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Hypoxia Expands Primitive Hematopoietic Progenitor Cells from Mouse Bone Marrow During *In Vitro* Culture and Preserves the Colony-Forming Ability

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Abstract

Self-renewal is a prerequisite for the maintenance of hematopoietic stem cells (HSCs) in the bone marrow throughout adult life. Cytokines are mainly providing pro-survival signals of HSC, whereas low oxygen levels (hypoxia) were recently shown to influence self-renewal. In contrast, the effects on other progenitor cell types is not clear. In the present work, we have analyzed whether hypoxia has any effects on mouse multipotent progenitors. When bone marrow-derived Lin⁻Sca1⁺c-kit⁺ (LSK) cells were kept in hypoxic cultures (1% O₂) for 4 days together with cytokines, the numbers of colony forming high-proliferative progenitors (HPP-CFC) and precursors for cobble-stone forming cells (CAFC) were increased compared to normoxic conditions. A similar effect was seen with pre-CFC_{multi} from unfractionated bone marrow, whereas more committed progenitors (CFU-GM) were expanded better in normoxia compared to hypoxia. The observed increase in numbers of primitive colony-forming progenitor cells was associated with maintenance of the c-kit/Sca-1 phenotype and a preferential expansion of immature blast-like appearing cells. The results suggest that a major function of hypoxia is to regulate differentiation by increased self-renewal. Furthermore, in cultures of limited cytokine supply, survival of the stem cell-like cell line FDCP-mix was increased during hypoxia. Thus, hypoxia allows for better survival and self-renewal of multipotent progenitors and HSCs from adult bone marrow. Such culture conditions may have beneficial clinical implications for *ex vivo* purposes and may improve the yields of stem cells and early progenitors.

Keywords: Hematopoiesis, Stem cells, Progenitor, Hypoxia, Survival, Self-renewal.

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Abbreviations

BM, bone marrow;

CAFC, cobble stone-area forming cell;
 CFC, colony-forming cell;
 CFU-GM, colony-forming unit for granulocytes
 and macrophages;
 CSF, colony stimulating factor;
 HPP, high proliferative progenitor;
 HSC, hematopoietic stem cell;
 KL; c-kit ligand;
 LSK, lineage⁻ Sca1⁺c-kit⁺.

Introduction

Hematopoietic stem cells (HSC) and multipotent progenitor cells are thought to reside in specialized niches within the bone marrow (BM) microenvironment [1-3]. In these niches, stromal cells and osteoblasts in close proximity provide necessary signals for the proliferation and maintenance of the stem cell pool. The pre-existing conditions of the microenvironment have not been fully identified but involve adhesions molecules, extracellular matrix, and membrane-bound and soluble cytokines. When HSCs and progenitor cells are purified from the bone marrow and cultured *in vitro*, some cytokines such as c-kit ligand (KL), Flt3 ligand (FL), thrombopoietin (Tpo), interleukin-3 (IL-3), and IL-6 have been demonstrated to support the short-term growth of HSCs. Extended studies of long term-repopulating HSCs have revealed that they are not expanded significantly when cultured with cytokines only [4, 5], whereas the use of stromal cell lines results in significant self-renewal of murine HSCs assayed 4 to 6 weeks after transplantation [6]. Compelling data suggest that the main function of cytokines is to prevent apoptosis and to increase the survival of HSCs without affecting self-renewal. This has led to the notion that the conditions in the bone marrow must be further identified in order to make the amplification of HSCs in culture a plausible goal.

Hematopoiesis is developmentally as well as spatially linked to angiogenesis. Both hematopoietic and endothelial cells originate from the same common stem cell-precursor, the hemangioblast [7]. The level of oxygen is known to be a critical regulator of angiogenesis, and hypoxia promotes blood vessel formation. Hypoxic areas are present

in the bone marrow and measurement of oxygen tension yields figures equating to 1-5% oxygen. It has been postulated that HSCs and progenitors may be distributed along an O₂ gradient with HSCs residing in the most hypoxic areas [8, 9]. Accordingly, hypoxia would have positive effects on stem cells, however experimental evidence are limited. One study applying whole marrow demonstrated that the repopulating ability of mouse bone marrow cells when transplanted into recipient mice was conserved better if the cells were preincubated in hypoxia compared to ambient oxygen levels [10]. However, since the study was not performed with purified stem cell-enriched populations it cannot be ruled out that some of the effects were dependent of bystander cells in crude marrow. It was recently reported that low levels of oxygen (1.5% O₂) have effects on human HSCs when cultured *in vitro* together with cytokines [11]. Thus, Danet *et al.* demonstrated that long term-reconstituting human HSCs from bone marrow expressing CD34 but lacking lineage markers (CD34⁺Lin⁻) could repopulate immunodeficient *scid* mice to a larger extent after preincubation in hypoxia. Few studies have been performed with murine HSCs enriched for stem cell-associated phenotypes, although mice deficient for the stem cell marker *Brcp1*/*ABCG2* show impaired colony survival under conditions of low oxygen [12, 13]. These results are not surprising since the *Brcp1* gene is induced by hypoxia-inducible factor (HIF)-1, but indicates that purified HSCs thrive under hypoxic conditions.

If HSCs and immature progenitors are residing in true hypoxic areas whereas committed progenitors and precursors are located close to the blood vessels, the functional response of HSCs to cultures at low O₂ levels is expected. However, since bone marrow transplantations are relying on both HSCs and different progenitors, it is important to elucidate whether distinct progenitor populations behave differently and if primitive progenitors are maintained better when grown in hypoxia.

Various functional assays to quantify stem cells have been developed, and can be used to measure the frequency and characteristics of HSCs. The precursor cells for these assays are primitive progenitors which mimic to some extent true HSCs.

Examples are long term culture-initiating cells (LTC-IC), cobble stone-area forming cells (CAFC), and high proliferative progenitor (HPP)-CFC. The objectives of this study were to evaluate the response of primitive progenitors to hypoxia and if this has any beneficial effects on clonogenic *in vitro* assays. We utilized mouse bone marrow cells enriched for HSCs by the expression of c-kit and Sca-1 and lack of lineage markers ($\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$: LSK). These LSK cells are functionally heterogeneous and consist of both long-term and short-term reconstituting HSCs. Our data suggest that maintenance of precursors for $\text{CFC}_{\text{multi}}$, HPP-CFC, and CAFC are all favoured by hypoxia. Compared to freshly isolated cells, normoxic cultures failed to expand HPPs while hypoxia led to an increase in numbers. Whereas hypoxia increases survival of the multipotent stem cell-like cell line FDCP-mix, subsequent analyses with single LSK cells indicated that hypoxia is mainly enhancing the self-renewal ability. In contrast, precursors for more committed colony-forming cells (CFC-GM) were better selected for in normoxia.

Materials and Methods

Hematopoietic cytokines. All cytokines were purified recombinant proteins and used at pre-determined optimal concentrations as follows: murine (m) KL, 100 ng/ml; human (h) FL, 50 ng/ml; hG-CSF, 25 ng/ml; mIL-3, 10 ng/ml; and hIL-6, 50 ng/ml, all from Peprotech (London, UK). Also used were hIL-1 β , 1 ng/ml (Glaxo IMB, Geneva, Switzerland) and hEpo, 2 units/ml (Cilag, Sollentuna, Sweden).

Normoxic and hypoxic culture conditions. Cells were incubated at either standard conditions in a tissue incubator at 37°C in 5% CO₂ and 95% air (i.e., 21% O₂), or 5% CO₂ and 95% N₂ until an atmosphere containing 1% O₂ was achieved. The level of O₂ was measured twice a day with a MiniOX1 oxygen meter (Mine Safety Appliances Company, Pittsburgh), and adjusted if necessary.

Pre-CFC_{multi}. Bone marrow from 6-12 weeks old normal C57Bl/6 mice were prepared from femurs and tibiae. The use of mouse bone marrow was approved by the ethical committee on animal

research at Linköping university. Unfractionated BM cells (2×10^5 cells) were cultured in 24-well culture plates in 1 ml of IMDM supplemented with 10% FCS and 0.1% deionized fraction V BSA (Sigma-Aldrich) at 37°C in 1% or 21% O₂. IL-1, IL-3, and KL were added to the culture medium at pre-determined concentrations. After 4 days, small aliquots of non-adherent cells from liquid cultures (25-50 μl) were plated in 1 ml of IMDM containing 0.9% methylcellulose (Fluka/Sigma-Aldrich), 21% FCS, 0.65% BSA fraction V, 5.5×10^{-6} M β -mercaptoethanol, h-transferrin (Roche Diagnostics, Mannheim), bovine insulin (10 $\mu\text{g}/\text{mL}$; Sigma-Aldrich), 20 units IL-3 and 4 units Epo in IMDM. After 8-10 days, all colonies were counted and scored for morphology as multilineage colonies when containing erythroid cells and at least two additional myeloid cell types ($\text{CFC}_{\text{multi}}$) and CFU-GM when containing granulocytic and monocytic cells but not erythroid cells.

Purification of $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$ bone marrow cells. Isolation of $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$ (LSK) cells was performed with a lineage cell depletion kit as described previously [14-16]. Briefly, isolation of lineage depleted (Lin^-) BM cells from 6-12 weeks old C57Bl/6 mice was performed with a lineage cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. BM cells were incubated with a cocktail of biotinylated purified lineage specific rat anti-mouse monoclonal antibodies (CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7/4, and Ter-119) and anti-biotin-conjugated immunomagnetic beads, and separated on an AutoMACS device (Miltenyi). Lin^- cells were incubated with PE-labelled goat anti-rat IgG (eBioscience, San Diego, CA) and subsequently blocked with normal mouse serum (Serotec, Raleigh, NC) for 10 minutes. Next cells were incubated with FITC-labelled anti-Sca-1 and allophycocyanine- (APC-) labelled rat anti-mouse c-kit antibodies (Becton Dickinson, Franklin Lakes, NJ). Isotype antibodies labelled with the same fluorochromes were used as controls. LSK cells were sorted on a FACS Aria (Becton Dickinson) for cells expressing Sca-1 and c-kit at high levels and lacking expression of lineage markers. Reanalysis of sorted LSK cells showed high purity of 97% to 99%.

Primary and secondary cultures with Lin⁻Scal⁺c-kit⁺ cells. One thousand LSK cells were seeded on 96-well plates with IMDM supplemented with 10% FCS, 0.1% deionized fraction V BSA, 5.5×10^{-6} M β -mercaptoethanol and pre-determined concentrations of cytokines (KL, FL, IL-3, IL-6, and G-CSF). In some experiments, 10,000 LSK cells were seeded on 24-well plates under the same conditions. The plates were incubated in humidified chambers placed inside tissue incubators at 37°C in either 5% CO₂ and 95% air (21% O₂), or 5% CO₂ and 95% N₂ until an atmosphere containing 1% O₂ was achieved. After 4 days, cell aliquots (20-40 μ l) from primary cultures were seeded in 1 ml IMDM containing 0.9% methylcellulose (StemCell Technologies), 20% FCS, 0.65% BSA fraction V, 5.5×10^{-6} M β -mercaptoethanol, transferrin, insulin, IL-3, Epo and KL (25 ng/ml). Dishes were incubated in a humidified atmosphere with 5% CO₂ at 37°C. On each plate, all colonies were counted after 14 days using an inverted microscope. Colonies more than 0.5 mm in size were scored as high-proliferative potential colony-forming cells (HPP-CFC).

Cobblestone Area-Forming Cells. To quantitate the number of cobblestone area-forming cells (CAFC), primary LSK cells were after exposure to 1% O₂ or 21% O₂ plated at limiting dilution in 24-well plates on irradiated monolayers of murine bone marrow cultures. After 2-3 weeks of culture in MyeloCult (StemCell Technologies) at 33°C with weekly changes of half the media, the total numbers of CAFC in each plate were scored.

FDCP-mix cells. FDCP-mix cells [17, 18] were maintained in Iscove's modified Dulbecco's medium (IMDM; PAA Laboratories, Pasching, Austria) supplemented with IL-3-containing supernatant [19] and 20% horse serum (JRH Biosciences, Lenexa, KS) at 37°C in 5% CO₂ and 95% air.

Assessment of apoptosis using FACS analysis. An apoptosis detection kit labelling cells with FITC-conjugated annexin V was used as recommended by the manufacturer (RD Systems, Minneapolis, MN). Briefly, cells were harvested, washed twice with cold PBS, and stained with FITC-conjugated annexin V and propidium iodide. After incubation for 15 min at room temperature,

cells were analyzed by flow cytometry on a FACS Calibur device (Becton Dickinson).

Single cell assays of Lin⁻Scal⁺c-kit⁺ cells on Terasaki plates. Single FACS-sorted LSK cells were seeded into Terasaki plates in 20 μ l of IMDM supplemented with 10% FCS, KL, FL, IL-3, IL-6, and G-CSF as described above. The plates were incubated in a humidified atmosphere at 1% or 21% O₂. After 8 days, the individual culture wells were analyzed for growth according to the following criteria: 50 cells-10% (between 50 cells up to 10% of the area covered with cells), 10-49%, 50-99%, and 100%. Identification of cell morphology was confirmed by transferring individual colonies to cytocentrifuge slides with subsequent May-Grünwald-Giemsa staining.

Results

Expansion of Pre-CFC_{multi} Progenitor Cells Is Enhanced by Hypoxia

Measurements of multipotential colony-forming cells CFC_{multi} (colonies containing erythroid cells and at least two additional myeloid cell types) and the cells providing long term marrow reconstitution *in vivo* have previously revealed that the precursor given rise to both these populations co-purifies in the same fraction and represents one close approach for quantitative detection of reconstituting stem cells by an *in vitro*-assay [20, 21]. To analyze the effects of hypoxia on these, we applied a 4-day short-term liquid culture system with bone marrow cells cultured in IL-1, IL-3, and KL [22] in either normoxia (21% O₂) or hypoxia (1% O₂). After 4 days the increase in the numbers of pre-CFC_{multi} was measured by replating cells from the liquid cultures to methylcellulose, IL-3, KL, and Epo. The results from four experiments are summarized in Figure 1A. When bone marrow cells were plated directly in methylcellulose without any preincubation, the number of CFC_{multi} was 26 ± 1 CFC_{multi}. Four day incubations in "normal" culture conditions (21% O₂) led to a 3.3-fold induction (85 ± 4). In 1% O₂ the increase was further enhanced corresponding to a 7.8-fold induction (202 ± 8). In the same cultures we also analyzed the effects of

hypoxia on less primitive progenitors. The number of CFU-GM increased 15-fold in normoxia, whereas in hypoxia only a seven-fold increase was seen (Figure 1B). These results suggest that the hypoxia-driven effects are more substantial on primitive progenitor cells.

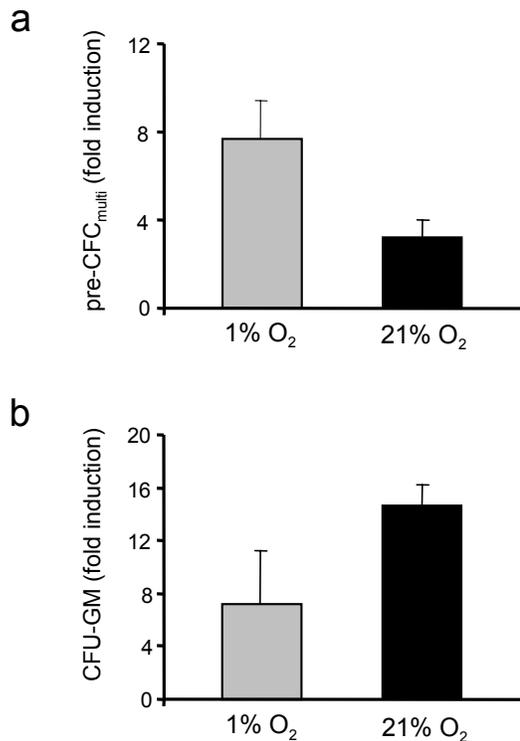


Figure 1. Effect of hypoxia on the expansion of precursors for multilineage erythroid colonies (CFC_{multi}) during 4-day liquid cultures. Total bone marrow (2×10^5) were incubated for 4 days in 1 ml cultures with IL-1 (1 ng/mL), IL-3 (10 ng/mL), and KL (100 ng/ml) under normoxic or hypoxic conditions. 25-50 μ l from each cultures were then plated in methylcellulose in the presence of IL-3 (20 units/ml) and Epo (4 units/ml) for evaluation of CFC_{multi} (a) and CFU-GM (b). Data represent the mean value \pm SD of fold induction compared to the number of colonies on day 0. Data are from four independent experiments, each performed as duplicates.

Self-Renewal of Precursors for HPP-CFC from $Lin^-Sca1^+c-kit^+$ Cells by Hematopoietic Cytokines Is Enhanced under Hypoxic Culture Conditions

The culture system above detecting expansion of pre-CFC_{multi} was performed much in the same manner as previous reports on hypoxic effects on reconstituting bone marrow cells [10, 23]. We therefore decided to repeat the experiments with FACS-sorted bone marrow cells with high expression of c-kit and Sca-1. These LSK cells represent less than 0.1% of the total bone marrow cells and contains all long-term and short-term repopulating HSCs. In addition, they are enriched for primitive high-proliferative potential colony-forming cells (HPP-CFC) with extended replating and multilineage colony-forming capacity [24].

LSK cells were incubated in normoxia or hypoxia and cultures containing KL, FL, IL-3, IL-6, and G-CSF, known to support partial rescue of long-term repopulating HSCs [25]. After 4 days, aliquots were transferred to methylcellulose cultures and further incubated at ambient oxygen (21%) for an additional 14 days. One thousand LSK cells plated directly after FACS-sorting gave rise to 12 ± 1 HPP-CFC, whereas 53 ± 4 HPP-CFC were detected from the same numbers of LSK cells grown for 4 days in 1% O₂, corresponding to a four-fold expansion (Figure 2A). In contrast, we observed failure by normoxia (21% O₂) to support expansion of HPP-CFC, and numbers were reduced to 8 ± 1 .

Hypoxia Increases the Numbers of Cobble-Area Forming Cells from $Lin^-Sca1^+c-kit^+$ Cells

We then attempted to determine if precursor numbers of cobble stone-area forming cells (CAFC) also was affected by pre-exposure to hypoxia. Limited numbers of LSK cells grown for 4 days in hypoxia or normoxia were transferred to pre-established irradiated bone marrow monolayers. After 2-3 weeks of culturing at ambient oxygen, the total number of CAFC in cultures were counted. We

observed a modest but measurable increase of CAFC from LSK cells in hypoxic cultures compared to normoxia (Figure 2B), again

suggesting that hypoxia influences multiple primitive progenitor cell types including CAFC.

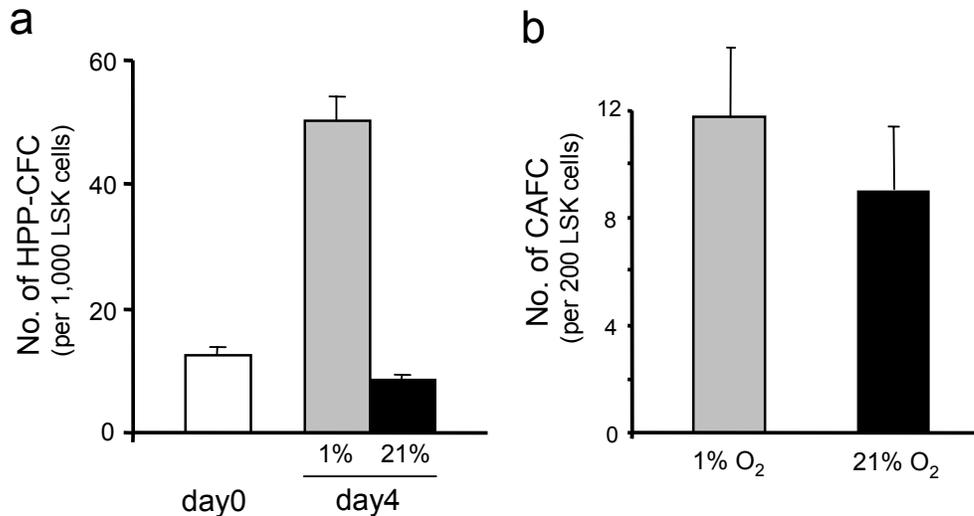


Figure 2. Hypoxia increases numbers of HPP-CFC and CAFC from Lin-Sca1+c-kit+ cells. One thousand LSK cells, purified from mouse bone marrow by FACS sorting, were incubated in IMDM containing KL (100 ng/ml), FL (50ng/ml), IL-3 (10 ng/ml), IL-6 (50 ng/ml), Epo (4 U/ml), and G-CSF (25 ng/ml) in either normoxic (21% O₂) or hypoxic (1% O₂) conditions for 4 days. (a) 20 μ l cells from primary cultures were transferred to secondary cultures containing methylcellulose, 20% FCS, 0.65% BSA, IL-3 (20 units/ml), KL (25 ng/ml), and Epo (4 U/ml). The numbers of HPP-CFC were counted as macroscopic colonies (>0.5mm in size) after 14 days of culture. Results are the mean value (\pm SD) from three independent experiments, performed as duplicates. (b) Aliquots (10 μ l and 20 μ l) of LSK cells were transferred to 2-weeks old irradiated monolayers of bone marrow stroma cells from C57BL6 mice. After two weeks of cultures, the numbers of cobble area-forming cells were scored microscopically. Results are mean value (\pm SD) from three independent experiments, each performed as quadruplicates.

Hypoxia Enhances the Survival of the Multipotent Progenitor Cell Line FDCP-Mix

One alternative possibility to the increase in numbers of multipotent colony-forming cells and HPP-CFC is that exposure to hypoxia increases survival. To determine this we next studied whether survival of multipotent FDCP-mix cells is increased when the cells are incubated in hypoxia. The receptor tyrosine kinase c-kit is expressed on FDCP-mix cells which render them responsive to c-kit ligand (KL). However, c-kit is heterogeneously expressed on these cells allowing only some of the cells to be responsive to KL stimulation. Thus, when FDCP-mix cells are grown in KL more than 50% of the cells die within 48 hours [26, 27]. KL can also synergize with other cytokines to increase survival and proliferation of FDCP-mix cells. Likewise, the addition of low concentrations of IL-3

to other cytokines leads to similar effects. Thus, we decided to test if hypoxia has any synergistic effects on the survival of FDCP-mix cells by stimulating the cells with either KL or suboptimal concentrations of IL-3 during hypoxic exposure.

When FDCP-mix cells were incubated with KL during hypoxia, an increase in survival compared to incubation at ambient oxygen was seen. The number of viable cells increased from 35% at normoxia to 63% at hypoxia (Figure 3). Similarly, suboptimal concentrations of IL-3 (0.01ng/mL) increased the number of viable cells from 18% at normoxia to 39% when grown in hypoxia. This indicates that hypoxia can enhance survival of FDCP-mix cells. No effects on proliferation of FDCP-mix by hypoxia was seen (not shown).

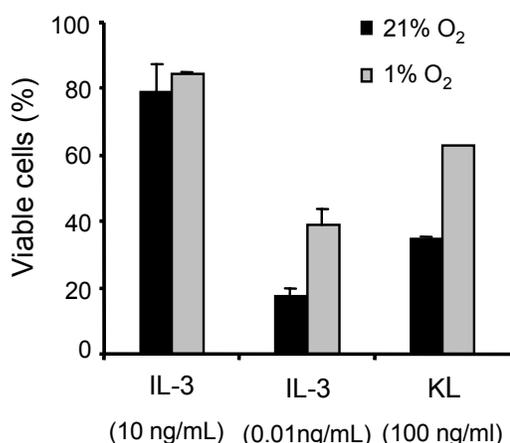


Figure 3. Hypoxia increases the survival of multipotent progenitor FDCP-mix. Cells were incubated in 21% or 1% O₂ in either IL-3 at 10 ng/mL or 0.01 ng/mL, or KL at 100 ng/mL. After 48 hours, the proportion of dead cells was determined by annexin V staining and propidium iodide.

Hypoxia Preferentially Expands Lin⁻Sca1⁺c-kit⁺ Cells with Immature Phenotype

To assess whether hypoxia affects self-renewal or leads to increased survival of LSK cells, we plated single FACS-sorted cells in cytokine-containing media (FL, KL, IL-6, IL-3, and G-CSF) and analyzed clonal growth after 8 days of culture.

In these experiments, we also tested the effects of hypoxia during longer time periods (8 days). In the absence of growth factors, LSK cells rapidly died with similar kinetics independently of the oxygen level. Cells placed either in normoxia or hypoxia with cytokines recruited similar numbers of clonable LSK cells, thus no preferential survival benefit of hypoxic conditions could be demonstrated (not shown). However, normoxic conditions led to a higher proportion of individual wells with more than 50% of the culture area covered with cells (Table 1). Culture wells containing limited numbers of cells were found overrepresented on plates incubated in hypoxia and contained cells with a small round appearance, indicative of an immature differentiation stage (Table 1). When cells from such wells were analyzed for morphology they mainly contained blast-like cells with typical immature appearance (Figure 4A). In contrast, wells with more than 50% cells consisted of differentiated myeloid cells (Figure 4B) and appeared at a higher frequency during normoxic conditions. FACS-analysis of LSK cells after 3 days of culture revealed that hypoxia maintained the immature c-kit Sca-1 phenotype better compared to normoxia (Figure 4C).

Table 1. **Mouse bone marrow-derived Lin⁻Sca-1⁺c-kit⁺ cells maintain immature blast-like morphology better in hypoxia than normoxia.**

Hypoxia	Incubation		Clonal size			Blasts
	Normoxia		I	II	III	IV
-	8d		4 (3)	14 (5)	81 (8)	4 (1)
4d	4d		8 (4)	27 (7)	65 (8)	18 (4)
8d	-		18 (6)	35 (11)	47 (5)	36 (10)

LSK cells were seeded at 1 cell/well in IMDM, 10% FCS, and pre-determined optimal concentrations of cytokines (KL, FL, IL-3, IL-6, and G-CSF) on Terasaki plates. After 8 days of incubation as indicated, cultures were analyzed for clonal growth. Size criteria were: I, wells with at least 50 cells up to 10% of the well covered with cells; II, 10-49% covered; III, 50%-100% covered. In addition, wells were analyzed for the presence of a homogenous population of immature small, round blast cells, IV. Results are presented as the mean (\pm SD) percentage from three independent experiments.

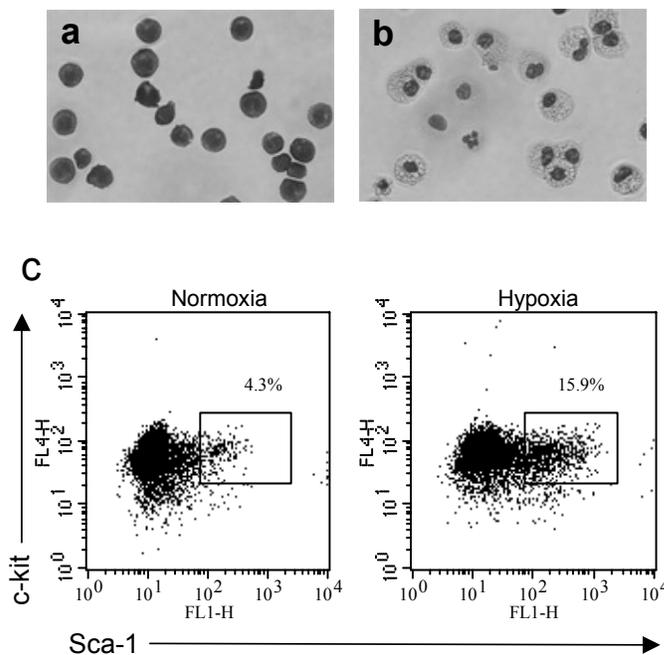


Figure 4. *Hypoxia maintains the immature phenotype of LSK cells.* Single LSK cells were grown on Terasaki plates in hypoxia or normoxia for 8 days in IMDM, 10% FCS, and cytokines, after which cell morphology was analyzed by transferring individual colonies to cytocentrifuge slides with subsequent May-Grünwald-Giemsa staining. Shown are typical morphology of (a) small round cells harvested from wells containing 50 cells up to 10% of the area covered and (b) from wells covered to 50-100% with cells, of which many were adherent. (c) Ten-thousand LSK cells were grown for 3 days under the same conditions, after which cells were harvested and restained for FACS analysis of c-kit (FL4; APC) and Sca-1 (FL1; FITC).

Discussion

Apart from the well-known effects on erythroid development, low O₂ levels influence hematopoiesis in particular by acting on the stem cell compartment favouring measurable expansion of reconstituting HSCs. Some studies have indicated that hypoxia cannot preserve the colony-forming ability of more committed progenitors [11], while others have claimed that progenitors are equally expanded as HSCs during conditions of 1-3% O₂ [10, 28]. Because previous studies applying mouse progenitor cells were performed with unfractionated bone marrow and not phenotypically defined cell populations enriched for HSCs and primitive progenitors, it is difficult to conclude that hypoxia has direct effects. Hematopoietic progenitor cells in the bone marrow are very heterogenous, and the majority are committed but some are truly self-renewing and can in part be used to study optimal *in vitro* conditions and effects on

the HSC compartment. In this study, we analyzed the effects of lowered O₂ levels on different primitive progenitor cells. We also extend previous studies by using HSC-enriched Lin⁻Sca-1⁺c-kit⁺ cells to measure the response to hypoxia. Favourable effects of pre-incubation in hypoxia could be measured for precursors of colony forming cells giving rise to multipotent colonies (CFC_{multi}), for progenitors with the ability to form large colonies (HPP-CFC), and for cobble stone-area forming cells (CAFC). Interestingly, expansion of precursors for HPP-CFC increased fourfold when LSK cells were grown for 4 days in hypoxia, whereas normoxia had deleterious effects in which precursor numbers decreased during the same incubation time. In addition, by seeding individual FACS-sorted LSK cells we could demonstrate that cells with an immature morphology persisted to a larger degree in cultures exposed to hypoxia. In contrast to a previous report, in which hypoxia sustained the proliferative state of human

CD34⁺CD38⁻ HSCs [11], we did not observe any significant difference in cell numbers on mouse LSK cells placed in normoxia or hypoxia. If any difference, normoxia gave rise to a higher proportion of culture wells covered to 50% or more with cells, indicating that hypoxia did not have any advantage of supporting proliferation compared to normoxia.

Similar to a study demonstrating that human HSCs maintain the CD34⁺38⁺Lin⁻ phenotype when cultured in hypoxic cultures [11], we show that mouse bone marrow cells expressing stem cell-associated markers, *i.e.*, high expression of *c-kit* and *Sca-1*, persist to a higher degree in hypoxia compared to normoxic conditions. These results suggest that primitive progenitors possess an intrinsic molecular response to hypoxia in favour for their maintenance and self-renewal which counteracts differentiation.

Recent observations have shown that hypoxia influences differentiation of various stem and progenitor cell populations in different tissues. For example, it has been shown that neuronal stem cells exhibit a conserved response to reduced oxygen levels which can promote their survival [29]. Culturing of neural crest stem cells at low O₂ results in the formation of more multipotent clones as compared to culturing at 21% O₂ [30, 31]. Human embryonic stem cells show minimized differentiation under hypoxia (1-4% O₂) and expression of the stem cell marker gene *Oct-4* is maintained in such cultures [32]. Furthermore, *Notch1* is upregulated when neuroblastoma cells are grown under hypoxic conditions, and based on expression pattern of differentiation markers hypoxia induces a more undifferentiated phenotype [33]. Because HSCs and multipotent progenitor cells exhibit *Notch1* expression [34, 35], similar mechanisms could be at play during hematopoiesis. Recently, it was shown that hypoxia led to an inhibition of differentiation in cortical neural stem cells, and that pharmacological blocking of *Notch* signaling alleviated this inhibition and restored differentiation, indicating that hypoxia maintains the undifferentiated cell state [36]. From our experiments with LSK cells assayed at limiting dilution it seems that hypoxia is executing vital functions at the single cell level. Since *Notch*

activation is likely to require ligand interaction on adjacent cells [37], this may suggest that other mechanisms apart from the *Notch* pathway may be involved.

Interestingly, the *Tie-2* receptor for angiopoietin-1 and receptors for other angiogenic factors are expressed on HSCs [11] and are targets for the transcription factor *HIF-1α*. This constitutes the primary molecular response to hypoxia via the stabilization of *HIF-1α* protein at low oxygen levels. Thus, important interactions between HSCs and supporting cells in the stem cell niche are likely to be maintained by hypoxia. Our future studies will aim to reveal the individual contribution of *HIF-1α* and other hypoxia-regulated transcription factors in the regulation of stem cell maintenance.

In conclusion, the data presented here suggest that lower levels of oxygen than normally applied in cell culture allow for better maintenance and self-renewal of both multipotent progenitors and HSCs from adult bone marrow. As a consequence, culture conditions relying on hypoxia should have beneficial implications for experimental studies as well as for *ex vivo* propagation of HSCs and immature progenitors.

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