative phosphorylation cells. Notably, although quiescence has been proposed to relate to the hypoxic/metabolic phenotype of HSPCs^{37, 38}, we demonstrate that hypoxic features are not exclusive of quiescent HSPCs. Further studies are required to determine the physiological implications of the referred metabolic profile, as well as how it functionally relates to other HSPC-specific properties such as, self-renewal and multipotency.

Although not crucial for the metabolic phenotype of HSPCs, it is still reasonable to assume that regional differences in BM environmental oxygen tensions may participate in the control of differentiation pathways, cell cycle, and oxidative stress in hematopoietic cells in general^{21, 39}. Nonetheless, the assumption that endosteal areas are less oxygenated relative to central portions of the marrow is not sustained from an anatomical standpoint. As previously reported for BM cavities of mouse calvaria⁴⁰, we provide evidence that endosteal surfaces of long bones are as highly vascularized as central BM regions, and contain the transition from arteriolar to sinusoidal/venous circulation, where most of the oxygen exchange from blood to tissues is thought to occur⁴¹. A future challenge lies in

determining the thus far poorly understood dynamics of oxygen availability in BM parenchyma and its impact in hematopoietic physiology.

Anatomical subdivision in diverse functional microenvironments is thought to underlie the complexity and plasticity of diverse blood cell production in the BM. Here we employ a technology that proves invaluable to quantitatively study cell-specific niche distributions and analyze cellular features of rare populations in highly complex tissues. We offer unequivocal anatomical evidence to support an alternative view to the widely accepted notion of a super hypoxic HSPC niche in the BM. The application of these quantitative and 3D imaging techniques to BM and other organs sustained by rare stem cell populations will provide important insights into the sophisticated regulation of tissue homeostasis.

Methods

Mice

Adult C57/B6 mice (6–12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) or Taconic. All animal experiments were approved and monitored by the Children's Hospital Animal Care and Use Committee. In all experiments mice were euthanized by CO₂ asphyxiation.

Antibodies

A complete list of antibodies and protocols used for flow cytometry and immunofluorescent staining of HSPCs, vascular structures and hypoxypore Pimonidazole in BM cryosections is provided in Supplementary Tables 1 and 2.

Immunofluorescence of BM sections

To achieve rapid *in situ* fixation of tissues, mice were perfused post-mortem with 10ml paraformaldehyde-lysine-periodate (PLP) fixative through the vena cava. Bones were isolated, further fixed in PLP for 4–8 hours, rehydrated in 30% sucrose solution for 48 hours and snap frozen in OCT (TissueTek). Whole longitudinal single cell-thick (5µm) femoral cryosections were obtained using a Leica Cryostat and the Cryojane tape transfer system (Leica microsystems). Detailed experimental procedures employed for immunofluorescent staining of different HSPC populations, hypoxic markers and vascular structures are provided in Supplementary Table 1.

Laser Scanning Cytometry analysis

Laser Scanning Cytometry was performed with a iCys Research Imaging Cytometer 4-laser system (Compucyte) equipped with 4 laser lines (405, 488, 561 and 633 nm) and 4 detectors with the following filter sets 450/40, 521/15, 575/50 and 650/LP. Cryosections displaying damage or alteration of the microstructure of the BM parenchyma were discarded for scanning or analysis. Sections displaying preserved integrity in all regions of the BM (both metaphysis and diaphyseal cavity) were selected, scanned and subsequently analyzed entirely. Each section was first scanned with a 10x objective using the 405nm laser to generate low resolution images of the DAPI stained nuclei and obtain a general view of the BM. Subsequently, sections were divided in small regions that were scanned with a 40x dry

objective lens at a spatial resolution of 0.25 mm stage step size to create high resolution field images of the whole BM cavity (Supplementary Fig. 1). Lasers were selected according to fluorescent dyes used in each individual staining (Supplementary Table 1) and used to scan BM sections in independent passes to avoid the need of signal compensation. Detector levels were set according to background fluorescence of isotype control stained sections, which were always scanned in parallel to stained samples. Data was analyzed from raw, unprocessed images, post-acquisition using iCys Cytometric Analysis Software (Compucyte). BM autofluorescent cells were typically identified by collection of the red signal (filter set 575/50) after excitation with the 405nm laser. This strategy allowed exclusion of autofluorescent events (~ 3–5 %) of total BM, from our analysis. Images were saved in iCys and exported into Photoshop (Adobe Inc) for processing.

Generation and whole mount immunostaining of femoral BM slices for microscopy

To generate thick femoral bone slices, femurs were harvested and processed as described above. OCT-embedded frozen femurs were iteratively sectioned using a cryostat until the BM cavity was fully exposed along the longitudinal axis. Bone was then reversed and the procedure was repeated on the opposite face of the bone until a thick slice of BM was obtained. OCT freezing medium covering the sample was removed, slices were washed with PBS and blocked o.n at 4°C in blocking solution (0.2% Triton/1% BSA/10% donkey serum/PBS). BM slices were stained with rabbit anti-Laminin and rat anti-Sca-1 for 3 days in blocking solution, washed o.n in PBS and stained with DyLight488 donkey anti-rabbit IgG and DyLight549 or DyLight649 donkey anti-rat IgG. Whole-mount stained slices were then washed in PBS and incubated o.n in FocusClear (CelExplorer Lab). For observation under the confocal microscope, BM slices were mounted in glass slides embedded in FocusClear.

Confocal and multiphoton microscopy

Multiphoton microscopy (MPM) was performed with an Olympus BX50WI microscope equipped with a Bio-Rad radiance 2000 MP confocal/multiphoton microscopy system controlled by Laser-Sharp software (Bio-Rad Laboratories). For multiphoton excitation a MaiTai Ti:Sapphire laser (Spectra-Physics) was tuned to 800nm and stacks of x-y optical sections were sequentially acquired along the z-axis at variable step sizes. Second harmonic generation of bone tissue was detected through a 400/40 nm bandpass filter and fluorescent emitted light through 525/50 nm and 620/100 nm bandpass filters with non-descanned detectors to generate multi-color images. Confocal microscopy was performed with a Zeiss LSM510 system. Image stacks were processed and rendered into 3D volumes using Volocity Software (Improvision) and Image J.

Flow cytometric assessment of the hypoxic profile of BM, spleen and peripheral blood populations

Hypoxia of BM cells was assessed using Hypoxyprobe-1 Plus Kit (HPI Inc, Burlington, MA) as previously described¹³. In brief, 6–12 week old mice were i.p injected with 60–120mg/kg Pimo 90 min before euthanasia. One femur and one tibia were prepared for cryosectioning and immunofluorescent staining while the contra-lateral limb bones were used for FACS analysis. BM cell suspensions were stained for cell-surface markers and permeabilized using IntraPrep Kit (Beckman Coulter) or Cytotfix/cytoperm Kit (BD

biosciences). Intracellular Pimo adducts were labeled with FITC-conjugated mouse anti-Pimo. PBS-injected mice were used as controls to detect baseline levels of anti-Pimo antibody binding. Intracellular expression of HIF-1 α was determined by staining with PE-conjugated mouse anti HIF-1 α (R&D) compared to PE-conjugated mouse IgG. Stained cell suspensions were analyzed by FACS. For simultaneous cell cycle analysis, PE or APC anti-Ki67 (BD biosciences) were added after permeabilization and cells were incubated in DAPI (2 μ M) 20 mins prior to analysis. In some cases, for assessment of blood and spleen LSK populations, mice were treated with AMD3100 (10mg/kg) 60 mins before euthanasia. In some experiments mice were treated with 250 μ g/g of 5-fluorouracil (APP Pharmaceuticals) to determine the effect of chemotherapy in the hypoxic profile of HSPCs.

Colony-forming unit assay

For CFU-C assays freshly harvested BM cells were fluorescently stained for HSPC markers and populations of interest were sorted using BD FACSAria sorter. Lin⁻CD48⁻c-kit⁺ (300 cells per 35mm petri dish), Lin-Sca-1⁺c-Kit⁺ (LSK, 300 cells per 35mm petri dish), were cultured for colony forming units (CFU-Cs) in methylcellulose IMDM medium (MethoCult GF M3434, StemCell Technologies) containing glutamine, 2-mercaptoethanol, IL-3, SCF, IL-6, and erythropoietin according to manufacturer's instructions. Fresh whole BM (WBM, 20,000 cells per 35mm petri dish) cells were used as a control. Colonies were counted after 7 to 12 days of culture at 37°C, 5% CO₂ in a humidified incubator.

Labeling of intravascular populations of the BM

Specific labeling of BM intravascular populations *in vivo* was performed as previously described {Pereira, 2009 #858}. In brief, mice were injected with 1 μ g PE or PECy7-conjugated rat anti-mouse CD45 (BD Biosciences) in a total volume of 100 μ l of PBS. Mice were euthanized 2 minutes after injection and BM from femurs and tibias was harvested. Bones were crushed with mortar and pestle, single cell suspensions were obtained in PBS 2% FCS and red blood cells removed with ACK lysis buffer. BM cell suspensions were then blocked using anti-mouse CD16/32 (BD Pharmingen) and stained with mAbs against HSPC markers. In some cases, bones were processed for analysis under the confocal microscope.

Statistical analysis

Statistical significance was determined using two tailed unpaired student-t test (Prism Software, Graphpad Inc). Data are always expressed as mean $\pm$ SEM. For graphs representing LSC data, single femurs from different mice analyzed were considered as independent data points (n= number of mice). In cases in which multiple sections from a single femur were analyzed, the results were treated as technical replicates, which were averaged and considered as one single independent sample for statistical purposes. The number of independent samples, and the total number of sections analyzed in each experiment are provided in the Figure legends. Statistical significance and p values are indicated by asterisks and specified in the Figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HSPCs	hematopoietic stem and progenitor cells
BM	bone marrow
LSC	laser scanning cytometry

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Figure 1. Quantitative image analysis of the spatial distribution of c-kit⁺ cells in whole longitudinal femoral sections

(a) Low magnification image of a longitudinal femoral BM cryosection immunostained for DAPI (blue) and c-kit (green) (PM=proximal metaphysis, DIA=diaphysis, DM=distal metaphysis). (b) Representative LSC scattergrams of isotype control and c-kit stained BM sections used to quantify percentages of c-kit⁺ cells in different regions of the BM in **d** and **e**. Intensity in the DAPI channel is shown in the X axis. BM cells contained in the c-kit⁺ gate in (b) can be automatically identified and visualized in high-resolution field images (c). Representative image of a BM stained for DAPI (blue) and c-kit (green) (top panel). Cells gated as positive based on fluorescence intensity in the c-kit channel, are automatically marked by the LSC analysis software (green rectangles, bottom panel). (d) Representative tissue maps and frequencies of c-kit⁺ hematopoietic progenitors in the metaphysis (MET) and diaphysis (DIA) of femoral BM (Data are represented as Mean±SEM calculated from n= 9 mice, a total of 17 sections were analyzed). (e) Frequencies of c-kit⁺ progenitors in longitudinal gates, spanning the width of the diaphysis of femoral sections (representative gates in left panel). ER=endosteal regions, CMR= central medullary region (*p< 0,05 Mean ±SEM calculated from n= 7 mice, a total of 11 sections). (f) Image of the diaphysis of a BM section co-stained for c-kit and Laminin. See also Supplementary Video 1. (g) LSC analysis of perivascular cells in BM sections. Laminin fluorescent signal is thresholded and an integration contour in which the original vascular structure is expanded by 20 pixels (10 μm in the original 40x magnification) is defined to generate spatial gates containing only perivascular spaces (green outline right panel). (h) Representative LSC dot plots of total BM, perivascular and non-perivascular cell populations used to quantify cells in (i) Frequency of c-kit⁺ cells in perivascular and non-perivascular fractions (dashed line frequency of total BM) (**p< 0,01 Mean±SEM calculated from n=7 mice, a total of 10 sections analyzed).

Figure 2. The vast majority of HSPCs lie in direct contact with BM microvessels
 Mapping of three subsets of committed progenitors and HSPCs in BM cavities via LSC. Representative dot plots of BM femoral sections stained for lineage markers and c-kit (**a**) Sca-1 and c-kit (**c**), or lineage, CD41, CD48 and c-kit (**e**). Isotype control stained sections were used to determine specific gates. Individual cells falling in the target gates were systematically visualized and confirmed. Representative images of examples of Lin⁻c-kit⁺, SK, and Lin⁻CD48⁻CD41^{lo/-}-c-kit⁺ cells are shown in **b**, **d** and **f**, respectively. (**g**) Frequency of perivascular (<10μm from nearest blood vessel) Lin⁻c-kit⁺, SK and Lin⁻CD48⁻CD41^{lo/-}-c-kit⁺ cells. (**h**) Histogram showing the distribution of the distances of Lin⁻c-kit⁺ (Mean±SEM calculated from n=3 mice, a total of 3 sections and 1915

individually validated cells) SK (n= 8 mice, 8 sections and 1589 individually validated cells) and Lin⁻CD48⁻CD41^{lo/-}c-kit⁺ HSPC (n=4 mice, 8 sections and 312 individually validated cells) populations to nearest bone surface, * p< 0,05.

Figure 3. Three-dimensional study of BM microvasculature heterogeneity

(a) Representative 3D reconstruction of the femoral diaphysis stained with Laminin (green) and Sca-1 (red). Sca-1⁺ arteries run centrally along the diaphysis, emitting branches of smaller arterial vessels, which gradually narrow as they run laterally towards endosteal surfaces. Within endosteal regions Laminin^{hi}Sca-1⁺ endosteal vessels give rise to Sca-1⁻ sinusoids (in **b** and **c**), which return towards the central area of the diaphysis and coalesce in the draining central sinus (shown in **c**). a=arteries, s=sinusoid, cs=central sinus. Bone surfaces are shown in blue as revealed by second harmonic generation signals. (d) 3D overview of arterial and sinusoidal networks in the femoral metaphysis. (e) Transition from arterial to sinusoidal vessels (white arrow) in the endosteal surface of trabecular bone areas. See also Supplementary Videos 3, 4, 5, 6 and 7.

Figure 4. HSPCs directly interact with structurally and phenotypically diverse types of BM microvessels

(a) Low magnification immunofluorescent image of a whole femoral section stained for Laminin (red), DAPI (blue), c-kit (green) and Sca-1 (magenta). Lower panel shows the enlarged image of a section of the diaphysis in which central arteries ($\text{Sca-1}^+ \text{Laminin}^+$), sinusoids ($\text{Laminin}^{+/lo} \text{Sca-1}^{-/lo}$) and endosteal vessels ($\text{Sca-1}^+ \text{Laminin}^+$) can be visualized. **(b)** High-resolution images enlarged from boxed areas in (a) depicting examples of SK progenitors interacting with arteries, sinusoids and endosteal vessels. **(c)** Quantification of the percentages of perivascular SK progenitors and $\text{Lin}^- \text{CD48}^- \text{CD41}^{\text{lo/-}} \text{c-kit}^+$ cells in contact with sinusoidal and non-sinusoidal endothelium (Mean $\pm$ SEM calculated from $n = 8$

mice, a total of 8 sections and 1589 cells and n=4 mice, a total of 8 sections and 312 individually validated cells, respectively).

Figure 5. *c-kit*⁺ progenitors display a hypoxic status regardless of their perivascular localization and distribution in the BM cavity

(a) Representative flow cytometry histograms of Pimo incorporation (green) of total BM cells, B220⁺ cells, LS-K (Lin⁻*c-kit*⁺Sca-1⁻) progenitors, ST-HSCs (LSKCD34⁺) and LT-HSCs (LSKCD34⁻). Grey histograms depict background levels of Pimo staining in PBS-injected control mice. (b) Quantification of the shift in the mean fluorescence intensity of the different BM populations in the Pimo channel (MFI, in fluorescence arbitrary units) from mice injected with Pimo compared to non-injected Pimo controls (n = 7 mice ** p < 0,01). (c) Images of femoral sections of control PBS-injected mice and Pimo-injected mice stained with anti-Pimo. (d) Representative images showing multiple examples of perivascular *c-kit*⁺ cells brightly stained for Pimo (white arrowheads) in both endosteal and central marrow regions. Pimo^{hi}*c-kit*⁺ cells are found in many cases adjacent to B220⁺Pimo⁻ cells (orange arrowheads). (e) LSC analysis of sections stained for Pimo. Representative histograms used to quantify the mean fluorescence intensity (MFI) in the Pimo-specific channel of total BM cells, B220⁺, and *c-kit*⁺ cells in total BM sections. (f) MFI of the Pimo fluorescent signal of total BM cells, B220⁺ cells and *c-kit*⁺ cells localized in the diaphysis (DIA) and the proximal and distal metaphysis (PM and DM respectively) (Mean±SEM calculated from n = 5 mice, a total of 9 sections * p < 0,05 ** p < 0,01). (g) Quantification of Pimo signal (MFI) of *c-kit*⁺ cells present in consecutive longitudinal regions of the diaphysis as shown in Fig. 1e. ER = endosteal regions, CMR = central medullary region (Mean±SEM calculated from n = 6 mice, a total of 9 sections). (h) Representative histograms of the fluorescence intensity (left) and quantification of the MFI (right) in the Pimo channel of non-perivascular and perivascular *c-kit*⁺ cells (Mean±SEM calculated from n = 7 mice, a total of 14 sections).

Figure 6. The hypoxic status of HSPCs is independent of their localization in BM regions and their cycling status

(a) Representative images of perivascular SK cells that strongly stained for Pimo. (b) LSC-quantified Pimo specific signal for total BM cells, c-kit⁺ progenitors and SK cells (Mean $\pm$ SEM calculated from n=3 mice, a total of 3 sections* p<0,05) (c) Quantification of Pimo fluorescent signal of SK cells located at various distances from nearest bone surface (Mean $\pm$ SEM calculated from n=3 mice, a total of 3 sections 742 cells individually validated SK cells, *p<0,05). (d) Pimo incorporation of LSK HSPCs present in BM, circulating blood and spleen after mobilization with AMD3100. (e and f) Flow cytometric analysis of Pimo incorporation of LSKCD34⁺ and LSKCD34⁻ at different stages of the cell cycle. (e) Bottom panels show representative histograms of Pimo specific fluorescence for fractions of each population at G₀ and G₁. (f) Quantification of Pimo intensity for LSKCD34⁺ and LSKCD34⁻ HSPCs in G₀ and G₁ (n=6 mice). For source data for graphs presented in panels 6b and 6c, see SI table 3.

Figure 7. Stable HSPC expression of HIF-1α is not dictated by localization in the BM
(a) Flow cytometric analysis of intracellular HIF-1α expression in subsets of hematopoietic progenitors and HSPCs. **(b)** Representative LSC-obtained histograms depicting HIF-1α expression of total BM cells and c-kit⁺ progenitors in immunostained BM femoral sections. **(c)** MFI of the Pimo fluorescent signal of total BM cells, and c-kit⁺ cells localized in the diaphysis (DIA) and the proximal and distal metaphysis (PM and DM respectively) (Mean ±SEM calculated from n= 5 mice, a total of 10 sections *** p< 0,005) **(d)** Quantification of HIF-1α expression (MFI) of c-kit⁺ cells present along consecutive longitudinal regions of the diaphysis (Mean±SEM calculated from n= 5 mice, a total of 10 sections). **(e)** Representative images depicting multiple examples of HIF-1α⁺c-kit⁺ cells (white arrowheads) in direct contact with isolectin B4⁺ vessels in the context of a BM femoral section. **(f)** Images of perivascular HIF-1α-expressing, Sca-1⁺c-kit⁺ cells in central and endosteal areas of the BM (marked by asterisks). Distances to the closest endosteal surface are indicated.

Figure 8. HIF-1α expression on cycling and actively proliferating HSPCs

(a) Expression of HIF-1α in HSPCs and hematopoietic progenitors from BM and spleen. (b) Flow cytometric analysis of HIF-1α expression in HSPCs in different stages of cell cycle. (c and e) Representative histograms and quantification of Pimo incorporation (c) and HIF-1α expression (e) of LSK cells in control and 5-FU-treated mice (7–9 days post-treatment). (c) n= 8 mice *** p<0,005, and (e) n= 5 mice * p<0,05. (d and f) Representative images of BM sections from control and 5FU-treated mice stained for Pimo, HIF-1α and vascular markers. Pimo⁺ and HIF-1α⁺ cells localize in close contact with disrupted vascular structures during the recovery phase post 5-FU treatment.