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Hypoxia mediates low cell cycle activity and increases the proportion of long term-reconstituting hematopoietic stem cells during in vitro culture

Pernilla Eliasson [a], Matilda Rehn [b], Petter Hammar [a], Peter Larsson [c], Oksana Sirenko [d], Lee A. Flippin [d], Jörg Cammenga [b] and Jan-Ingvar Jönsson [a]

[a)] Experimental Hematology unit, Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden

[b)] Lund Strategic Center for Stem Cell Biology and Cell Therapy, Lund University, SE-221 84 Lund, Sweden

[c)] Radiation Physics, Department of Medical and Health Sciences, Faculty of Health Sciences, Linköping University, SE-581 85 Linköping, Sweden

[d)] FibroGen Inc., San Francisco, CA

Offprint requests to: Pernilla Eliasson, PhD, Experimental Hematology Unit, Department of Clincial and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden. E-mail: pernilla.m.eliasson@liu.se; Phone: +46 13 22 23 92; Fax: +46 13 22 13 75

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Abstract

Objective. Recent evidence suggests that hematopoietic stem cells (HSCs) in the bone marrow (BM) are located in areas where the environment is hypoxic. Although previous studies have demonstrated positive effects by hypoxia, its role in HSC maintenance has not been fully elucidated, neither has the molecular mechanisms been delineated. Here, we have investigated the consequence of *in vitro* incubation of HSCs in hypoxia prior to transplantation and analyzed the role of hypoxia inducible factor (HIF)-1α.

Materials and Methods. HSC and progenitor populations isolated from mouse BM were cultured in 20% or 1% O_2, and analyzed for effects on cell cycle, expression of cyclin-dependent kinase inhibitors (CDKI) genes, and reconstituting ability to lethally irradiated mice. The involvement of HIF-1α was studied using methods of protein stabilization and gene silencing.

Results. When long-term FLT3$^-$CD34$^-$ Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells were cultured in hypoxia, cell numbers were significantly reduced in comparison to normoxia. This was due to a decrease in proliferation and more cells accumulating in G_0. Moreover, the proportion of HSCs with long-term engraftment potential was increased. Whereas expression of the CDKI genes p21^{cip1}, p27^{Kip1}, and p57^{Kip2} increased in LSK cells by hypoxia, only p21^{cip1} was upregulated in FLT3$^-$CD34$^-$LSK cells. We could demonstrate that expression of p27^{Kip1} and p57^{Kip2} was dependent of HIF-1α. Surprisingly, overexpression of constitutively active HIF-1α or treatment with the HIF stabilizer agent FG-4497 led to a reduction in HSC reconstituting ability.

Conclusions. Our results imply that hypoxia, in part via HIF-1α, maintains HSCs by decreasing proliferation and favouring quiescence.

Introduction

In the bone marrow (BM), hematopoietic stem cells (HSCs) reside in specialized niches in close proximity to bone and endosteal osteoblasts [1, 2]. Here, under normal physiological conditions, HSCs are kept in a relatively low proliferative, quiescent state, protecting them from stress, such as accumulation of reactive oxygen species (ROS) and DNA damage, and preventing their depletion due to excessive proliferation [3-5]. Recent data imply that HSCs reside in areas that are hypoxic, i.e., niches low in oxygen content. Furthermore, it has been demonstrated that vascular cells can secrete signals that regulate HSC maintenance [6-8]. Despite that the BM is extensively vascularized, it appears that the nature of the vasculature where HSCs reside is mainly of a sinusoidal type, characterized by slow blood flow that contains venous and poorly oxygenated blood. In this way, HSCs would benefit from the local hypoxic microenvironment by being protected from potentially harmful oxygen species. Indeed, two recent studies agree with this notion, and thus HSCs from mouse BM are the most positive for binding of the hypoxic probe pimonidazole [9], which coincides with low levels of intracellular ROS [10]. Using pimonidazole as a marker for hypoxia, it has also been shown that hypoxic areas in the BM seem to correlate with the endosteal space [11]. Furthermore, slow-cycling cells are located in hypoxic regions containing sinusoids without capillary structures [12]. Collectively, these studies suggest that HSCs are predominantly located at the lowest end of an oxygen gradient in the BM.

At oxygen levels above 5%, the transcription factor subunit HIF-1α is sensitized for degradation by hydroxylation of two prolyl residues (P402 and P563) mediated by oxygen dependent prolyl hydroxylases [13, 14]. Once stabilized in

hypoxia, HIF-1α translocates to the nucleus where it associates with the constitutively expressed HIF-1β subunit and activates transcription of target genes such as vascular endothelial growth factor (VEGF) involved in angiogenesis as well as genes involved in cell cycle regulation, cell survival, and cellular metabolism. Several lines of evidence indicate that HIF-1 inhibits cell growth in certain cell types including embryonic stem cells, possibly by affecting the expression of cyclin-dependent kinase (CDK) inhibitor genes, also implied in the maintenance of HSCs [15, 16].

While concordant studies have shown distinct effects of hypoxia on some HSCs and progenitors [17-21], no study has been performed with phenotypically defined mouse HSCs, neither has the molecular programme of HIF-1α been investigated in highly enriched HSCs. In the present study, we cultured FACS-sorted long-term and short-term reconstituting $FLT3^{-}CD34^{-/+}$ $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells in 20% or 1% O_2 for 2-4 days and analyzed the effects on cell proliferation and ex vivo expansion as well as engraftment potential. The major finding is that hypoxia decreases HSC proliferation and supports exit from cell cycle by upregulation of CDK inhibitors. Despite that lower cell numbers evolved in hypoxic cultures, the long-term reconstituting ability was efficiently preserved.

Materials and Methods

Cell culture and hypoxic treatment

Ba/F3 cells were cultured in RPMI-1640 supplemented with 2 nM L-glutamine, 25 mM HEPES, 5 µM β-mercaptoethanol, 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), and 5% IL-3 supernatant. The multipotent progenitor cell line FDCP-mix, established from mouse BM [22] was

maintained in Iscove's modified Dulbecco's medium (IMDM; PAA Laboratories) supplemented with IL-3 and 20% horse serum (JRH Biosciences, Lenexa, KS), which favours proliferation and counteracts the ability to differentiate to myeloid and erythroid lineages [23]. Conditions for normoxia were 37°C in 5% CO_2, whereas hypoxia was reached by incubation in a CO_2/O_2 incubator (Innova CO-14 incubator, New Brunswick Scientific CO, Edison, NJ) at 37°C adjusted to 5% CO_2 and 1% O_2. In some experiments, hypoxia was achieved by flushing with 5% CO_2/95% N_2 through a sealed culture chamber. When 1% O_2 was attained, monitored with a GMH 3690 Digital Oxymeter (Greisinger Electronic GmbH, Regenstauf, Germany), leak-proof inlet and outlet valves were closed.

Isolation of Lin-Sca1$^+$kit$^+$ (LSK) BM cells and FLT3$^-$CD34$^{-/+}$LSK cells

BM cells were harvested by crushing femurs and tibiae from 8-14 weeks old C57BL6/J mice. Hematopoietic stem and progenitor cells (HSPC) were enriched by depletion of lineage cells or positive selection of c-kit (CD117) expressing cells using immunomagnetic beads on an AutoMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). After magnetic separation, HSPC were incubated with Fc-block (CD16/CD332) before staining with PE-Cy5-conjugated antibodies to mouse-Gr-1 (RB6-8C5), -Mac-1 (M1/70), -B220 (RA-3-6B2), -CD3 (145-2c11), -CD4 (H129.19), and -Ter119 (Ter119) (all were purchased from BD Biosciences, San Jose, CA), -CD117-APC (BD Biosciences), FITC- or Alexa 700-conjugated anti-mouse Sca-1, -FLT3 (A2F10)-PE, and -CD34 (RAM-34)-FITC (eBioscience, San Diego, CA). Lin$^{-/low}$Sca-1$^+$c-kit^{hi} (LSK) cells or FLT3$^-$CD34$^{-/+}$ LSK cells were sorted on a FACSAria™ cell sorter (BD Biosciences). Re-analysis of sorted LSK cells showed >96% purity. FACS-sorted cells were cultured in IMDM with 10% FBS or in

StemSpan serum-free medium (Stem Cell Technologies, Vancouver, BC) and 50 ng/mL of murine stem cell factor (SCF), human Thrombopoietin (TPO), and human IL-6 (all from Peprotech Inc., Rocky Hill, NJ).

CFSE staining

To compare cell divisions between normoxic and hypoxic cultures, freshly FACS-sorted FLT3^{-}CD34$^{-/+}$LSK cells were labeled with 10 µM carboxyfluorescein succinimidyl ester according to the manufacturer´s protocol (CFSE: Molecular Probes, Eugene, OR) prior to culture. FACS analysis of CFSE expression was performed 4 days later.

Real time PCR

Total RNA isolated from cultured cells using RNeasy mini kit (Qiagen, Hilden, Germany) was reverse transcribed to cDNA by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). To quantify transcripts we used SYBR green PCR mastermix (Roche Diagnostics, Indianapolis, IN) containing DNA polymerase, dNTPs, and buffer. Primers used are listed in supplementary Table SI. Reactions were performed in triplicates with 0.5 µM of forward and reverse primers and 4-12 ng template. β-actin was used for sample normalization. Quantitative PCR was performed using 7500 or 7900 FAST Real-Time System (Applied Biosystems). The ct values were analyzed using the $2^{-\Delta\Delta Ct}$ method [24].

Cell-cycle analysis

Cells were ethanol-fixed and resuspended in 3.5 mM Tris, 1 mM NaCl, 0.1% NP-40, 50 µg/mL propidium iodide (Sigma Aldrich, St Louis, MO), and 10 µg/mL RNaseA (Sigma Aldrich). After incubation for 15 minutes at room temperature (RT), cells were analyzed using a FACS Calibur. For analysis of cells in the G_1/G_0 stage, cells were fixed and permeabilized in BD Cytofix/Cytoperm™ solution (BD Biosciences) for 20 minutes on ice, labeled with anti-Ki-67 FITC (clone B56, BD Biosciences) at RT for 20 minutes, and with 50 µg/mL 7AAD (Sigma Aldrich) at 4°C overnight. Cell cycle analysis was assessed by flow cytometry.

Retroviral vectors and transductions

The cDNA encoding constitutively active murine HIF-1α, carrying two point mutations P402A/P563A rendering the protein insensitive to oxygen dependent degradation (a gift from L. Poellinger, Stockholm [25]), was cloned into the retroviral vector pMy-GFP (a gift from C. Stocking, Hamburg). For RNA-interference experiments, HIF-1α complementary DNA hairpin oligonucleotides were designed by Broad Institute – The RNAi consortium (cloneID TRCN0000054450). Oligos were ordered PAGE purified from Invitrogen and are listed in supplementary Table SII. Annealed oligos were cloned into the lentiviral vector pLKO.1-EGFP (provided by Dr. J. Larsson, Lund). Viral supernatants were obtained by calcium phosphate transfection of 293T cells together with helper plasmids. Ba/F3 cells were transduced using spin-infection in 4 µg Sequabrene/mL (Sigma Aldrich). BM cells were transduced using Retronectin™ (Takara, Tokyo, Japan). GFP^+ cells were sorted on a FACSAria (BD Biosciences).

5-FU Treatment

5-fluorouracil (5-FU) was administrated to mice intraperitoneally at a dose of 150 mg/kg 4 days prior to harvest of BM.

In vivo Reconstitution Assay

LSK cells from B6.SJL mice (CD45.1) were cultured for 4 days in normoxia or hypoxia. For transplantation, the culture equivalent to 2,000 initially plated LSK cells along with 2 x 10^5 freshly isolated BM competitor cells from C57BL6/J mice (CD45.2) were injected in the lateral tail vein. C57BL6/J mice were lethally irradiated with a single dose of 9 Gy using a linear accelerator (Varian Clinac 600C) (described in supplementary data). 5-FU treated BM cells from B6.SJL or H2K-BCL2 mice [26] were transduced with pMy and pMy-ca-HIF-1α. For reconstitution assays, 5x10^5 – 2x10^6 transduced cells were transplanted together with 2x10^5 BM competitor cells. Peripheral blood was collected by lateral-tail vein bleeding post transplant and stained with anti-CD45.1 (A20)-PE and anti-CD45.2 (104-2)-FITC (Beckman Coulter, Fullerton, CA) or CD45.2-APC, anti-B220/CD19-PE-Cy5 and anti-Mac-1/Gr1-PE-Cy5 (BioLegend, San Diego, CA). Mouse experiments were approved by the ethical committees at Linköping University and Lund University.

FG-4497 treatment of HSPCs

5-FU treated BM from C57BL6/J and C57BL6/J x B6.SJL mice were plated in IMDM supplemented with 3% FBS, 0.1 mM β-mercaptoethanol, mSCF, hFlt-3L (Peprotech),

hTPO, and 20 μM FG-4497 (FibroGen Inc, San Francisco, CA) or dimethylsulphoxide (DMSO) (Sigma Aldrich) control. After 3 days FG-4497- and DMSO-treated cells were simultaneously transplanted to recipient mice using the equivalent of $5x10^5$ input cells from each condition. To avoid any skewing in competitive repopulation due to strain variations between the C57BL6/J and C57BL6/J x B6.SJL strains, cells from both strains were treated with both conditions and transplanted in two different combinations.

Statistical analysis

To determine the statistical significance unpaired t-test or non-parametric Mann-Whitney u-test was used. Statistics were calculated with GraphPad Prism (GraphPad Software, La Jolla, CA)

Results

Hypoxia diminishes the proliferation of both FLT3$^-$CD34$^-$ and FLT3$^-$CD34$^+$ LSK cells and accumulates more cells in G_0

We first decided to compare proliferation of LSK cells stimulated with SCF, TPO, and IL-6 cultured in serum-free media for 4 days in normoxia (20% O_2) or hypoxia (1% O_2). As seen in Fig 1A, cell numbers increased only 28-fold (± 6.5) in hypoxia compared to 168-fold (±31) in normoxia. To determine the effects of hypoxia on proliferation, we labeled freshly FACS-sorted long-term (FLT3$^-$CD34$^-$ LSK) reconstituting HSCs with CFSE and then split the cells for growth in hypoxia or normoxia. After 4 days we determined the CFSE fluorescent intensity and could demonstrate that HSCs exposed to hypoxia had divided fewer times compared to cells in normoxia (Figure 1B). We next analyzed the cell cycle distribution of cells grown in hypoxia compared to normoxia. This was first done in the multipotent progenitor cell line FDCP-mix cultured for 48 hours in 1% O_2, after which cells were stained with propidium iodide. A decreased fraction of FDCP-mix cells in S phase was seen after hypoxic exposure (9% versus 16% in normoxia) (Figure 1C), which was accompanied by an increased fraction of cells in G_0/G_1 (not shown). To investigate if this effect was true for long-term HSCs, we stained FLT3$^-$CD34$^-$ LSK cells grown for 48 hours either in hypoxia or normoxia for the proliferative marker Ki-67. As seen in Figure 1D, in hypoxia less cells stained for Ki67 (21%) compared to normoxia (29%), indicating that hypoxic exposure leads to accumulation of HSCs in G_0.

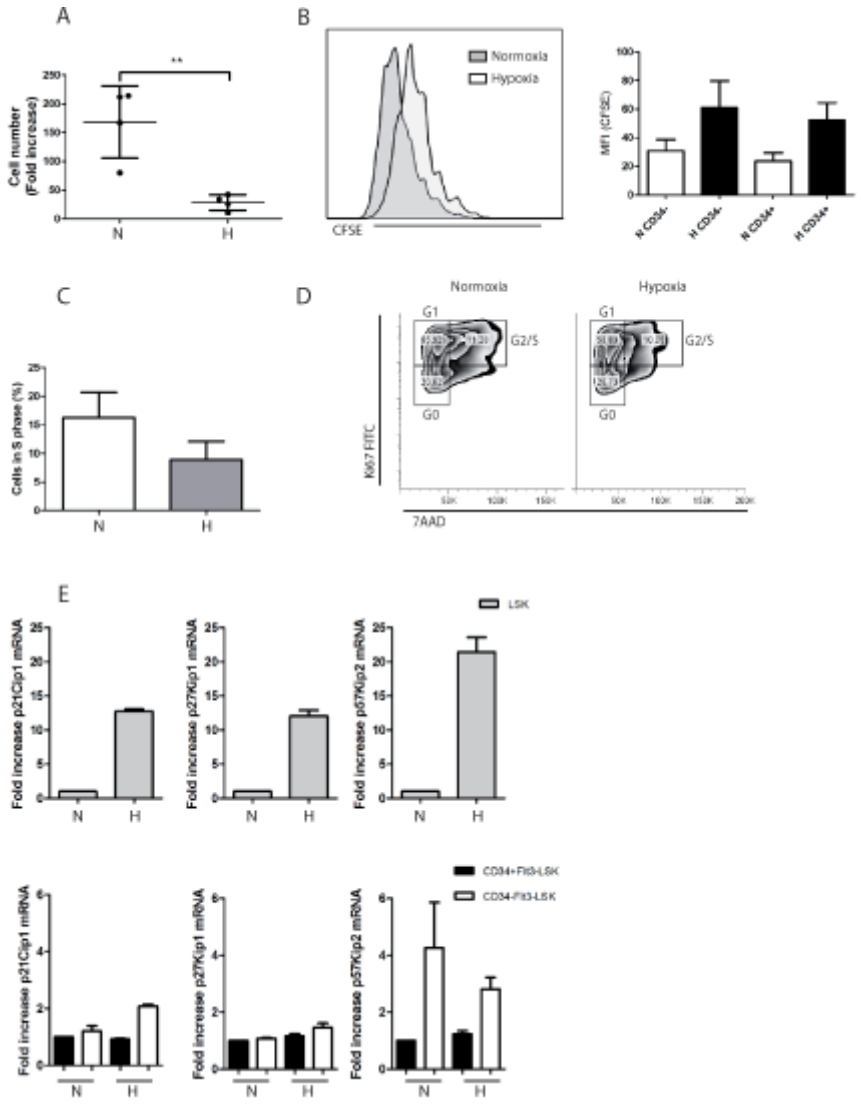

Figure 1. ***Hypoxia diminishes proliferation of HSPCs.*** *(**A**) LSK cells were cultured in 20% O_2 (normoxia) or 1% O_2 (hypoxia) for 4 days in serum-free StemSpan media supplemented with SCF, IL-6, and TPO. The total number of viable cells was determined by trypan blue exclusion. Error bars indicate the SD (**p< 0.005). (**B**) Freshly isolated $CD34^{-/+}FLT3^{-}LSK$ cells were labeled with CFSE and analysed by flow cytometry after 4 days. The FACS histogram to the left shows CFSE labeled $CD34^{-}FLT3^{-}LSK$ cells (left line: normoxia, dark grey; right line: hypoxia, light grey). The right panel shows the mean fluorescence intensity (MFI) and SD for either $CD34^{-}$ or $CD34^{+}$ $FLT3^{-}LSK$ cells from three independent experiments. (**C**) FDCP-mix cells were cultured for 48 hours in normoxia or hypoxia. Cell cycle analysis was performed after labeling DNA with propidium iodide. Bars represent mean (±SD) values of percent cells in S phase. (**D**) Cell cycle analysis of $CD34^{-}FLT3^{-}LSK$ cells cultured for 48 hours in StemSpan media supplemented with SCF and TPO in normoxia or hypoxia. Cells were stained with anti-Ki67 FITC and 7AAD. Results shown are from one representative experiments of two performed. (**E**) Quantitative RT-PCR for $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ was performed 24 hours after culture of LSK cells or $CD34^{-/+}FLT3^{-}LSK$ cells in normoxia or hypoxia. Samples were normalized to β-actin and the ΔΔCt method was used to calculate fold induction. Bars show results from one representative experiment.*

Expression of cyclin-dependent kinase inhibitor genes is upregulated by hypoxia

To investigate whether the CDKI genes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ are involved in hypoxia-mediated anti-proliferative effects, we decided to perform quantitative real time-PCR on LSK cells cultured in hypoxia or normoxia. After 24 hours in hypoxia, the expression of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ increased by 12.7-, 12.0-, and 21.2-fold, respectively (Figure 1E, upper). To investigate the situation of CDKI gene expression in ST- and LT-HSCs, we next cultured $FLT3^{-}CD34^{+}$ LSK and $FLT3^{-}CD34^{-}$ LSK cells in hypoxic conditions. In this case, $p21^{Cip1}$ was increased 2-fold by hypoxia in $FLT3^{-}CD34^{-}$ LSK cells (Figure 1E, lower), whereas expression of $p27^{Kip1}$ was similar in both cell populations and remained unchanged upon hypoxic exposure. Finally, whereas higher expression of $p57^{Kip2}$ was seen in freshly FACS-sorted $FLT3^{-}CD34^{-}$ LSK cells compared to $FLT3^{-}CD34^{+}$ LSK cells, the expression was unaffected by hypoxia.

Hypoxia maintains the stem cell potential of LSK cells and increases the proportion of long term-reconstituting HSCs in vitro

Because it has been demonstrated that the G_0 fraction of mouse BM HSCs transplant better than cells in G_1 or S phase [27], we next wanted to determine whether the decreased proliferation of HSPCs cultured in hypoxia preserved the stem cell potential. LSK cells were cultured in normoxic or hypoxic conditions for 4 days. Thereafter, a fraction of cells corresponding to 2 $x10^3$ LSK cells on day 0 from either culture were transplanted into recipient mice together with $2x10^5$ BM competitor cells. The reconstitution capacity of the cells was similar regardless of hypoxic or normoxic exposure and both for short-term (4 weeks) and long-term (16 weeks) engraftment (Figure 2A). However, since the increase of total cell numbers were not as

pronounced in hypoxia as compared to normoxia (Figure 1A), these results indicate that the proportion of HSCs with long-term engraftment potential was in fact increased in hypoxic cultures (Figure 2B). As for contribution of myeloid and B-cell lineage reconstitution, no difference between the conditions could be demonstrated (Figure 2C).

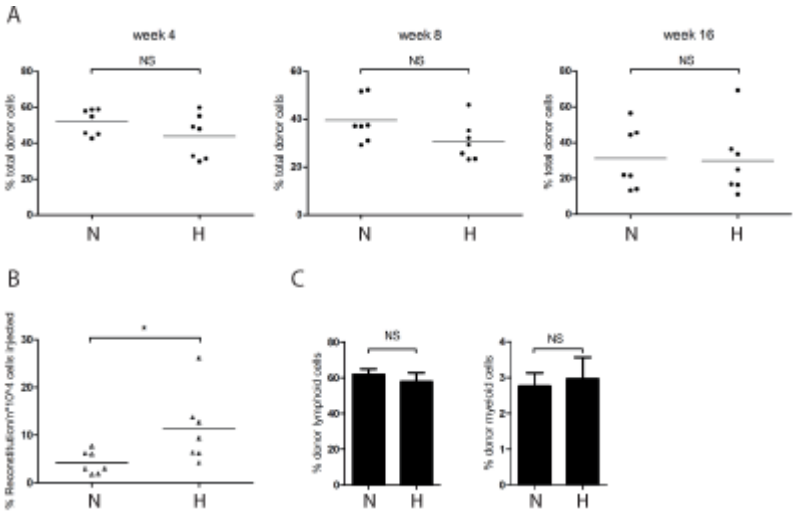

***Figure 2**. **Hypoxia increases the proportion of long term-reconstituting HSCs in vitro.** (**A**) LSK cells from CD45.1 mice (B6SJL) were cultured for 4 days in SCF, TPO, and IL-6 in normoxia or hypoxia. CD45.2 mice (C57BL6/J) were lethally irradiated before lateral injection of 2,000 (input) LSK cells. 2x10^5 BM cells from C57BL6/J mice were used as competitor cells. After 4, 8, and 16 weeks peripheral blood analysis for CD45.1 and CD45.2 markers was performed. Each symbol represents an individual recipient. Data shown is from one representative experiment out of three performed. (**B**) Same experiments as in (A), but the engraftment potential 16 weeks post transplant was considered in relation to the number of cells injected (*p<0.05). (**C**) Lymphoid and myeloid engraftment in peripheral blood 14 weeks post transplant of equal input cell number. Lymphoid cells were detected with anti-CD19 and myeloid cells were detected with anti-Mac-1 and anti-Gr-1 antibodies.*

Stabilization of HIF-1 elicits anti-proliferative effects

To clarify if HIF-1 was involved in the hypoxic effects on proliferation, we constructed a retroviral GFP vector containing constitutively active HIF-1α (caHIF-1). This cDNA carries two point mutations at positions P420 and P563, which when substituted to alanine renders the protein stable in normoxia, as demonstrated by Western blotting

of GFP$^+$ caHIF-1α-transduced Ba/F3 cells (Supplementary Figure 1). In these cells, a significant decrease in the fraction of cells in S phase could be demonstrated (Figure 3A). A potent role of HIF-1 on proliferation was also verified by exposing cells to the novel HIF-PHD inhibitor FG-4497, inducing HIF-1α activity during normoxic conditions. CFSE-labeled LSK cells were treated with FG-4497 for 3 days in pre-determined FBS concentrations to avoid inhibitory effects of serum proteins on FG-4497 (see Supplementary data figure S2). As shown in Figure 3B, FG-4497 significantly decreased the proliferation of LSK cells in normoxia, demonstrating that HIF-1 is sufficient to mediate anti-proliferative effects.

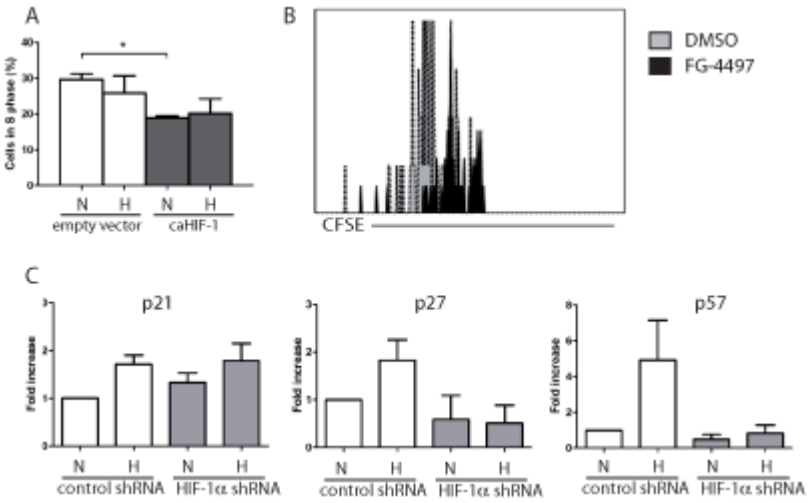

***Figure 3**. **The effect of hypoxia on HSPC proliferation is mediated through HIF-1.** Ba/F3 cells retrovirally transduced with constitutively active HIF-1α or empty vector were cultured in hypoxia or normoxia for 24 hours. Cell cycle analysis was performed after labeling with propidium iodide by flow cytometry. The figure shows mean (SD) values of three experiments. (**B**) CFSE-labeled LSK cells were cultured in IMDM with 3% FBS and treated with 20μM FG-4497 (filled black) or DMSO (filled grey, dotted) for 3 days. (**C**) Quantitative RT-PCR for p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} was performed on LSK cells stably transduced with shRNA to HIF-1α, sorted for GFP expression, and then cultured for 24 hours in normoxia or hypoxia. Bars represent mean (SEM) values from triplicates from 2-3 independent experiments.*

HIF-1α is required for expression of $p27^{Kip1}$ and $p57^{Kip2}$

We next analyzed the consequence of HIF-1α-silencing on proliferation by constructing a GFP lentiviral vector containing a short hairpin RNA (shRNA) targeting HIF-1α. This construct suppressed HIF-1α expression in LSK cells by 75±5% in normoxia and by 68±9% in hypoxia (Supplementary figure S3). We then determined if upregulation of CDKI genes during hypoxia was mediated by HIF-1. While expression of $p27^{Kip1}$ and $p57^{Kip2}$ was notably decreased in both normoxic and hypoxic conditions in HIF-1α-silenced LSK cells, no difference in $p21^{Cip1}$ expression was detected (Figure 3C), implying both HIF-1-dependent and -independent hypoxic effects on cell cycle regulation.

Persistent stabilization of HIF-1 reduces the reconstituting ability

Because pharmacologically stabilized or constitutively active HIF-1 was able to execute hypoxic effects in normoxia, we decided to investigate the reconstitution ability of HSPCs transduced with caHIF-1α or after FG-4497 treatment. The culture equivalent of $5x10^5$ BM cells from 5-FU treated mice, cultured for 3 days with either FG-4497 or DMSO, were simultaneously transplanted to recipient mice, allowing for a competitive assay to test the reconstituting ability of the two populations. While the engraftment potential was significantly lower for cells treated with FG-4497 compared to control after 4 weeks post transplant, the difference levelled out over time and after 20 weeks, FG-4497-treated cells showed the same level of reconstitution as control cells (Figure 4A). No difference was observed in contribution to the B-cell, T-cell, and myeloid lineages in peripheral blood (Figure 4B). When the effect of caHIF-1α-transduced Lin^- BM cells was evaluated, the percentage GFP^+ cells in peripheral blood of recipients transplanted with cells transduced with empty vector dropped only

marginally. In contrast, the contribution of caHIF-1α transduced cells decreased dramatically already after 4 weeks (Figure 4C). A similar rapid loss of GFP expressing caHIF-1α-transduced cells could be observed *in vitro* (Figure 4D).

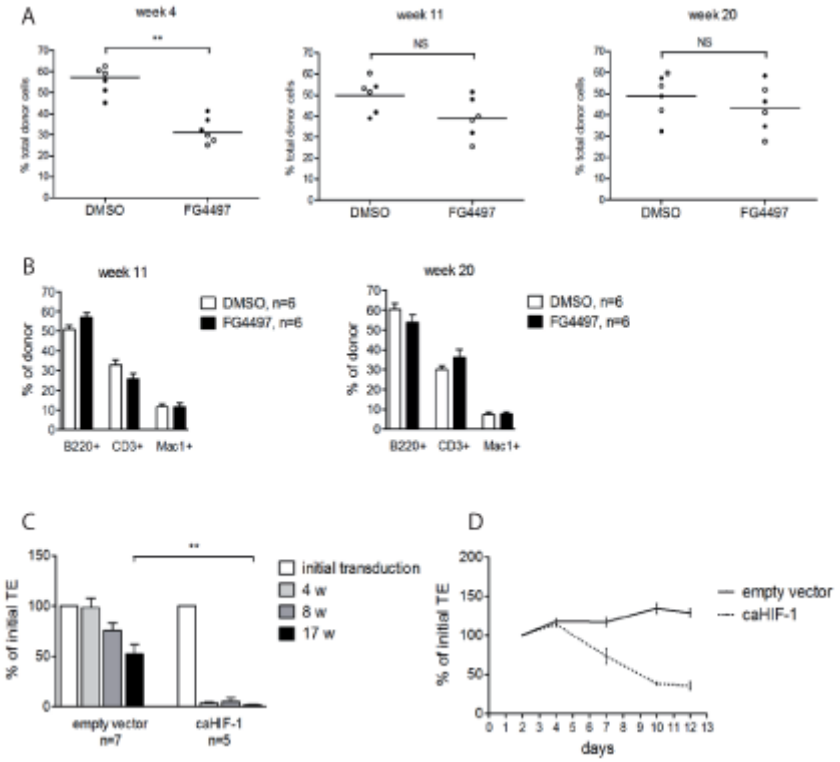

Figure 4. Transient activation of HIF-1 decreases progenitor potential, whereas stable overexpression affects engraftment potential of both progenitors and long-term HSCs. *(**A**) 5-FU treated BM cells were cultured for 3 days with SCF, TPO, and Flt3 ligand in the presence of FG-4497 or DMSO. After culture, cells were competitively transplanted against each other in the same recipient. Relative contribution to peripheral blood was measured at 4, 11, and 20 weeks post transplant. Results shown are two separate experiments performed with 3 recipients each, presented as open or filled dots. (**p<0,005). (**B**) The distribution between B-cells (B220$^+$), T-cells (CD3$^+$), and myeloid cells (Mac1$^+$) within the DMSO- or FG-4497-derived populations in peripheral blood was measured at 11 and 20 weeks post transplant. Mean (SEM) values for n=6 recipients per group. (**C**) 5x10^5 Lin$^-$ BM cells transduced with caHIF-1α or vector alone were transplanted to lethally irradiated recipients and the relative contribution of transduced cells was measured at 4, 8, and 17 weeks post transplant. Data are represented as percent GFP$^+$ cells within the donor compartment in peripheral blood relative to the initial transduction efficiency (TE) obtained at transduction (33% and 25 % for control and caHIF-1 transduced cells, respectively). Mean (SEM) values for n=7 (empty vector) and n=5 (caHIF-1) recipients (**p>0,005). (**D**) Lin$^-$ BM cells transduced with caHIF-1α or vector alone were kept in liquid culture for 12 days and percentage GFP$^+$ cells was monitored every 2-3 days. Data are represented as percent GFP$^+$ cells in culture relative to the initial transduction efficiency (20% and 10% for control and caHIF-1 transduced cells, respectively). Mean (SEM) values of duplicate samples from a representative experiment.*

Bcl-2 fails to rescue engraftment of caHIF-1α expressing HSPCs

To exclude that cells expressing HIF-1α were lost in transplanted animals due to apoptosis, we performed transplantation experiments with caHIF-1α-transduced cells from H2K-BCL2 mice. Similar to the experiments using caHIF1α-transduced BM cells from wild type mice, caHIF-1α-expressing Bcl-2 transgenic cells had an *in vivo* disadvantage compared to cells transduced with empty vector. The contribution of cells transduced with empty vector increased initially as seen by higher percentage of GFP^+ cells in the peripheral blood compared to the percentage measured at transduction (Figure 5A). However, caHIF-1α transduced Bcl-2 cells gradually declined and contribution decreased over time (Figure 5A). In addition, when cells transduced with caHIF-1α or empty vector were maintained *in vitro*, caHIF-1 transduced Bcl-2 cells again showed a decrease in relative GFP expression over time (Figure 5B). Thus, the negative effect of ca-HIF1α overexpression cannot be rescued by Bcl-2 and is unlikely due to apoptosis.

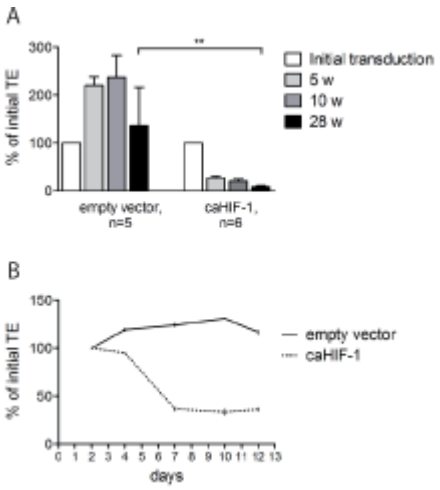

Figure 5. Stable overexpression of HIF-1α negatively affects progenitors and HSCs by a mechanism other than apoptosis. *(**A**) $5x10^5$ 5-FU treated BM cells from H2K-BCL2 transgenic mice transduced with empty vector or caHIF-1α were transplanted to lethally irradiated recipients. The relative contribution of transduced cells to hematopoiesis was measured at 5, 10, and 28 weeks post transplant. Data are represented as percent GFP^+ cells within the donor compartment in peripheral blood relative to the initial transduction efficiency (TE), which was 17% and 9% for control and caHIF-1 transduced cells, respectively. Mean (SEM) values for n=5 (pMy) and n=6 (caHIF-1) recipients (**p<0,005). (**B**) Lin^- BM cells from BCL2 transgenic mice transduced with empty vector or caHIF-1α were kept in liquid culture for 12 days and GFP expression was monitored every 2-3 days. Data represent percent GFP^+ cells in culture relative to the initial transduction efficiency obtained at transduction, 20% and 10% for control and caHIF-1 transduced cells, respectively. Mean (SEM) values of duplicate samples from a representative experiment.*

Discussion

In this study, we have investigated the consequence of hypoxia on *in vitro* culture of transplantable HSCs as well as the molecular mechanisms by which HIF-1α affects HSCs. We found that hypoxic culture of HSPCs in serum free media with TPO, SCF, and IL-6 preserved the stem cell capacity. Although beneficial effects of hypoxia on mouse HSCs have been suggested before [17, 20], previous studies were performed with total BM cells and with cytokines known to negatively affect HSCs expansion such as IL-3. We now extend this to include purified LSK cells, containing both long and short-term HSCs as well as primitive progenitor cells. When LSK cells were grown in 1% O_2, and the same number of input cells were transplanted to recipient mice, the reconstitution potential of HSCs was preserved, but not enhanced compared to normoxic conditions. However, when the numbers of cells injected were taken into account, fewer cells from hypoxic cultures were needed to reconstitute to the same extent as normoxia, indicating that although both conditions can preserve HSCs during cell culture, progenitors are expanded more vigorously in normoxia. These results are in agreement with a recent study on $CD34^{+}38^{-}$ cells from human cord blood [18, 21], where Shima *et al.* showed that human transplantable HSCs from umbilical cord blood remain quiescent in hypoxia. In contrast, Danet *et al.* found that when human BM HSCs were cultured for 4 days in hypoxia, SCID-repopulating cells actually increased 4-6-fold. In the later study, cell cycle distribution was analyzed which showed that hypoxia preferentially promoted the transition of cells from G_0 to G_1. However, these experiments were performed with $Lin^{-}CD34^{+}$ cells, a population that mainly contain progenitor cells and few LT-HSCs. Instead, we have

used highly-purified, functionally tested, LT- and ST-HSCs from mouse BM to study cell cycle effects by hypoxia. With these cells, we could demonstrate a clear decrease in proliferation and increased numbers of LT-HSCs ($FLT3^-CD34^-$ LSK) in G_0 in hypoxic cultures, as measured by higher CFSE-staining and larger fraction of $Ki\text{-}67^-$ cells. In addition, we could demonstrate that, at least in part, HIF-1 is mediating these anti-proliferative effects since treatment with the HIF-1 stabilizer agent FG-4497 led to similar results.

Because quiescent cells in the BM are most abundant in the hypoxic region close to the endosteal surface, and in association with sinusoids and distant to capillaries [12], it seems that hypoxia could be a critical regulator to maintain HSCs. We detected an upregulation of $p21^{Cip1}$ in LT-HSCs cultured in hypoxia. Similar to our results, Shima *et al.* observed that quiescence of human $CD34^+38^-$ cells cultured in hypoxia was associated with increased levels of $p21^{Cip1}$ [21]. The ability of $p21^{Cip1}$ to regulate quiescence of mainly HSCs have been reported earlier [3, 28-30]. In contrast to $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ were not affected by hypoxia, although higher levels of $p57^{Kip2}$ were seen in $CD34^-$ LT-HSCs compared to $CD34^+$ ST-HSCs, as previously described [31-33]. It is possible that the expression of $p57^{Kip2}$ in LT-HSCs is already high due to its BM localization and in part by cytokine-induced expression of $p57^{Kip2}$ [31-33]. Thus, in our study, exposure to hypoxia of $FLT3^-CD34^-$ LSK cells would not further increase $p57^{Kip2}$ expression.

Previous studies in primary fibroblasts and B lymphocytes have shown that HIF-1α induces cell cycle arrest by activating $p21^{Cip1}$ and $p27^{Kip1}$ expression [16, 34]. However, it has also been suggested that $p27^{Kip1}$ is a key hypoxic regulator of cell cycle arrest independently of HIF-1 [35]. In our study, all three CDKI genes analyzed were upregulated in LSK cells by hypoxia. However, only $p27^{Kip1}$ and

$p57^{Kip2}$ expression were affected by silencing of HIF-1α. This implicates that hypoxia-induced quiescence of HSCs, associated with increased $p21^{Cip1}$ levels, is not necessarily dependent on HIF-1α, whereas proliferation of progenitor cells are inhibited by HIF-1α through upregulation of $p27^{Kip1}$.

From our findings that hypoxia and HIF-1α promoted slower proliferation and an increase of cells in G_0, we speculated that treatment with the HIF-1 stabilizing agent FG-4497 during *in vitro* normoxic cultures could potentially give rise to more efficient reconstitution of recipient mice. This was based on the previous report that HSCs from the G_0 fraction reconstitute more efficiently compared to cells in G_1 or $S/G_2/M$ [36]. Similarly, we expected that constitutively active HIF-1α should provide an *in vivo* advantage of engraftment when permanently expressed in HSCs. However, initial reconstitution was lower after treatment of HSPCs with FG-4497, whereas the long-term repopulation ability was unaffected. One explanation is that excessive activation of HIF-1 could lead to a decrease in proliferation of progenitor cells, but not affecting the number of long-term reconstituting HSCs. When these experiments were performed with constitutively expressed HIF-1α, a reduction of repopulation ability was seen already after 4 weeks, and hematopoietic reconstitution with HSCs expressing caHIF-1α never recovered. This rapid cell loss most likely represents an *in vivo* disadvantage of caHIF-1α transduced cells relative to control cells, possible due to anti-proliferative effects. Alternatively, too high level of caHIF-1α leads to detrimental cellular effects on viability. However, overexpression of the anti-apoptotic protein Bcl-2 failed to rescue donor-derived reconstitution of caHIF-1α transduced cells.

Taken together, the results presented in this study imply that hypoxia, in part mediated by HIF-1α, plays an important role in preserving stemness and sustaining HSC quiescence by decreasing the proliferative rate via modulation of cyclin-dependent kinase inhibitor genes.

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Supplementary detailed experimental procedure

Titration of serum level for FG-4497 treatment

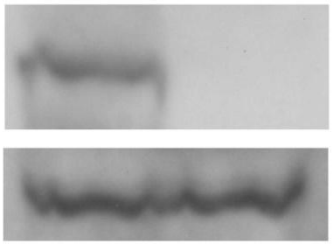
FG-4497 (kind gift from FibroGen Inc, San Francisco, CA) is a small molecule that inhibits the activity of prolyl-hydroxylase domain proteins (PHD), the oxygen sensing proteins responsible for degradation of HIF-α subunits in normoxia. FG-4497 thereby induces HIF-activity. The activity of FG-4497 is sensitive to protein concentration with optimal activity at 0.5% serum, and thus a dose-response curve to determine the activity of FG-4497 in FBS at a concentration that can support culture of HSPCs was performed. 3T3 cells were transduced with a lentiviral vector expressing a reporter construct that is induced by HIF-activity. The reporter consists of 5 copies of the hypoxia response element (HRE) driving the expression of GFP. 3T3-HRE-GFP cells were cultured in DMEM+Glutamax supplemented with 1-10% FCS or 1% BSA for 48 hours in normoxia. The reporter activity, indicated by mean fluorescence intensity (MFI), was measured by FACS analysis.

Lethally irradiation of mice

Mice were irradiated in a cage with two opposed fields, using a linear accelerator (Varian Clinac 600C) with a 6MV spectra to a total absorbed dose to water of 9Gy, single fraction. The absorbed dose was precisely determined, in accordance with IAEA TRS 398, ref [1], by measurements in a small water phantom positioned at the centre of the cage. The expanded combined uncertainty was 3.0% (coverage factor

2), assessed according to guidelines in ref [2]. Outside the centre the dose increased up to 2.3%.

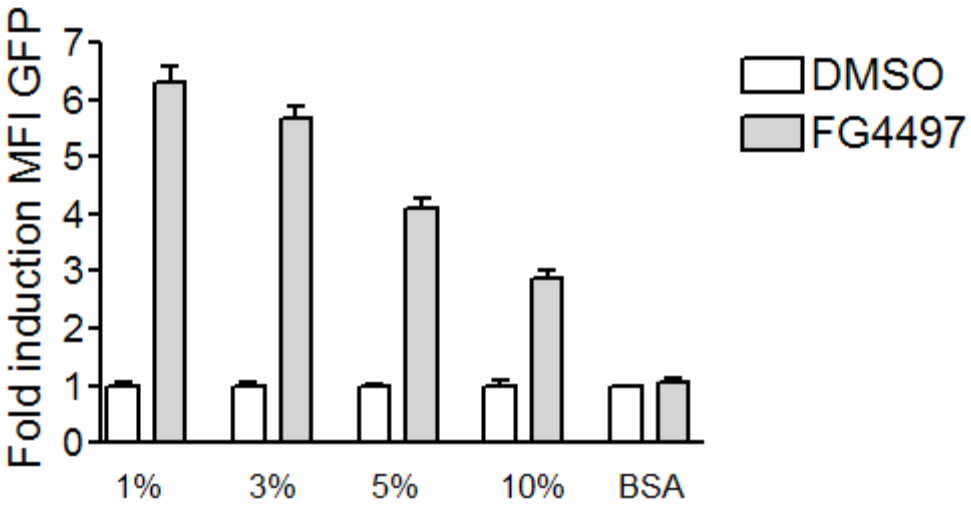
1. IAEA 2000, Absorbed Dose Determination in External Beam Radiotherapy. Technical report series no. 398, International Atomic Energy Agency, Vienna, Austria.
2. International Organization for Standardization (ISO) 1995, Guide to the Expression of Uncertainty in Measurement, Geneva, Switzerland.



caHIF empty vector

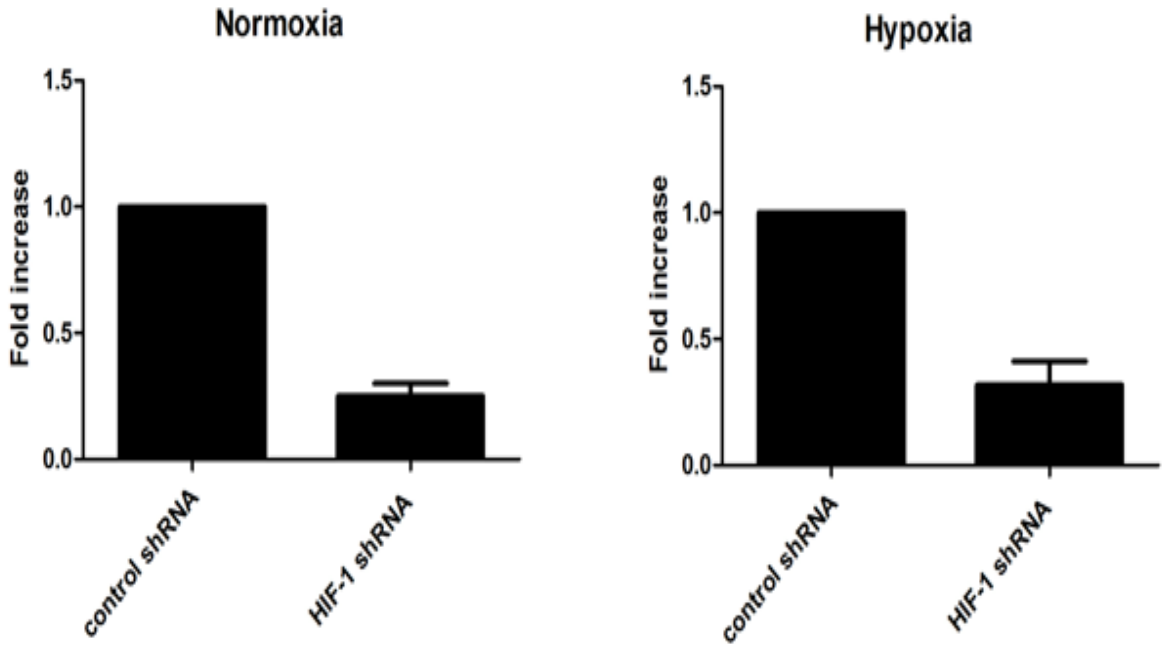
Constitutively active HIF-1α protein is expressed in normoxia. Protein levels of HIF-1α in Ba/F3 cells transduced with constitutively active (ca) HIF-1α or empty vector were analysed with Western blot on a NuPAGE Bis-Tris gel (invitrogen). Loading control used was antibodies against the housekeeping gene α-actin.

Supplementary Figure S1



FG-4497 induces HIF-activity in an HRE-GFP reporter assay. 3T3 cells transduced with a lentiviral HRE-GFP were treated with FG-4497 or DMSO and grown in different concentrations of FBS or BSA (1%). Reporter activity was measured by mean fluorescence intensity (MFI) of GFP expression. Results are displayed as fold induction compared to the DMSO treated controls. The figure shows mean (SEM) values of duplicate samples.

Supplementary figure S2



LSK cells transduced with control shRNA or shRNA to HIF-1α. The HIF-1α shRNA suppressed HIF-1α expression in LSK cells by 75±5% in normoxic and by 68±9% in hypoxic conditions after 24 hours measured by Quantitative RT-PCR.

Supplementary figure S3

Supplementary Table SI

Sequences of primers used in PCR amplification, sequencing reactions, and RT-real time PCR analysis.

Primer name	**Sequence 5'->3'**	**For use in**
Forward primer pLKO.1	TCTTTCCCCTGCACTGTACC	PCR
Reverse primer pLKO.1	ATGGACTATCATATGCTTAC	PCR, sequencing
Forward primer HIF-1α	CACCGATTCGCCATGGA	Real time PCR
Reverse primer HIF-1α	ACGTTCAGAACTCATCTTTTTC TTCTC	Real time PCR
Forward primer $p21^{Cip1}$	GGACCACGTGGCCTTGTC	Real time PCR
Reverse primer $p21^{Cip1}$	GAATCTTCAGGCCGCTCAGA	Real time PCR
Forward primer $p27^{Kip1}$	TCTTCGGCCCGGTCAA	Real time PCR
Reverse primer $p27^{Kip1}$	CCGGCAGTGCTTCTCCAA	Real time PCR

Forward primer $p57^{Kip2}$	GGCCAATGCGAACGACTT	Real time PCR
Reverse primer $p57^{Kip2}$	CCTTGTTCTCCTGCGCAGTT	Real time PCR
Forward primer β-actin	GCTGTATTCCCCTCCATCGTG	Real time PCR
Reverse primer β-actin	CACGGTTGGCCTTAGGGTTCAG	Real time PCR

Supplementary Table SII

21bp sequences of sense and anti-sense regions of the shDNA oligonucleotides used in this study.

shDNA oligonucleotides	**sequence**	**Genetic location**
HIF-1 α sense sequence	CCAGTTACGATTGTGAAGTTA	
HIF-1 α anti-sense sequence	TAACTTCACAATCGTAACTGG	bp2674-2694
Scramble sense sequence	CCTAA GGTTA AGTCG CCCTC G	
Scramble anti-sense sequence	CGA GGGCG ACTTA ACCTT AGG	