Oxygen tension plays a critical role in the hematopoietic microenvironment *in vitro*

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Acknowledgments: the authors would like to thank Katrin Müller, University Hospital Dresden, for her excellent technical assistance.

Funding: this work was supported by a DFG grant, "Collaborative Research Center 655; Cells into tissues: stem cell and progenitor commitment and interactions during tissue formation" (SFB 655, projects B2 and B7).

Manuscript received on July 4, 2011. Revised version arrived on October 14, 2011. Manuscript accepted on October 24, 2011.

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

In the bone marrow mesenchymal stromal cells and osteoblasts form functional niches for hematopoietic stem and progenitor cells. This microenvironment can be partially mimicked using *in vitro* co-culture systems. In this study, we examined the oxygen tension in three distinct compartments in a co-culture system of purified CD34$^+$ cells and mesenchymal stromal cells with regard to different spatial localizations.

Design and Methods

Hypoxic cells in the co-culture were visualized by pimonidazole staining. Hematopoietic cell distribution, and functional and phenotypic characteristics were analyzed by flow cytometry. The secretion of vascular endothelial growth factor and stromal-derived factor-1 by mesenchymal stromal cells in low oxygen co-cultures was determined by an enzyme-linked immunosorbent assay. The effect of co-culture medium on the hematopoietic cell migration potential was tested in a transwell assay.

Results

In co-cultures under atmospheric oxygen tension, regions of low oxygen tension could be detected beneath the feeder layer in which a reservoir of phenotypically more primitive hematopoietic cells is located *in vitro*. In low oxygen co-culture, the adhesion of hematopoietic cells to the feeder layer was decreased, whereas hematopoietic cell transmigration beneath mesenchymal stromal cells was favored. Increased vascular endothelial growth factor-A secretion by mesenchymal stromal cells under low oxygen conditions, which increased the permeability of the monolayer, was responsible for this effect. Furthermore, vascular endothelial growth factor-A expression in low oxygen mesenchymal stromal cells was induced via hypoxia-inducible factor signaling. However, stromal cell-derived factor-1 secretion by mesenchymal stromal cells was down-regulated under low oxygen conditions in a hypoxia-inducible factor-independent manner.

Conclusions

We demonstrate for the first time that differences in oxygen tension cause selective modification of hematopoietic cell and mesenchymal stromal cell interactions in a co-culture system, thus confirming that oxygen tension plays a critical role in the interaction between hematopoietic cells and the niche environment.

Key words: hematopoietic microenvironment, oxygen tension, C34$^+$ cells, mesenchymal stromal cells.

Citation: Jing D, Wobus M, Poitz DM, Bornhäuser M, Ehninger G, and Ordemann R. Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. *Haematologica 2012;97(3):331-339. doi:10.3324/haematol.2011.050815*

Introduction

Hematopoietic stem and progenitor cells (HSPC) give rise to all types of blood cells.[1-3] HSPC transplantation is used in the treatment of various hematologic malignancies and non-malignant disorders.[4;5] To improve the clinical outcome of HSPC transplantation, many groups are working on the *ex vivo* expansion of HSPC, particularly in cases in which graft sizes are limited.[6] Several strategies have been developed to expand HSPC *in vitro* by utilizing cytokines[7;8] and mesenchymal stromal cells (MSC).[9-13] However, the *in vivo* regulation of HSPC remains poorly understood and the maintenance of HSPC *in vitro* is difficult to accomplish.

In the bone marrow, HSPC interact with a specific microenvironment called the "stem cell niche," which regulates the fate of HSPC in terms of quiescence, self-renewal, and differentiation.[14-16] For decades, these niches were believed to be hypoxic regions in which only cells requiring less oxygen were able to survive.[17] Furthermore, the oxygen concentration in the bone marrow of healthy volunteers is lower than that in the peripheral blood.[18] Recently, HSPC were reported to be predominantly located in a sinusoidal hypoxic niche at the lowest end of the oxygen gradient in the bone marrow.[19;20] Several *in vitro* studies have revealed that hypoxia facilitates the maintenance of HSPC.[21-23] Oxygen tension does, therefore, appear to be critical for establishing the stem cell niche *in vitro*.

Co-culture of CD34$^+$ cells with a MSC layer *in vitro* is a simplified system to investigate the interactions between HSPC and the stem cell niche.[9-13] Recently, we identified three distinct compartments in a hematopoietic cell (HC)/MSC co-culture system which regulate the HC fate in distinct ways: (i) non-adherent cells in the supernatant, (ii) phase-bright cells on the MSC surface, and (iii) phase-dim cells beneath the MSC layer.[24] The MSC surface is the predominant site of proliferation, whereas the compartment beneath the MSC layer appears to mimic the stem cell niche for immature cells, indicating that even *in vitro* spatial localization has an important effect on the fate of stem cells.[24]

In the present study, we identified that the compartment beneath the MSC layer had the lowest oxygen concentration in the co-culture system, which may contribute to the maintenance of CD34$^+$ cells. This observation prompted us to investigate the effects of oxygen tension on CD34$^+$ cells and MSC in detail. We, therefore, analyzed immunophenotypic characteristics, cell proliferation, and migration of CD34$^+$ cells as well as cytokine secretion by MSC under low oxygen conditions. Both oxygen tension and interactions between CD34$^+$ cells and MSC were assumed to contribute significantly to the complex process of niche regulation.

Design and Methods

Purification of CD34$^+$ cells from mobilized peripheral blood

Mobilized peripheral blood of healthy donors was obtained from leukapheresis products after the donors had been treated with 7.5 μg/kg granulocyte colony-stimulating factor for 5 days. Informed consent was obtained in accordance with a research protocol approved by the local institutional review board. CD34$^+$ HC were purified from leukapheresis samples using CD34 antibody-conjugated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Germany). CD34$^+$ HC have a purity of more than 95% as assessed by flow cytometry (FACS) and a vitality of more than 96% as measured by trypan blue exclusion.

Isolation of mesenchymal stromal cells

MSC were isolated from bone marrow aspirates that were derived and cultured from healthy donors after receiving informed consent and approval from the local ethics committee as described previously.[25] The phenotypes of all the MSC batches were examined by FACS: presence of CDw90, CD105, CD166, and CD73 and absence of CD34 and CD45 were required. MSC of passage two were then seeded in a 12-well plate at a density of 1×10^4/cm^2 in MSC medium. All MSC batches were examined for their potential for osteogenic and adipogenic differentiation.[25] The medium was changed every third day until the MSC layer reached confluence.

Co-culture of CD34$^+$ cells with the mesenchymal stromal cell layer

CD34$^+$ cells were suspended in CellGro® SCGM medium (CellGenix, Germany) containing 10% fetal calf serum (Biochrom, Cambridge, UK), 150 ng/mL fetal liver tyrosine kinase-3 ligand (FLT3-L, Biosource, USA), 150 ng/mL stem cell factor (Biosource, USA), and 50 ng/mL interleukin-3 (Miltenyi Biotec, Germany). The CD34$^+$ cells were plated at a density of 1×10^4/cm^2 on the confluent MSC layer at 37°C.

Cell culture under low oxygen conditions

Cells were cultured under low oxygen conditions using a low oxygen chamber (Biospherix, USA) which was placed in an incubator. The oxygen tension was set at 0.5% O_2 by flushing the chamber with a gaseous mixture of 95% N_2 and 5% CO_2.

Cell collection from the three distinct compartments

HC from the three distinct compartments of the co-culture system were collected separately as described previously.[24] In brief, the co-culture supernatant was vigorously rinsed and the cells in the supernatant (non-adherent cells) were collected. The MSC layer was then washed gently twice with phosphate-buffered saline (PBS) to remove any remaining non-adherent cells. The cells remaining on the MSC layer (phase-bright cells) were collected by further vigorous washing with PBS. When no phase-bright cells could be observed under a phase-contrast microscope, the MSC layer and the cells beneath it (phase-dim cells) were trypsinized and collected. To exclude the effects of trypsin, non-adherent and phase-bright cells were also trypsinized for 5 min. The number of cells in each fraction was counted after staining with trypan blue (vitality more than 96%).

Assessment of hypoxic cells in co-culture

Hypoxic cells in the co-culture were detected using the Hypoxyprobe-1 Plus Kit (NPI Inc., USA) according to the manufacturer's instructions. In brief, at day 5 of co-culture, pimonidazole was added to the co-culture at a final concentration of 400 μM. After 2 h of incubation, hypoxic cells were detected by FACS or fluorescence imaging.

Immunofluorescence microscopy

The pimonidazole-treated co-culture was subjected to immunofluorescence staining as described previously.[24] In brief, the MSC layer and the remaining HC were fixed in 3.8%

formaldehyde and permeabilized using 0.1% Triton X-100. After blocking, the cells were labeled with Hypoxyprobe Mab1-FITC (1:200; NPI Inc., USA) and CD45-PE (1:10; Miltenyi Biotec, Germany). Nuclei were labeled with DAPI (Sigma, USA). Finally, fluorescence imaging was performed using a confocal laser scanning microscope (LSM 510, Zeiss, Germany).

Flow cytometry analysis

HC subsets were labeled with CD34-APC, CD45-PE, CD11a-FITC, CD49d-APC, and CD49e-PE monoclonal antibodies (1:50; Miltenyi Biotec, Germany). After staining, the cells were examined using FACScalibur (BD Biosciences, Germany) and analyzed with CellQuest software (BD Biosciences, Germany). Gating on $CD45^+$ was performed to separate HC and MSC.

In order to detect hypoxic cells, pimonidazole-treated HC subsets were fixed in 3.8% formaldehyde in PBS for 15 min and blocked with 2% fetal calf serum in PBS. The samples were then labeled with hypoxyprobe Mab1-FITC and CD45-APC and examined as described above.

Cell proliferation

Generations of HC were identified using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Germany). In brief, $CD34^+$ cells were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions and co-cultured with MSC. On day 4, the HC subsets were collected and analyzed by FACS. The number of cell divisions was quantified according to the CFSE signal intensity using CellQuest software. As a control, CFSE-labeled $CD34^+$ cells were treated with 50 μg/mL mitomycin (Santa Cruz, USA) to arrest the cell cycle at G_0.

Transwell migration assay

A 600-μL aliquot of conditioned medium from either the atmospheric oxygen or low oxygen co-culture was added to the lower chamber of a 5-μm 24-well polycarbonate transwell culture dish (Corning Costar Corporation, USA). Subsequently, 2×10^5 fresh $CD34^+$ cells were suspended in 0.1 mL of the medium and added to the upper chamber. The cells were allowed to migrate for 4 h at 37 °C. The cells that migrated into the lower chamber were then counted.

Hematopoietic cell - mesenchymal stromal cell transmigration assay

The HC-MSC transmigration assay was performed to verify whether vascular endothelium growth factor (VEGF) induces MSC permeability. In brief, conditioned medium was collected from either the atmospheric oxygen or low oxygen MSC culture on day 4. Next, a freshly prepared confluent MSC monolayer was incubated with the conditioned medium with or without 3 μg/mL VEGF-blocking antibody (anti-human VEGF Mouse IgG, IBL Co., Japan) for 24 h. An MSC layer incubated in fresh medium for 24 h was used as a control. $CD34^+$ HC were purified with MACS and suspended in CellGro® SCGM medium with 10% fetal calf serum, 150 ng/mL FLT3-L, 150 ng/mL stem cell factor, and 50 ng/mL interleukin-3. After a 24-h pre-incubation, the MSC monolayer was washed with PBS and co-cultured with $CD34^+$ cells at a density of $5\times10^4/cm^2$. The co-culture was incubated under normal culture conditions (atmospheric oxygen, 37°C, 5% CO_2) for 5 h, which allowed $CD34^+$ cells to migrate in the co-culture. After incubation, the supernatant of the co-culture was discarded and the cells on the MSC surface (phase-bright cells) were removed by vigorous washing. When no phase-bright cells could be observed under a phase-contrast microscope, the MSC monolayer with HC beneath it (phase-dim cells), was trypsinized, collected, and analyzed by FACS. The number of transmigrated HC was determined after counting the total cell number and calculating the percentage of $CD45^+$ and $CD166^+$ cells by FACS.

Real-time reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated using Trizol reagent (Invitrogen, USA). cDNA was synthesized from 1 μg of total RNA in a 20-μL standard reaction mixture containing 200 U Superscript III RNaseH-reverse transcriptase (Invitrogen GmbH, Germany). Stromal cell derived factor-1α (SCDF-1α) or VEGF-A was amplified using a Taqman 7500 real-time polymerase chain reaction machine (Applied Biosystems, Darmstadt, Germany). The housekeeping gene *HPRT-1* was used as the reference gene. All primers were purchased from Applied Biosystems.

Western blots

For western blotting, 20 μg of protein were loaded onto 10% (w/v) polyacrylamide gels. After transfer, nitrocellulose membranes were incubated with mouse anti- hypoxia-inducible factor (HIF)-1α (1:500, BD Biosciences, Germany), rabbit anti-HIF-2α (1:1000, Novus Biologicals, USA), or mouse anti-β actin (Sigma-Aldrich, USA) antibodies. Sheep anti-mouse-HRP (1:10000, Amersham, UK) or goat anti-rabbit-HRP (1:2500, Santa Cruz, USA) antibodies were used as secondary antibodies. Signals were detected using the Western-Lightning reagent (Perkin-Elmer, USA) on Hyperfilms (Amersham, UK).

Enzyme-linked immunosorbent assay

VEGF-A and SDF-1 expression was detected in the culture medium using a human VEGF-A enzyme-linked immunosorbent assay (ELISA) kit (BioVendor, Czech Republic) and a human SDF-1 ELISA Kit (R&D Systems, Germany) according to the manufacturer's instructions.

Loss of function experiments

VEGF-A small interfering RNA (siRNA; Ambion, USA) was transfected into MSC using Lipofectamine™ 2000 (Invitrogen) in a 12-well plate. For each transfection, siRNA-Lipofectamine complexes were prepared with 200 pmol of siRNA and 2 μL of Lipofectamine in 200 μL Opti-MEM (Invitrogen, UK) for each well. Twenty-four hours after transfection, MSC were used in the subsequent experiments.

Adenovirus construction, purification, and infection

Recombinant adenoviruses were constructed using the Adeno-X Expression System 1 (Clonetech, Mountain View, USA) according to the manufacturer's instructions. Adenoviruses were purified using CsCl density gradient centrifugation. The viral titers were determined using an Adeno-X Rapid Titer Kit (Clonetech, Mountain View, USA). The infection efficiency was determined using an adenovirus encoding for the coral green fluorescent protein. The best results were obtained by infecting MSC at an MOI of 500. MSC were seeded in a 6-well plate at a density of 2×10^5/well. Cells were infected with an adenovirus encoding β-galactosidase or a dominant-negative mutant of HIF-2α (AdXHifαdn).[26] Forty-eight hours after infection the medium was changed and the cells were further cultivated under either atmospheric oxygen or low oxygen conditions.

Statistical analysis

All data were derived from at least three independent experiments. Data are represented as mean ± standard error of the mean and were analyzed using the two-sided paired Student's t

test. Differences were considered statistically significant if the *P* value was less than 0.05.

Results

Pimonidazole staining indicates low oxygen regions in atmospheric oxygen co-culture

Confocal microscopy of immunostained CD45$^+$ HC cultured on the MSC layer showed that some of the HC in the atmospheric oxygen co-culture were positive for pimonidazole, indicating that they were hypoxic (Figure 1A). In the low oxygen co-culture, HC and MSC were positive for pimonidazole (Figure 1B). As shown by FACS, the pimonidazole signal intensity of phase-bright and phase-dim cells co-cultured under atmospheric oxygen conditions demonstrated a positive shift compared to the negative control and non-adherent cells, indicating a decrease in oxygen tension in the co-culture system (Figure 1C). Comparison of the pimonidazole staining patterns of the three cell compartments revealed that phase-dim cells (HC beneath the MSC layer) constituted the highest proportion of hypoxic cells in the co-culture system (Figure 1E). As expected, high pimonidazole signal intensities were detected in all three HC subsets of the low oxygen co-culture (Figure 1D, E).

Oxygen tension influences hematopoietic cell distribution in the three compartments of the co-culture system

HC migration in the co-culture system involves two major processes: (i) adhesion of the cells in supernatant to the MSC surface and (ii) cell migration from the MSC surface to beneath the MSC layer. The ratio of cell adhesion on the MSC surface and that of cell migration through the MSC layer were defined according to the number of cells in each of the three co-culture compartments (Figure 2D and E). As shown in Figure 2A and B, the numbers of non-adherent and phase-bright cells were significantly lower in the low oxygen co-culture than in the atmospheric oxygen co-culture during the 7 days of co-culture. Consistently, the ratio of cell adhesion [phase-bright/(non-adherent + phase-bright)] was significantly lower in the low oxygen co-culture (Figure 2D). However, in the low oxygen co-culture there was a robust increase in phase-dim cells until day 6 (Figure 2C), resulting in a significant increase in the ratio of cells that migrated through the MSC layer (phase-dim/[phase-bright + phase-dim]) under low oxygen conditions (Figure 2E). These data suggest that the HC distribution in the three compartments was influenced by oxygen tension.

Oxygen tension influences the characteristics of hematopoietic cells in co-culture

In our co-culture system, the MSC surface was shown

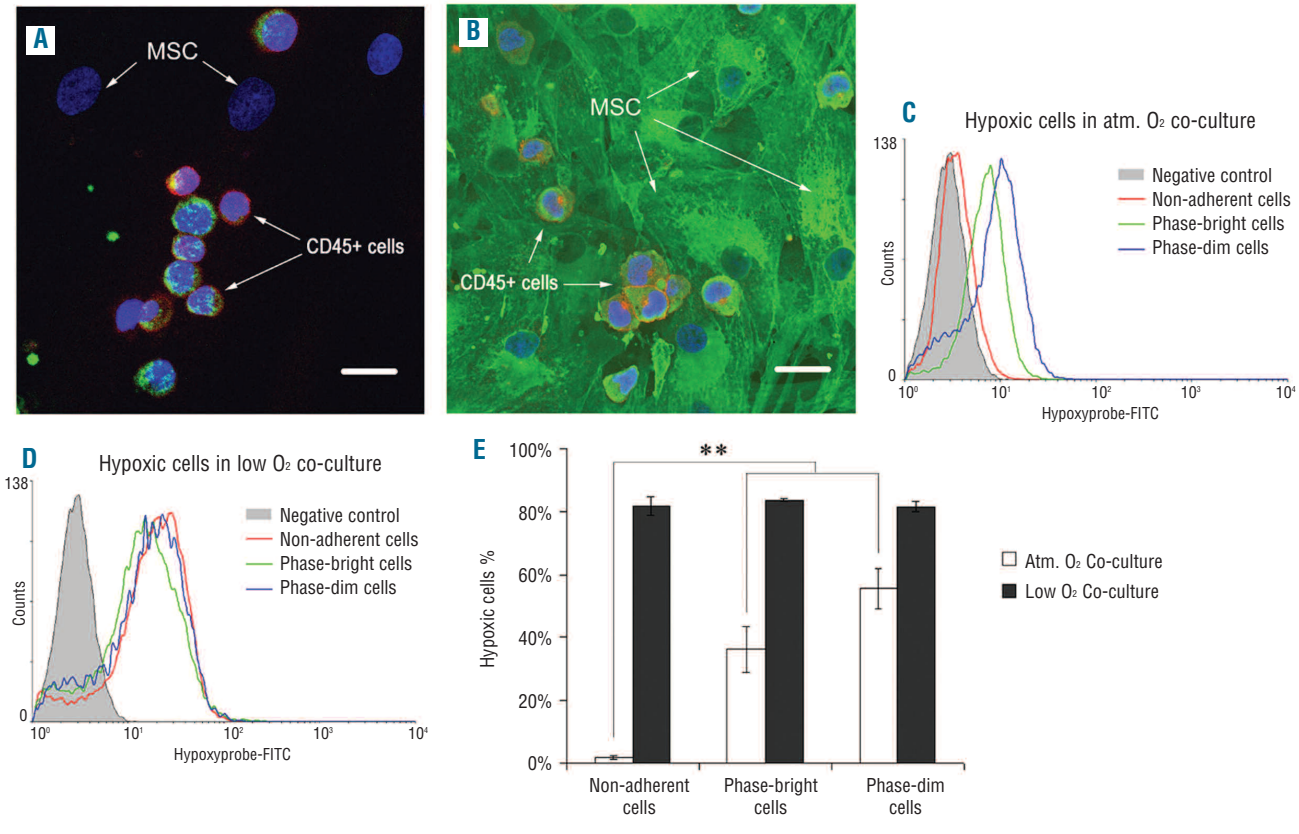

Figure 1. Localization of hypoxic HC in the co-cultures under atmospheric O_2 concentration (atmospheric O_2 co-culture) and 0.5% O_2 concentration (low O_2 co-culture). Hypoxic cells in atmospheric O_2 co-culture (A) and low O_2 co-culture (B) were stained with pimonidazole (green). Hematopoietic cells are CD45$^+$ (red). Bar=20 μm. The percentage of low oxygen HC in the distinct compartments of atmospheric O_2 co-culture and low O_2 co-culture were quantified according to fluorescence intensities by FACS, as shown in (C), (D), and (E). N=5; **P<0.01.

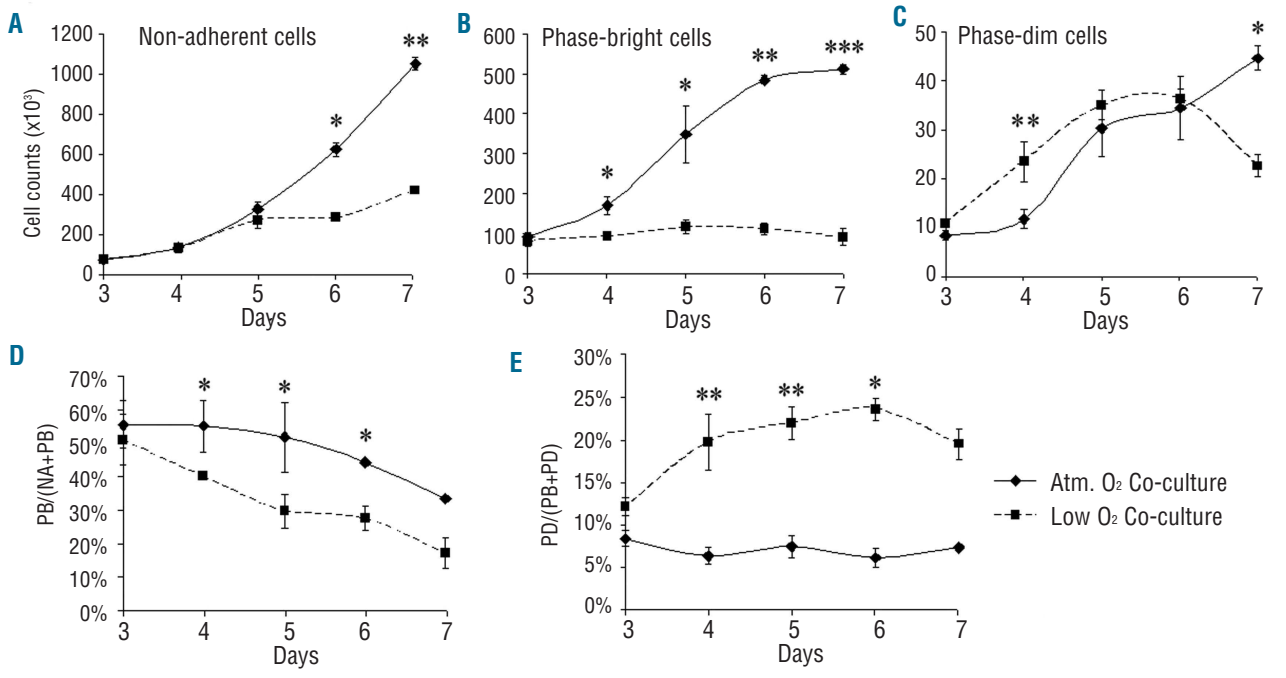

Figure 2. HC distribution in atmospheric O_2 co-culture and low O_2 co-culture. (A-C) Number of (number per well of a 12-well plate) non-adherent (NA), phase-bright (PB), and phase-dim (PD) cells in atmospheric O_2 co-culture and low O_2 co-culture on days 3–7. (D and E) Two ratios were used to evaluate the HC distribution in atmospheric O_2 co-culture and low O_2 co-culture. (D) The ratio of cell adhesion on the MSC surface was defined as the number of PB cells divided by the sum of NA and PB cells (PB/[NA + PB]). (E) The ratio of cell migration through the MSC layer was defined as the number of PD cells divided by the sum of PB and PD cells (PD/[PB + PD]). N=3; **P*<0.05; ***P*<0.01; ****P*<0.001.

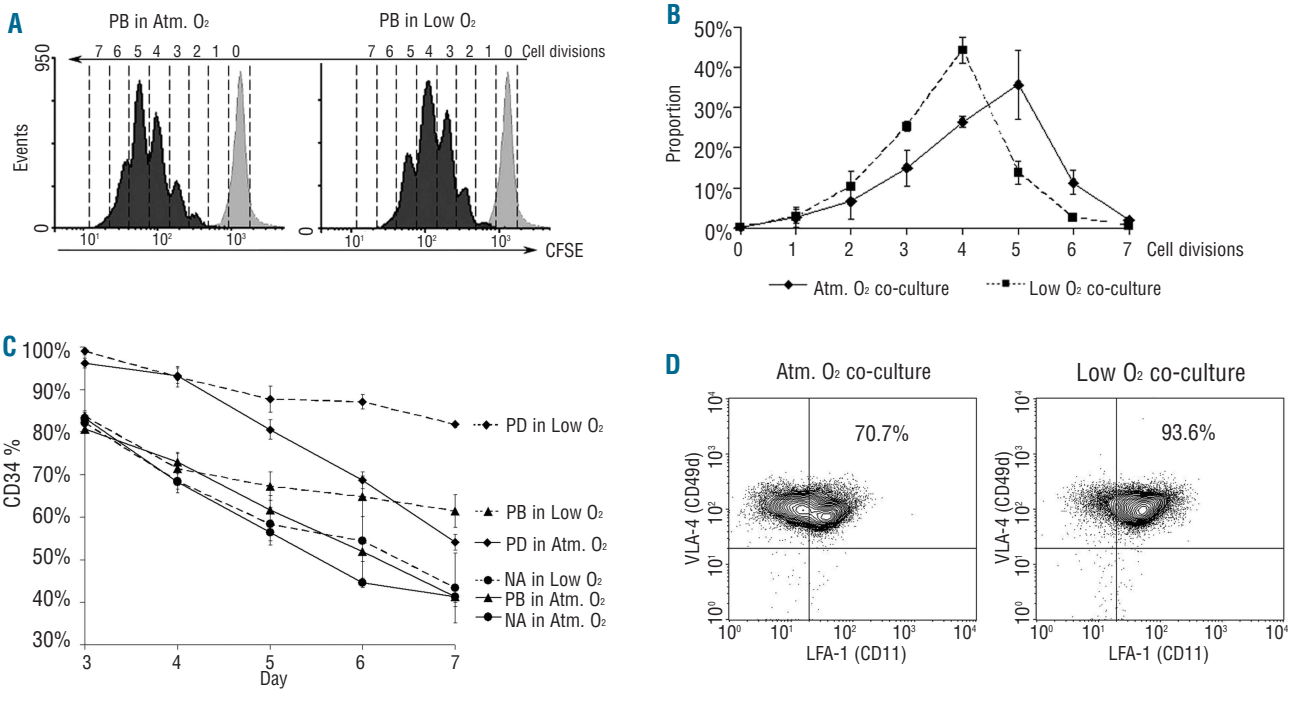

Figure 3. Effect of oxygen tension on HC in the co-culture system. (A) Cell division tracking showing the representative CFSE staining of phase-bright (PB) cells in atmospheric O_2 co-culture and low O_2 co-culture on day 4. (B) The statistics of (A), showing the cell division frequency under distinct oxygen tensions on day 4 (N=3). (C) CD34⁺ fraction of HC subsets in distinct compartments [non-adherent (NA), phase-dim (PD), and PD cells] of atmospheric O_2 co-culture and low O_2 co-culture on days 3–7 (N=3). (D) FACS analysis of VLA-4 (CD49d) and LFA-1 (CD11) expression in PB cells in atmospheric O_2 co-culture and low O_2 co-culture on day 4. N=4; *P*<0.05.

to be the predominant site of HC proliferation.[24] CFSE staining demonstrated that the cell division of phase-bright cells was prolonged under low oxygen conditions (Figure 3A and 3B).

Next, we compared the immunophenotypes of HC in the three compartments of the atmospheric oxygen and low oxygen co-cultures. Consistent with our previous report on atmospheric oxygen co-culture,[24] the stem cell marker CD34 was highly maintained in HC beneath the MSC layer (phase-dim) in the low oxygen co-culture (Figure 3C). Interestingly, under low oxygen conditions, phase-bright cells were also able to maintain their CD34 expression at levels between 65% and 80%. In contrast, non-adherent cells lost their CD34 expression under low oxygen conditions (Figure 3C).

Integrins were shown to be critical for the adhesion of HC to the MSC surface, which facilitates HC transmigration through the MSC layer.[24] In the present study, we measured the expression of two integrin α subunits, i.e., VLA-4 (CD49d) and LFA-1 (CD11), in HC in the co-culture by flow cytometry. VLA-4 expression levels on the HC surface did not differ between the two conditions, with almost 100% of the cells classified as positive (Figure 3D). Interestingly, the percentage of phase-bright cells expressing LFA-1 in the low oxygen co-culture was 88.5±3.0% while that in the atmospheric oxygen co-culture was 74.2±1.7% (representative FACS data are shown in Figure 3D). Non-adherent cells demonstrated a similar trend (*data not shown*).

CXCR4 is important for HSPC migration.[27] However, no significant changes in CXCR4 expression were observed in hypoxic HC by either FACS or real-time reverse transcriptase polymerase chain reaction (*data not shown*).

Vascular endothelium growth factor-A secretion by mesenchymal stromal cells is up-regulated in low oxygen co-culture

We detected consistently higher VEGF-A concentrations in the supernatant of the low oxygen cultures, including MSC monoculture and HC/MSC co-culture, whereas VEGF-A concentrations were very low in a suspension culture of CD34$^+$ cells without MSC (*Online Supplementary Figure S1A*). This suggests that VEGF-A is mainly produced by MSC in the co-culture and its expression can be regulated by oxygen tension.

A recent study demonstrated that VEGF increases the permeability of the endothelial monolayer, thus promoting *in vitro* transmigration of cells through the monolayer.[28] In order to examine the effect of VEGF on the MSC monolayer we performed a HC-MSC transmigration assay. In brief, the confluent MSC monolayer was pre-incubated with conditioned medium from either the atmospheric oxygen or low oxygen co-culture with or without anti-VEGF antibodies, as described in the *Design and Methods* section. As shown in *Online Supplementary Figure S1B*, HC transmigration through the MSC layer primed with conditioned medium was significantly increased, and the anti-VEGF antibody was found to block this increase. These data indicate that the permeability of the MSC monolayer increases upon VEGF stimulation.

Next, we assessed the role of VEGF in HC distribution in the co-culture using the anti-VEGF antibody and siRNA. As expected, 4 days after co-culture, the number of phase-dim cells decreased when MSC were treated with VEGF antibody or siRNA (*Online Supplementary Figure S1D and S1G*), which indicated that the permeability of MSC decreased due to the VEGF blockade. Interestingly, VEGF down-regulation caused a significant increase in the number of HC on the MSC surface (phase-bright cells, *Online Supplementary Figure S1C and S1F*). This may also be related to changes in the permeability of the MSC layer.

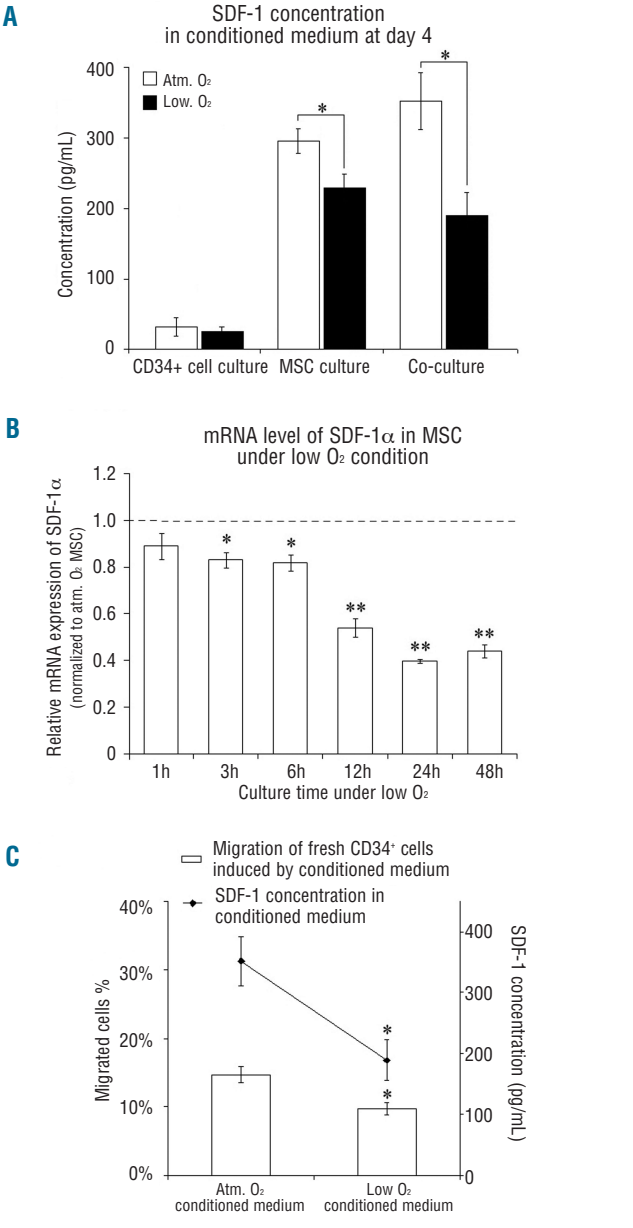

Figure 4. SDF-1 secretion by MSC influences HC migration. (A) SDF-1 concentrations in CD34$^+$ cell culture, MSC culture, and CD34$^+$ cell/MSC co-culture as determined by ELISA (N=6; *P<0.05). (B) Relative SDF-1α mRNA levels in MSC under low O_2 conditions at 1, 3, 6, 12, 24, and 48 h (N=3; *P<0.05; **P<0.01). Expression levels were normalized to MSC mRNA levels under atmospheric O_2 conditions at the same time points. Dashed line indicates SDF-1α mRNA levels in MSC under atmospheric O_2 condition set as 1. (C) Fresh CD34$^+$ cell migration induced by conditioned medium from atmospheric O_2 co-culture and low O_2 co-culture was observed using the *in vitro* transwell migration assay (N=7; *P<0.05); SDF-1 concentrations in the conditioned medium (N = 6; *P<0.05).

Stromal cell-derived factor-1 secretion by mesenchymal stromal cells is down-regulated in low oxygen co-culture

SDF-1 modulates the migration and mobilization of HSPC by mediating chemotaxis through an SDF-1/CXCR4 interaction.[27] Surprisingly, SDF-1 concentrations in both the MSC monoculture and HC/MSC co-culture were down-regulated under low oxygen conditions (Figure 4A). This observation was confirmed by real-time reverse transcriptase polymerase chain reaction which demonstrated that SDF-1α mRNA levels in MSC were remarkably decreased after 12 h of incubation under low oxygen conditions (Figure 4B).

Next, we studied the effect of the co-culture medium on HC migration using the transwell migration assay. Interestingly, freshly isolated CD34⁺ cells migrated significantly slower induced by the conditioned medium from the low oxygen co-culture than by the conditioned medium from the atmospheric oxygen co-culture (histogram in Figure 4C). This is consistent with the observation that SDF-1 expression was significantly lower in the low oxygen medium (189±33 pg/mL) than in the atmospheric oxygen medium (352±40 pg/mL) (shown as the line graph in Figure 4C).

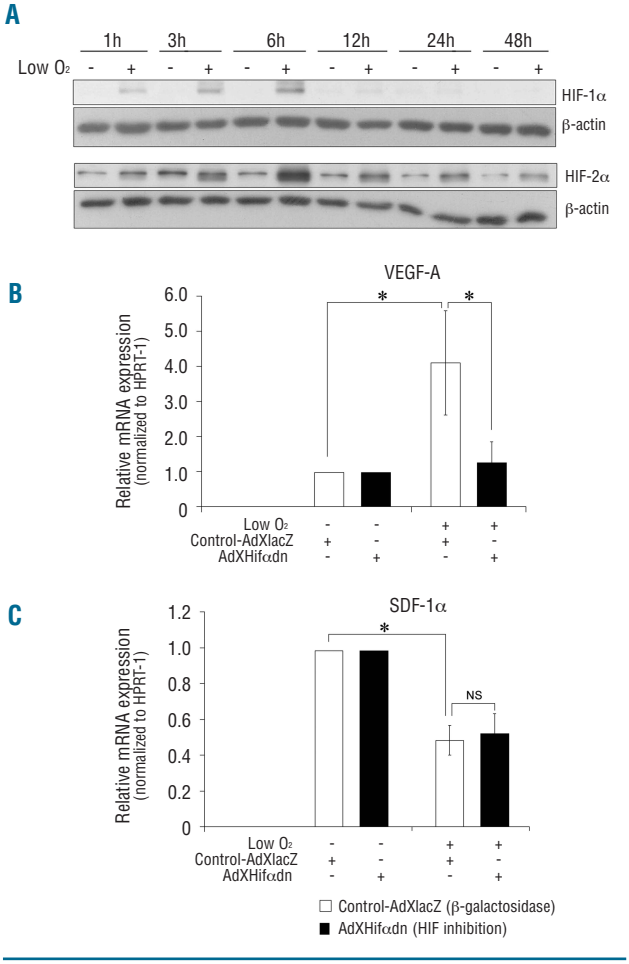

Figure 5. (A) HIF-1α and HIF-2α expression in MSC in atmospheric O_2 co-culture and low O_2 co-culture at 1, 3, 6, 12, 24, and 48 h. (B and C) The effect of HIF inhibition on (B) VEGF-A and (C) SDF-1α mRNA levels in MSC. MSC were infected with an adenovirus encoding either β-galactosidase (control; AdXlαcZ) or the dominant-negative mutant of HIF-2α (AdXHifαdn) for 48 h. After 24 h of incubation under atmospheric O_2 or low O_2 conditions, VEGF-A and SDF-1α mRNA levels were measured using real-time reverse transcriptase polymerase chain reaction (N=6; **P*<0.05; NS: not significant). Data are normalized to HPRT-1 expression levels.

Regulation of vascular endothelium growth factor-A and stromal cell-derived factor-1α by hypoxia inducible factor

HIF-1α and 2α proteins, the oxygen-sensitive subunits of HIF 1 and 2, respectively, were stabilized by hypoxia in a short period of time. The stabilization of both these subunits in MSC was detected within 1 h under low oxygen conditions, and the expression of both subunits peaked at 6 h (Figure 5A). After 12 h, HIF-1α was undetectable, whereas HIF-2α was down-regulated but continued to be expressed until 48 h (Figure 5A).

To examine whether the HIF pathway was involved in the regulation of VEGF-A and SDF-1 in hypoxic MSC, we used an inhibitory approach. The HIF signaling pathway was inhibited by the over-expression of a dominant-negative mutant of HIF-2α (AdXHifαdn), which functioned as a competitive inhibitor of both HIF-α isoforms (HIF-1α and -2α). An adenovirus encoding β-galactosidase (AdXlacZ) served as the control. VEGF-A mRNA expression after HIF silencing significantly decreased (Figure 5B). In contrast, down-regulation of SDF-1α mRNA levels in hypoxic MSC was still observed after HIF inhibition (Figure 5C).

Discussion

The hematopoietic microenvironment in the bone marrow, the so-called 'niche', is critical for stem cell regulation. To mimic the *in vivo* niche, we established an *in vitro* system in which CD34⁺ cells were co-cultured with MSC. In our previous study, we identified three distinct compartments in our HC/MSC co-culture system with regard to different spatial localizations.[24] HC subsets in the three compartments showed distinct immunophenotypes, cell proliferation, and migratory capacities, indicating that spatial localization has a significant effect on the fates of stem cells.

In the present study, we expanded our previous co-culture system by incorporating oxygen tension as an experimental variable. The *in vivo* hypoxic endosteal niche maintains HSPC in a quiescent state, while the oxygenated vascular niche is more proliferative.[29] This indicates that oxygen tension plays a critical role in the *in vivo* stem cell niche.

HSPC metabolism is regulated by oxygen tension,[30] and Simsek *et al.* recently reported that anaerobic glycolysis is required for the long-term maintenance of HSPC.[31] Eliasson *et al.* demonstrated that *in vitro* hypoxia mediates the low cell-cycle activities of HSPC and increases the long-term reconstitution of HSPC.[23] These observations were confirmed within the co-culture system by demonstrating that all HC subsets displayed reduced cell cycle activities under low oxygen conditions (Figure 3A). However, the profile of CD34⁺ HC preservation in the low oxygen co-culture was still dependent on spatial localization, as in the atmospheric oxygen co-culture (Figure 3C). Under both conditions, the phase-dim cell fraction contained many more primitive HC than did either the phase-bright or non-adherent fraction, indicating that both location and oxygen tension have significant effects on HC

fate. Our findings demonstrated that low oxygen influences the differentiation pattern of CD34+ cells in co-culture, as well as affecting HC distribution. As shown in Figures 2 and 3, no difference in the cell count could be detected at day 3. However at later time-points differences developed and became significant, indicating that in such a dynamic system oxygen tension may affect both proliferation and differentiation as well as migration of HC in co-cultures with MSC via various signaling pathways.

We assume that the localization of HC in the distinct compartments modulates their immunophenotypes, even in low oxygen co-cultures.

HC migration is a complex process mediated by many factors. LFA-1 and VLA-4 are important for HC adhesion to endothelial cells, passage through vessels, and *in vivo* engraftment in the bone marrow.[27] Using blocking experiments, we recently demonstrated that integrins are critical for HC adhesion to the MSC surface and further migration beneath the MSC layer.[24] In the present study, LFA-1 expression was significantly up-regulated in HC on the MSC surface in the low oxygen co-culture, which may have contributed to the high ratio of HC beneath the MSC layer.

MSC play a pivotal role in the stem cell niche for both *in vivo* and *in vitro* maintenance of HSPC. The effect of low oxygen on the proliferation and expandability of MSC has been demonstrated,[32] and the secretion of various morphogens has been shown to be modulated by oxygen tension.[33]

During HSPC mobilization, a local low oxygen region in the bone marrow was proposed to increase VEGF expression, which facilitates HSPC migration through blood vessels.[34] We confirmed the results of previous studies by demonstrating VEGF up-regulation in MSC under low oxygen conditions.[35] In a recent study, high VEGF concentrations were shown to increase the permeability of the endothelial monolayer, which promotes cells transmigration.[28] Since MSC can differentiate into endothelial-like cells *in vitro* under high VEGF concentrations,[25] it is speculated that MSC may behave similarly. Indeed, CD34+ HC transmigration through the MSC monolayer was significantly up-regulated after pre-incubating the MSC layer with conditioned medium that contained a high concentration of VEGF. This effect can be reversed by blocking VEGF with an anti-VEGF antibody or siRNA. As shown in *Online Supplementary Figure S1*, siRNA transfection led to a quantitatively and temporarily limited knock-down of VEGF-A mRNA. The blocking experiments performed on the protein level support the relevance of the VEGF-A signaling pathway for the quantitative changes observed in the co-culture model under low oxygen conditions. In conclusion, low oxygen tension induces higher VEGF secretion by MSC in co-cultures, increasing the permeability of the MSC monolayer, and hence, up-regulating CD34+ HC transmigration through the MSC layer.

The SDF-1/CXCR4 axis plays a key role in the homing and mobilization of HSPC.[27;36] The hypoxic gradient can regulate HSPC migration *in vivo* by inducing SDF-1 in endothelial cells.[37] Although numerous studies have demonstrated increased SDF-1 levels under low oxygen conditions,[37;38] other reports do not suggest a significant modulation of SDF-1 in other cell types.[39;40] We found no *in vitro* SDF-1 induction in hypoxic MSC in this study. In contrast, a decrease in SDF-1 was observed at both the mRNA and protein levels (Figure 4). To exclude that a reduced rate of MSC proliferation was the cause of the observed differences we performed independent culture experiments with MSC under atmospheric and low oxygen conditions. No significant differences in MSC numbers were observed on days 2, 4 and 7 of culture (*data not shown*).

The unchanged CXCR4 expression levels in hypoxic HC and decreased SDF-1 secretion levels in MSC support the role of this axis in active cell movement processes, such as stem cell homing. SDF-1/CXCR4 is less important for maintaining HC in their residing niche in the *in vitro* co-culture systems. Presumably, SDF-1 expression *in vivo* is dynamically regulated in the perivascular region in the bone marrow. Systemic treatment strategies, such as chemotherapy or irradiation, may lead to dramatic changes in bone marrow perfusion and oxygen tension.[41]

HIF is the key transcriptional factor that modulates the cellular response to hypoxia. HIF-dependent induction of VEGF and SDF-1 under low oxygen conditions is well known.[37;38;42] In addition to its HIF-dependent regulation under low oxygen conditions, SDF-1 has also been shown to be independent of HIF in some cases.[43] We were, therefore, prompted to examine whether VEGF and SDF-1 were regulated by an HIF-dependent mechanism in hypoxic MSC. We inhibited HIF by using adenoviral transduction of AdXHifαdn. As expected, VEGF expression was significantly blocked by HIF inhibition, confirming that the VEGF gene is targeted by HIF signaling. In contrast, SDF-1 expression was not affected by HIF inhibition, indicating that SDF-1 is down-regulated by an HIF-independent process.

Collectively, the data show that a condition of low oxygen is an important regulator within the stem cell niche, both *in vivo* and *in vitro*. Differences in the oxygen tension among the three compartments of the co-culture system support our previous speculation that different microenvironments can be formed in the same culture system and regulate HC in distinct manners. Importantly, the low oxygen condition governs HC distribution within the three compartments, allowing lower adhesion levels on the MSC surface and a higher number of CD34+ stem and progenitor cells beneath the MSC layer, accompanied by less division and better maintenance of HC in low oxygen co-cultures.

As a dynamic, interactive but controllable system, this co-culture model is a novel tool for understanding the parameters involved in *in vitro* niche regulation.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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