Molecular Cancer Research

Hypoxia Promotes Dissemination and Colonization in New Bone Marrow Niches in Waldenström Macroglobulinemia

Barbara Muz[1], Pilar de la Puente[1], Feda Azab[1], Irene M. Ghobrial[2], and Abdel Kareem Azab[1]

Abstract

Waldenström macroglobulinemia, a rare and indolent type of non–Hodgkin lymphoma, is characterized by widespread lymphoplasmacytic B cells in the bone marrow. Previous studies have shown that hypoxic conditions play a key role in the dissemination of other hematologic malignancies. In this study, the effect of hypoxia was tested on the progression and spread of Waldenström macroglobulinemia. Interestingly, tumor progression correlated with hypoxia levels in Waldenström macroglobulinemia cells and other cells in the bone marrow and correlated with the number of circulating tumor cells *in vivo*. Mechanistic studies demonstrated that hypoxia decreased cell progression and cell cycle, did not induce apoptosis, and reduced the adhesion between Waldenström macroglobulinemia cells and bone marrow stroma, through downregulation of E-cadherin expression, thus explaining increased egress of Waldenström macroglobulinemia cells to the circulation. Moreover, hypoxia increased the extravasation and homing of Waldenström macroglobulinemia cells to new bone marrow niches *in vivo*, by increased CXCR4/SDF-1–mediated chemotaxis and maintaining the VLA4-mediated adhesion. Re-oxygenation of hypoxic Waldenström macroglobulinemia cells enhanced the rate of proliferation and cell cycle progression and restored intercellular adhesion between Waldenström macroglobulinemia cells and bone marrow stroma. This study suggests that targeting hypoxic response is a novel strategy to prevent dissemination of Waldenström macroglobulinemia.

Implications: This study provides a better understanding of the biology of dissemination of Waldenström macroglobulinemia and opens new windows for investigation of new therapeutic targets in Waldenström macroglobulinemia based on tumor hypoxia mechanisms. *Mol Cancer Res; 13(2); 263–72. ©2014 AACR.*

Introduction

Waldenström macroglobulinemia is a rare low-grade B-cell lymphoma characterized by abnormal lymphoplasmacytic cells that overproduce monoclonal immunoglobulins M (IgM) and are spread widely in the bone marrow (1). The Waldenström macroglobulinemia cells infiltrate specifically into the bone marrow niche which implies the critical role of the bone marrow as a supportive microenvironment for the Waldenström macroglobulinemia cells due to the various interactions (2–4). We have previously shown in Waldenström macroglobulinemia that the bone marrow milieu promotes tumor progression including cell proliferation, survival, and drug resistance through production of cytokines, regulation of cell interaction, cell adhesion with stroma and endothelial cells, and cell trafficking (3, 5–7). We have also shown before that the bone marrow microenvironment plays a crucial role in the progression and drug resistance of other hematologic malignancies including multiple myeloma (8–10) and chronic myeloid leukemia (CML; refs. 11, 12).

Hypoxia plays an important role in the progression and dissemination of hematologic malignancies (12–16). Tumor hypoxia activates adaptive transcriptional programs, including HIF1α, that promote cell survival, motility, invasiveness, drug resistance, and neoangiogenesis in multiple myeloma (16, 17), associated with more aggressive tumor (18). We have shown that hypoxia induces egress of multiple myeloma cells from the bone marrow to the circulation and facilitates their homing to the new bone marrow niches in myeloma. These processes involved activation of epithelial-to-mesenchymal transition (EMT) machinery including decreased expression of E-cadherin and increased expression of CXCR4 in multiple myeloma cells (13). However, the role of hypoxia in the progression of Waldenström macroglobulinemia in particular and lymphoma in general was not assessed before. Moreover, the role of hypoxia in the progression of hematologic malignancies aside from cell trafficking including important cell processes, such as proliferation, apoptosis, cell cycle, and cell signaling, was never studied.

In this study, we focused on the role of hypoxia in dissemination of Waldenström macroglobulinemia cells including the cell–cell interactions in the bone marrow and egress to the circulation, chemotaxis, and homing to new bone marrow niches, adaptation of hypoxic Waldenström macroglobulinemia cells in the new bone marrow milieu, as well as hypoxic effects on proliferation, survival, apoptosis, and cell cycle in Waldenström macroglobulinemia cells.

[1]Department of Radiation Oncology, Cancer Biology Division, Washington University in Saint Louis School of Medicine, Saint Louis, Missouri. [2]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Abdel Kareem Azab, Department of Radiation Oncology, Cancer Biology Division, Washington University in Saint Louis School of Medicine, 4511 Forest Park Avenue, Room 3103, St. Louis, MO 63108. Phone: 314-362-9254; Fax: 314-362-9790; E-mail: aazab@radonc.wustl.edu

doi: 10.1158/1541-7786.MCR-14-0150

Materials and Methods

Reagents

Stromal-derived growth factor (SDF-1) for adhesion and chemotaxis assays was purchased from R&D. Transwell plates for chemotaxis assay were obtained from Corning. E-Cadherin blocking antibody and human fibronectin were purchased from EMD Millipore. Total protein concentration for immunoblotting was assessed by Quick Start Bradford dye reagent (BioRad). MTT solution for cell viability assay was purchased from Sigma-Aldrich. Annexin V/propidium iodide (PI) for apoptosis assay and BD BioCoat Human Fibronectin plates for adhesion assay were purchased from BD Biosciences. Cell trackers including calcein-AM, DiO, and DiD were purchased from Invitrogen (Life Technologies). Hypoxia marker pimonidazole hydrochloride (PIM) and its correlating antibody were obtained from HypoxyProbe. A 10× red blood cell (RBC) buffer was obtained from BioLegend.

Cell culture

The Waldenström macroglobulinemia cell lines (BCWM.1, BCWM.1-mCherry, MWCL.1) and HS-5 stromal cells used in this study were a kind gift from Dr. Irene Ghobrial from Dana-Farber Cancer Institute (Boston, MA). Waldenström macroglobulinemia cell lines were cultured in RPMI-1640 (Corning CellGro, Mediatech) supplemented with 10% FBS (Gibco, Life Technologies), 2 mmol/L of L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (CellGro, Mediatech). HS-5 cells were cultured in 20% FBS DMEM (Corning CellGro, Mediatech) containing L-glutamine and penicillin/streptomycin. Cells were incubated at 37°C under normoxic conditions (21% O_2) in the NuAire Water Jacket Incubator or hypoxic conditions (1% O_2) in the hypoxic chamber from Coy for 24 hours. In some cases, hypoxic Waldenström macroglobulinemia cells were re-oxygenated by exposing them to normal oxygen levels.

Animals

SCID-beige mice (female; 7 weeks old) were obtained from Taconic Farms and Balb/C mice from Charles Rivers Laboratories. Approval for these studies was obtained from the Ethical Committee for Animal Experiments at Washington University in St. Louis Medical School (St. Louis, MO).

Effect of tumor progression on hypoxia in Waldenström macroglobulinemia bone marrow

BCWM.1 cells were genetically engineered to express mCherry fluorescent protein and luciferase (Luc), as described previously (5). Human BCWM.1-mCherry cells were injected into 12 SCID mice intravenously at different concentrations of 0.5×10^6, 1×10^6, and 2.5×10^6 cells per mouse (4 mice per condition) and allowed to grow for 3 weeks. Mice were treated with PIM (100 mg/kg) for 4 hours, sacrificed and their bone marrow and peripheral blood were extracted. Mononuclear cells (MNC) were isolated, washed, fixed, permeabilized, and stained with anti-PIM antibody conjugated to allophycocyanin (APC) antibody. Waldenström macroglobulinemia cells were detected by flow cytometry by gating on mCherry-positive cells, and bone marrow stromal cells were mCherry-negative, and hypoxia in these cells was measured as mean fluorescence intensity (MFI) of APC. Circulating Waldenström macroglobulinemia cells were detected by gating on mCherry-positive cells in the peripheral blood samples after RBC lysis.

Effect of hypoxia on extravasation of Waldenström macroglobulinemia cells

BCWM.1-mCherry cells were exposed to normoxia or hypoxia for 24 hours and each population was injected via tail vein into 4 Balb/C mice (1×10^6 cells per mouse). Fifty microliters of blood was collected from the portal vein at 5, 15, and 30 minutes postinjection, RBCs were lysed, and MNCs were labeled with CD45-APC-Cy7 (BD Biosciences) for 1 hour on ice and analyzed by flow cytometry. The number of circulating Waldenström macroglobulinemia cells was analyzed as the percentage of $CD45^+/mCherry^+$ of the total $CD45^+$ population. The absence of these cells from the circulation reflected extravasation of the Waldenström macroglobulinemia cells.

Effect of hypoxia on homing of Waldenström macroglobulinemia cells to the bone marrow

BCWM.1-mCherry cells were first stained with DiD or DiO (5 μL per 1 mL medium) for 2 hours at 37°C, washed, and then exposed to normoxia (1 flask with DiD and 1 flask with DiO) or hypoxia (1 flask with DiD and 1 flask with DiO) for 24 hours. Two suspensions of cells were prepared: normoxic DiD mixed with hypoxic DiO and normoxic DiO mixed with hypoxic DiD. Each cell mix was injected into 3 SCID mice (6 mice in total) at a concentration of 3×10^6 cells per mouse. After 24 hours postinjection, the mice were sacrificed and the MNCs were isolated from the femurs, filtered, stained with DAPI (Sigma-Aldrich), washed and analyzed by the flow cytometry. Viable cells were gated as the population with negative DAPI staining, and the number of $mCherry^+/DiD^+$ or $mCherry^+/DiO^+$ cells was analyzed in each mouse. The number of positive cells reflected the number of Waldenström macroglobulinemia cells that homed to the bone marrow. The numbers of DiD versus DiO cells in each animal were compared, and the results were normalized to the average of the number of normoxic cells in all 6 mice. The purpose of the alternative staining was to eliminate the effect of the different fluorescent labels on the detection sensitivity of the cells in the bone marrow.

The effect of hypoxia on expression of E-cadherin, CXCR4, and VLA-4 using flow cytometry

BCWM.1 cells (1×10^6) were exposed to normoxic or hypoxic conditions for 24 hours, then aspirated, incubated on ice for 10 minutes, washed with PBS, and stained on ice with primary anti-human CXCR4-APC antibody (BD Biosciences), mouse-anti-human VLA-4 antibody (BD Biosciences), or rabbit-anti-human E-cadherin antibody (Cell Signaling Technologies), followed by staining with secondary anti-mouse or anti-rabbit conjugated with fluorescein isothiocyanate (FITC) antibody (Cell Signaling Technologies) for 1 hour. The cells were then washed with 1× PBS and analyzed by flow cytometry.

The effect of hypoxia on cell signaling using immunoblotting

BCWM.1 cells (5×10^6) were exposed to normoxic or hypoxic conditions for 24 hours or re-oxygenated for 24 hours, aspirated, incubated on ice for 10 minutes, washed with 1 × PBS, and lysed. Protein concentration in the cell lysates was normalized and 80 μg of protein was loaded per lane. Electrophoresis was performed using 8% or 10% precast Novex Tris-Glycine gels (Novex) and transferred to a nitrocellulose membrane using iBlot (Invitrogen, Life Technologies). Membranes were blocked with 5% nonfat dry milk in Tris-Buffered Saline/Tween20 (TBST) buffer and incubated with primary antibodies detecting E-cadherin, HIF1α, HIF2α,

pPI3K-P85, pAKT (Ser473), pS6R, pP70S6, cleaved caspase-3, -8, -9, p-Rb, cyclin D1, cyclin E1, pCDK2, pCDK6, p21, or α-tubulin overnight at 4°C. The membranes were then washed with TBST for 30 minutes, incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody, washed, and developed using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen). Primary and secondary antibodies used for immunoblotting were purchased from Cell Signaling Technologies apart from HIF2α antibody, which was purchased from Novus Biologicals.

The effect of hypoxia on cell proliferation and viability

Cell growth and cell viability were measured by using Vi-Cell Viability Analyzer (Beckman Coulter) which provides the exact number of viable cells counted and averaged from 100 fields providing the percentile of viable cells. Cell number and viability of BCWM.1 and MWCL.1 cells were registered at time 0 (t_0), then cells were cultured in normoxia or hypoxia for 24 hours, and the measurement was repeated. In addition, cell growth was assessed using MTT (Chemicon International), followed by absorbance readout at 570 nm using a spectrophotometer.

The effect of hypoxia on apoptosis

BCWM.1 and MWCL.1 cells were cultured in normoxic or hypoxic conditions for 24 hours. The cells were aspirated, kept on ice for 10 minutes, washed twice with ice-cold 1× PBS, and resuspended in 100 μL Annexin binding buffer. The cells were then stained with 5 μL of Annexin V for 15 minutes followed by staining with 5 μL of PI for 15 minutes at room temperature. A total of 400 μL of 1× binding buffer was added and the cells were analyzed by flow cytometry.

The effect of hypoxia on cell cycle

BCWM.1 and MWCL.1 cells were cultured in normoxic or hypoxic conditions for 24 hours or were re-oxygenated for the next 24 hours. First, the cells were aspirated and kept on ice for 10 minutes, washed with ice-cold 1× PBS, fixed with 70% ethanol/PBS, incubated on ice for 30 minutes, and washed twice. Next, the cells were incubated with 500 μL RNase/PBS at the concentration of 10 μg/mL for 30 minutes at 37°C, washed and stained with PI at the concentration of 5 μg/mL for 15 minutes at the room temperature, and analyzed by flow cytometry.

The effect of hypoxia on adhesion to stromal cells and fibronectin

Monolayer of HS-5 stromal cell line was prepared by plating 3×10^3 cells per well in 96-well plates overnight in normoxic or hypoxic incubator. Fibronectin plates were prepared the day before by resuspending 50 μg/mL of fibronectin stock solution in 1× PBS for 5 μg/cm^2 coating, 100 μL was added per well in a 96-well plate, and the plate was stored in 4°C. BCWM.1 and MWCL.1 cells were cultured in normoxia or hypoxia or were re-oxygenated, prelabeled with calcein AM at the final concentration of 1 μg/mL for 1 hour at 37°C and washed with PBS. In some cases, BCWM.1 cells were treated with E-cadherin blocking antibody (5 μg/mL) for 1 hour or SDF-1 (30 or 60 nmol/L) before plating the cells. The adhesion was conducted on the plates precoated with HS-5 or fibronectin for 90 minutes at 37°C. For fluorescent microscope analysis of cell adhesion, HS-5 cells were plated in the presence of DiD overnight (5 μL per 1 mL of medium) and MWCL.1 cells were prelabeled with calcein AM (as described above). Nonadherent cells were washed with PBS, and adherent cells were measured by detecting the fluorescent intensity signal using fluorescent reader (Ex/Em = 485/520 nm) or fluorescent microscope (objective ×5).

The effect of hypoxia on chemotaxis

The ability of cells to migrate through 8-μm pore size of filter was determined using Transwell migration plates (Costar) according to the manufacturer's instructions. BCWM.1 or MWCL.1 cells were cultured under normoxia or hypoxia for 24 hours, the cells were plated in the upper chamber and were allowed to transmigrate into the lower chamber containing medium with or without 30 nmol/L recombinant SDF-1. After 4 hours of incubation in normoxia or hypoxia, the cells which migrated to the lower chamber were counted using flow cytometry.

Statistical analysis

Experiments were performed in triplicates and repeated at least 3 times. Results are shown as mean ± SD and were analyzed using the Student t test or one-way ANOVA for statistical significance and were considered significantly different for $P < 0.05$.

Results

Progression of Waldenström macroglobulinemia cells in the bone marrow induces hypoxia in Waldenström macroglobulinemia cells and in other cells in the microenvironment

We tested the effect of tumor progression on hypoxic phenotype in Waldenström macroglobulinemia cells *in vivo*. Different numbers of BCWM.1-mCherry-Luc cells were injected into mice via tail vein to establish different tumor burdens in the bone marrow of the mice, which was confirmed by the percentage of mCherry-positive cells in the bone marrow by flow cytometry. We further characterized the hypoxic state of the BCWM1-mCherry cells by analyzing the MFI of anti-PIM-APC signal. A direct correlation was found between the tumor burden in the bone marrow and the hypoxia in the Waldenström macroglobulinemia cells (Fig. 1A). We also tested the hypoxic state of other cells in the bone marrow, mCherry-negative population, and we found that these cells were less hypoxic than Waldenström macroglobulinemia cells, but hypoxic signs were shown at higher tumor burdens (Fig. 1B). We tested the effect of tumor hypoxia in the bone marrow on the egress of Waldenström macroglobulinemia cells *in vivo* and found a direct linear correlation between the hypoxia in the bone marrow and the number of circulating Waldenström macroglobulinemia cells (Fig. 1C), which indicates that the mechanism of Waldenström macroglobulinemia cell entry to the circulation is regulated by hypoxia.

Hypoxia reduces cell proliferation and causes cell cycle arrest with no effect on apoptosis

We investigated the effect of hypoxia on proliferation, cell cycle, viability, and apoptosis of Waldenström macroglobulinemia cells exposed to hypoxic conditions for 24 hours. We found that after 24 hours of normoxia, the BCWM.1 and MWCL.1 cell number doubled; whereas the number of hypoxic cells increased only by approximately 1.3-fold (Fig. 2A). The induction of hypoxic response in the Waldenström macroglobulinemia cells by incubation in 1% O_2 for 24 hours was confirmed by HIF1α and HIF2α protein stabilization in BCWM.1 cells (Fig. 2B). Furthermore, hypoxia decreased the expression of proteins associated with PI3K signaling pathway,

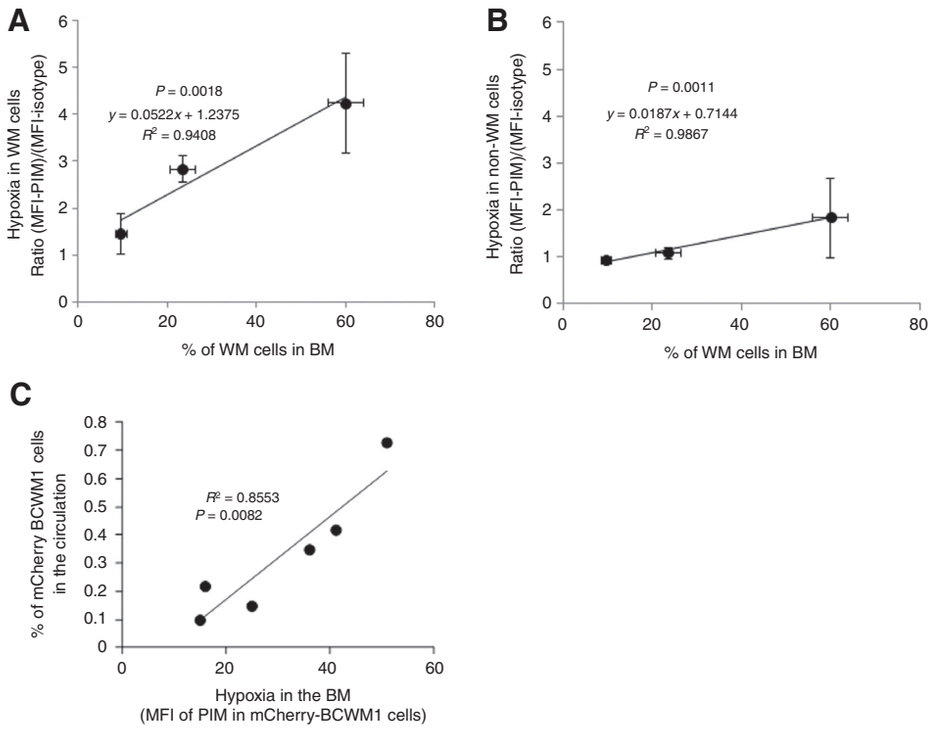

Figure 1.
Progression of Waldenström macroglobulinemia (WM) cells in bone marrow (BM) induces hypoxia in WM cells and in stromal cells in the microenvironment and induces WM cell egress into the circulation *in vivo*. The effect of tumor progression (% of BCWM.1-mCherry cells) in the bone marrow on their hypoxic status shown as a ratio of MFI of APC-PIM and MFI isotype of the mCherry-positive population analyzed by flow cytometry (A). The effect of tumor progression (% of BCWM.1-mCherry cells) in the bone marrow on hypoxic status of the BMSCs is shown as a ratio of MFI of APC-PIM and MFI isotype of the mCherry-negative population analyzed by flow cytometry (B). The effect of tumor hypoxia in the bone marrow (MFI of APC-PIM in BCWM.1-mCherry cells) on the number of circulating tumor cells (% of mCherry$^+$ BCWM.1 cells) analyzed by flow cytometry (C). The coefficient of determination (R^2) denotes a strong linear association between those variables.

involved in survival and proliferation, including decreased phosphorylation of PI3K-P85, pAKT, and pS6R in BCWM.1 cells (Fig. 2B). We further studied the cellular mechanism of the decreased proliferation of Waldenström macroglobulinemia cells in hypoxia by looking at the cell cycle status. Hypoxia increased the number of BCWM.1 and MWCL.1 cells in the G_1 phase and decreased DNA synthesis (S) and mitotic (G_2–M) phases of cell cycle (Fig. 2C). These results were confirmed by immunoblotting which showed that hypoxia reduced the expression of proteins associated with the G_1–S transition including pRb and pCDK6 and protein associated with the S–G_2 transition including cyclin E (Fig. 2D).

Then we tested the effect of hypoxia on apoptosis and viability of Waldenström macroglobulinemia cells. We found that incubation of BCWM.1 or MWCL.1 under hypoxic conditions for 24 hours induced no significant effect on the cell viability of Waldenström macroglobulinemia cells (Fig. 2E). We further investigated the effect of hypoxia on apoptosis of the Waldenström macroglobulinemia cells by using Annexin V/PI assay and found that BCWM.1 or MWCL.1 cells incubated under normoxic and hypoxic conditions for 24 hours had similarly around 5% of cells in total population undergoing apoptosis (Fig. 2F). We also tested the activation of intrinsic and extrinsic apoptotic pathways and found no difference in expression of cleaved caspase-9 (intrinsic pathway), cleaved caspase-8 (extrinsic pathway), or cleaved caspase-3 (which converge both pathways) in BCWM.1 cells (Fig. 2G).

Hypoxia regulates Waldenström macroglobulinemia cell–cell adhesion contributing to cell egress to the bone marrow

To examine the mechanism by which hypoxia contributes to cell egress from the bone marrow, we investigated the adhesion of BCWM.1 and MWCL.1 cells to the bone marrow stromal cells (BMSC) and also to each other, *in vitro*. Incubation of BCWM.1 or MWCL.1 in hypoxia reduced their adhesion to a BMSC monolayer by 50% and 25%, respectively (Fig. 3A). Adhesion results were confirmed by using fluorescent microscopy as shown on a representative image of MWCL.1 cell line (Fig. 3B). The decrease in adhesion of Waldenström macroglobulinemia cells was mediated by reduced expression of E-cadherin in hypoxia as demonstrated by immunoblotting and flow cytometry (Fig. 3C). To further confirm that this effect was mediated by E-cadherin, BCWM.1 cells were incubated with E-cadherin blocking antibody for 1 hour before plating onto stromal cells. We observed a significant reduction of adhesion of normoxic WM cells to HS-5 cells due to blocking of E-cadherin while no difference in adhesion in hypoxia (Fig. 3D). Similarly, we found that the interaction between BCWM.1 cells was reduced and the size of clumps formed by BCWM.1 cells exposed to hypoxia was visibly smaller that these formed in normoxia (Supplementary Fig. S1A and S1B). Treatment with E-cadherin blocking antibody in normoxia significantly decreased the size of clumps but had no additional effect in hypoxia (Supplementary Fig. S1A and S1B). Together, these data suggest that the Waldenström macroglobulinemia cells are losing cell–cell interactions between each other and with the bone marrow stroma due to hypoxia, which promotes cell dissemination.

Hypoxia facilitates extravasation and homing of Waldenström macroglobulinemia cells to the bone marrow niche by increased chemotaxis and maintaining VLA-mediated adhesion in response to SDF-1

We have tested the effect of hypoxia on the ability of Waldenström macroglobulinemia cells to extravasate and home to bone marrow niche. The extravasation of Waldenström macroglobulinemia cells from the circulation to the bone marrow was

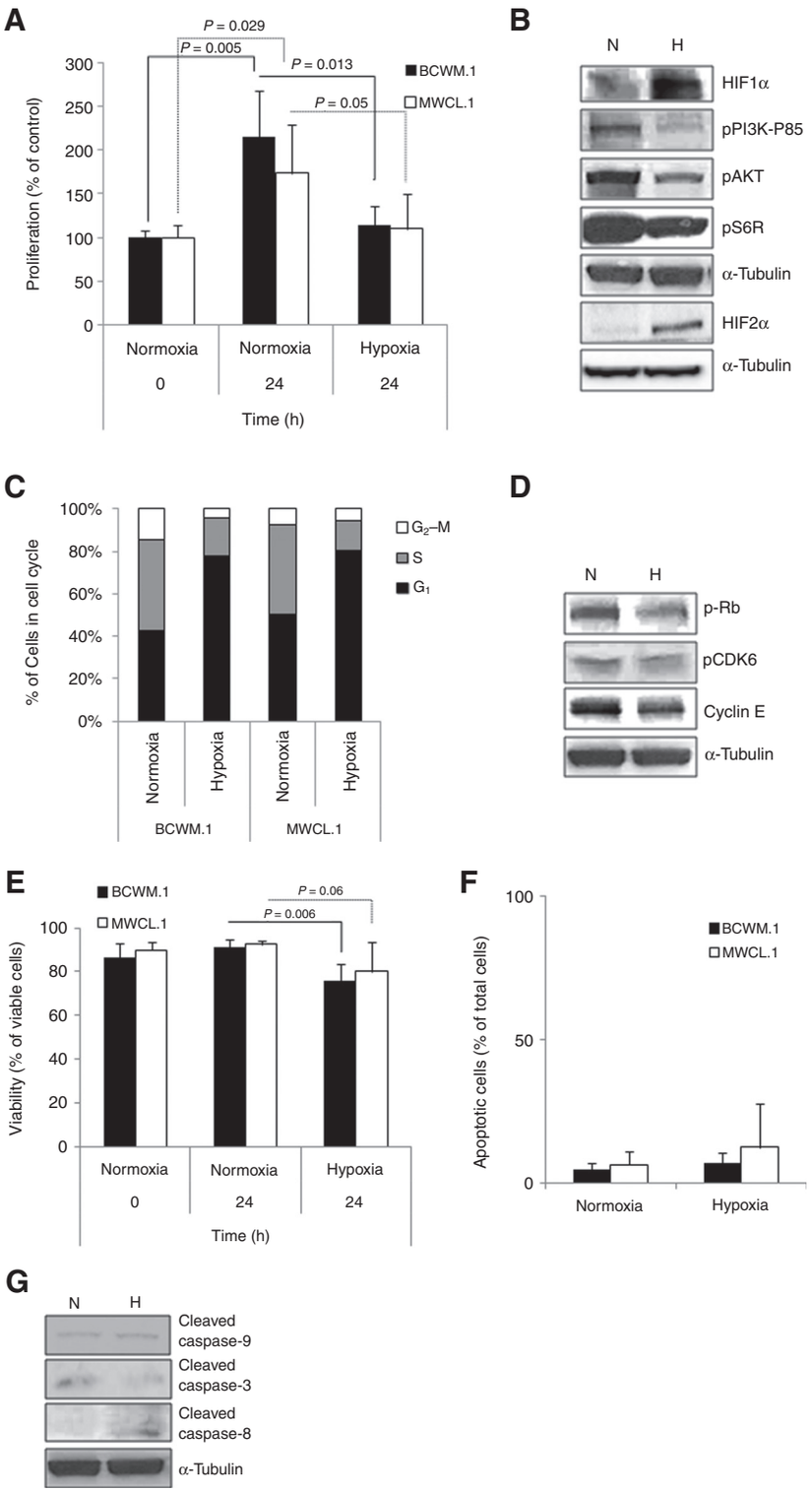

Figure 2.
Hypoxia decreases Waldenström macroglobulinemia (WM) cell proliferation, cell cycle, and viability without any effect on cell apoptosis *in vitro*. The effect of hypoxia (1% O_2, 24 hours) on proliferation of WM cells after 24-hour incubation normalized to 0 hour (A); expression of HIF1α, HIF2α, PI3K pathway proteins, and α-tubulin (loading control) detected by immunoblotting in BCWM.1 (B); cell cycle analysis after PI staining analyzed by flow cytometry in WM cell lines (C); expression of cell cycle–related proteins in BCWM.1 (D); viability of WM cells measured by trypan blue staining showing percentage of viable cells (E); cell apoptosis of WM cells based on Annexin V/PI staining and flow cytometry analysis showing the percentage of apoptotic cells which are Annexin-V–positive and PI-positive (F); and apoptosis-related proteins in BCWM.1 with α-tubulin (loading control) detected by immunoblotting (G). H, hypoxia; N, normoxia.

monitored by looking at the number of circulating BCWM.1-mCherry cells at 5, 15, and 30 minutes after intravenous injection. As soon as 5 minutes postinjection, there were 2.5-fold more normoxic cells than hypoxic cells observed in the circulation, meaning that hypoxic cells extravasated faster than normoxic cells (Fig. 4A). The homing of Waldenström macroglobulinemia cells to bone marrow niches was tested by injection of labeled hypoxic and normoxic BCWM.1-mCherry cells to mice and direct

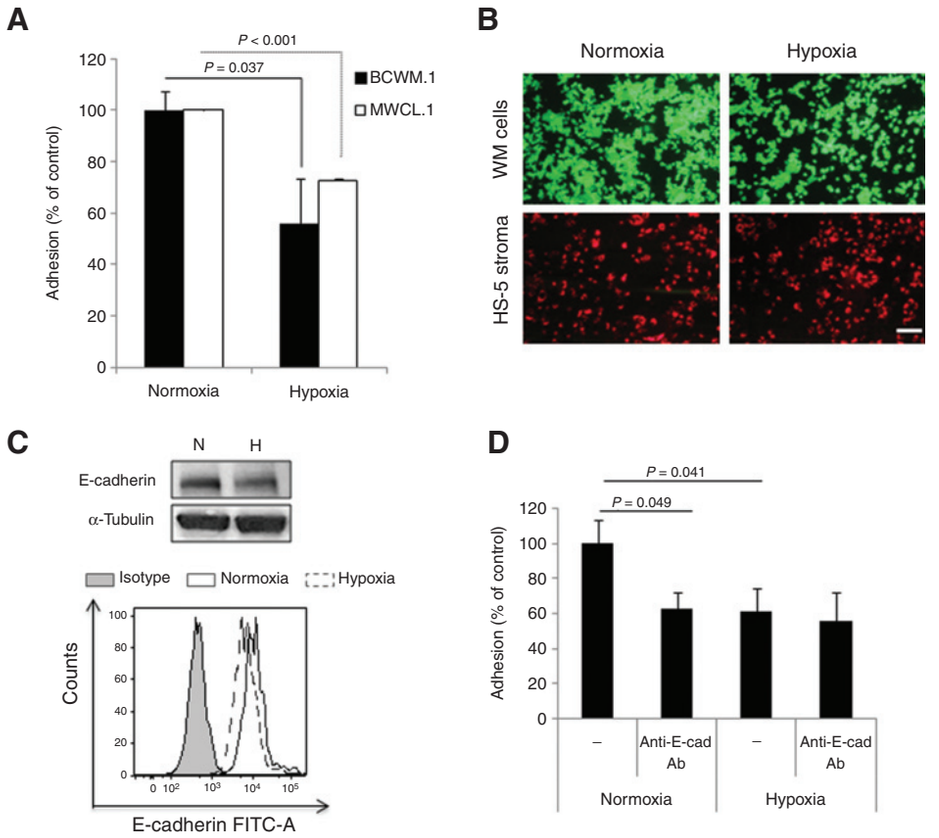

Figure 3.
Hypoxia regulates cell adhesion in an E-cadherin–dependent manner. The effect of hypoxia (1% O_2, 24 hours) on BCWM.1 and MWCL.1 cell adhesion to a monolayer of BMSCs (HS-5) normalized to normoxic cells as measured by fluorescent reader of the signal of adherent calcein-AM–positive Waldenström macroglobulinemia (WM) cells (A) and as shown by fluorescent microscope (scale bar represents 1 μm) and representative pictures of adherent calcein-positive MWCL.1 cells (green) adherent to DiD-positive stromal cells (red; B); E-cadherin protein expression in BCWM.1 shown by immunoblotting with α-tubulin (loading control) and on a histogram analyzed by flow cytometry (C). The effect of E-cadherin blocking antibody (5 μg/mL) on BCWM.1 cell adhesion in normoxia or hypoxia normalized to normoxic untreated cells (D). H, hypoxia; N, normoxia; anti-E-cad Ab, anti-E-cadherin blocking antibody.

detection of the number of each population in the bone marrow 24 hours postinjection. We found that hypoxic cells homed more than normoxic cells to the bone marrow (2-fold increase), suggesting that hypoxic Waldenström macroglobulinemia cells have enhanced homing capabilities (Fig. 4B).

To explain the rapid extravasation and homing *in vivo*, we examined the effect of hypoxia on chemotaxis and integrin-mediated adhesion *in vitro*. We found that hypoxia increased the chemotaxis of BCWM.1 and MWCL.1 cells toward SDF-1 (Fig. 4C). These results were in agreement with hypoxia-induced overexpression of CXCR4 on the surface of BCWM.1 cells, as detected by flow cytometry (Fig. 4D). Moreover, we tested the effect of hypoxia on VLA-4–mediated adhesion of Waldenström macroglobulinemia cells and found that it did not have an effect on the adhesion of BCWM.1 cells to fibronectin and that hypoxic BCWM.1 cells maintained their ability to increase VLA-4–mediated adhesion to fibronectin in response to SDF-1 chemotactic signals (Fig. 4E). These results were confirmed by the finding that hypoxic cells maintained their expression of VLA-4 compared with normoxic cells (Fig. 4F).

Re-oxygenation boosts the proliferation of Waldenström macroglobulinemia cells by increasing cell cycle progression and adhesion augmented by E-cadherin expression

The last step of cell trafficking after homing is recovery in the bone marrow site. Therefore, we investigated the effect of re-oxygenation of hypoxic cells in different cellular aspects including proliferation, cell cycle, and adhesion. We found that re-oxygenation of hypoxic BCWM.1 cells for 24 hours significantly increased the proliferation of Waldenström macroglobulinemia cells (2.4-fold), compared with hypoxic cells in hypoxia (1.5-fold increase), and re-oxygenated MWCL.1 cells (2-fold) compared with hypoxic cells (1.2-fold; Fig. 5A). The phosphorylation of PI3K-associated proteins involved in cell proliferation such as pAKT, p-S6R, and p-P70S6 was increased after re-oxygenation compared with their hypoxia-driven reduction (Fig. 5B).

Analysis of the cell cycle revealed that the re-oxygenated BCWM.1 and MWCL.1 hypoxic cells exited the hypoxia-induced G_1 cell-cycle arrest and increased their DNA synthesis phase more than normoxic cells as shown in Fig. 5C. The proteins involved in the cell cycle, including cyclin D1 and p-Rb, were increased after re-oxygenation compared with their hypoxia-driven reduction (Fig. 5D).

Re-oxygenation of hypoxic BCWM.1 and MWCL.1 cells increased cells adhesion to BMSCs (Fig. 5E) due to restored expression of E-cadherin (Fig. 5F). Moreover, re-oxygenation of BCWM.1 improved cell–cell adhesion between BCWM.1 cells and increased the size of clumps significantly (Supplementary Fig. S1A and S1B).

Discussion

The driving force for the metastatic process in Waldenström macroglobulinemia is not well understood. Accumulating evidence indicates that egress of Waldenström macroglobulinemia cells from one site of the bone marrow to a new site is a complex

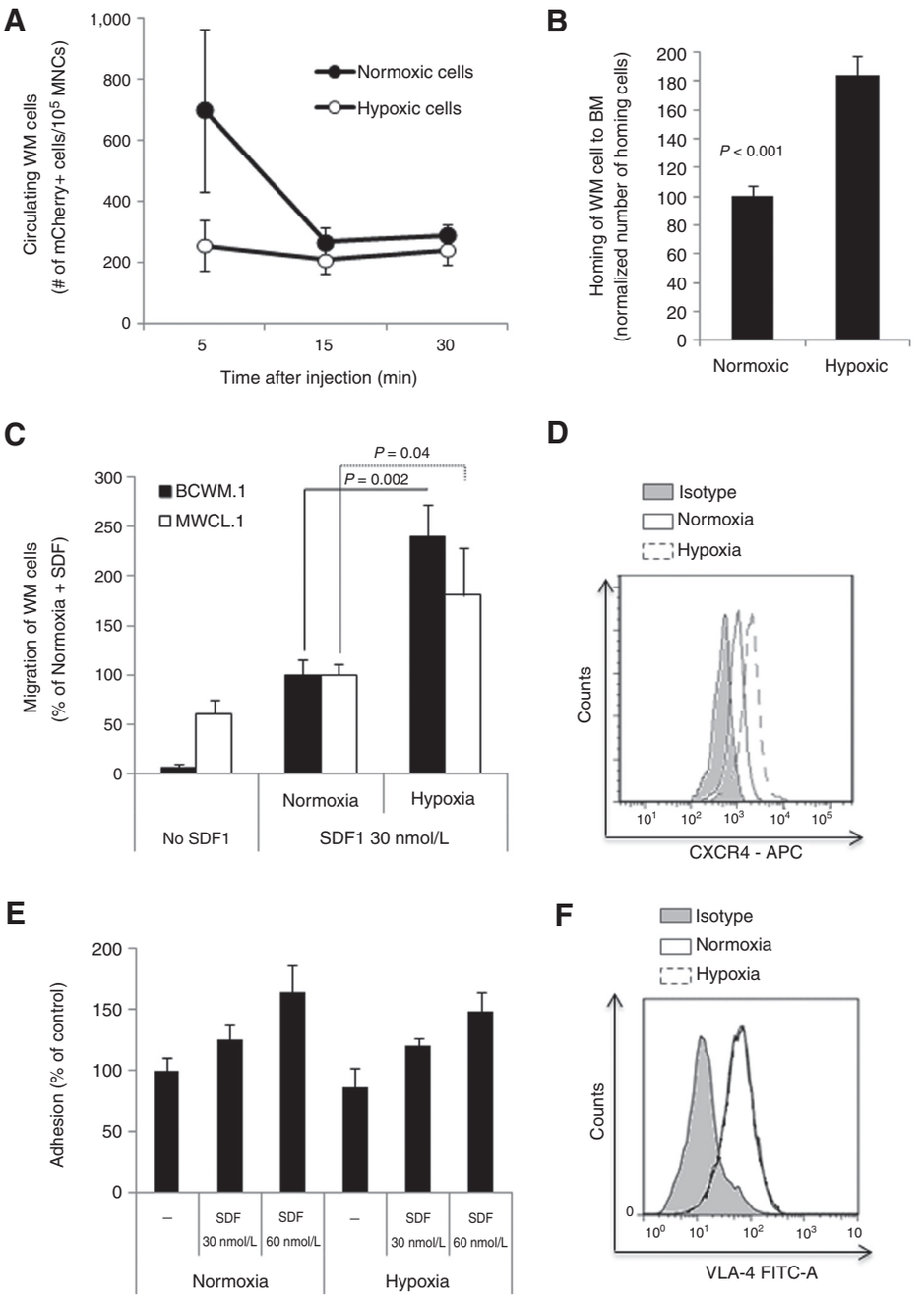

Figure 4.
Hypoxia promotes extravasation and homing of Waldenström macroglobulinemia (WM) cells to the bone marrow (BM) niches. The effect of hypoxia (1% O_2, 24 hours) on the number of circulating WM cells detected as mCherry-positive cells per 100,000 $CD45^+$ MNCs analyzed by flow cytometry at 5, 15, and 30 minutes of blood aspiration postinjection (A). The effect of hypoxia (1% O_2, 24 hours) on WM cell homing to the bone marrow is shown as the number of BCWM.1-$mCherry^+$ (DiD^+ or DiO^+ cells) detected in the bone marrow, analyzed by flow cytometry and normalized to normoxic cells (B). The effect of hypoxia (1% O_2, 24 hours) in the presence of SDF-1 (30 nmol/L) on BCWM.1 and MWCL.1 cell chemotaxis analyzed by flow cytometry and shown as a percentage of migrated cells normalized to cells which migrated toward SDF-1 in normoxia (C). The effect of hypoxia on the expression of CXCR4 in BCWM.1 cells shown as histogram analyzed by flow cytometry (D). The effect of hypoxia (1% O_2, 24 hours) with or without SDF-1 (30 and 60 nmol/L) on adhesion to fibronectin (FN) of BCWM.1 cells stained with calcein AM and analyzed by fluorescent reader and shown as the percentage of adherent cells normalized to normoxic untreated cells (E). The effect of hypoxia on the expression of VLA-4 in BCWM.1 cells shown as histogram analyzed by flow cytometry (F).

process that involves intercellular interactions with extracellular matrix (3), soluble growth factors (5), endothelial cells (6), and stromal cells (7). Hypoxia is a decreased oxygenation of tissue which was shown to develop due to uncontrollable tumor cell proliferation. Hypoxia was shown to play a key role in metastasis of solid tumors (19) and to promote resistance to therapy by reducing proliferation and inducing cell cycle arrest (20). It has been shown by a direct *in vivo* measurement that the bone marrow is a hypoxic tissue and the oxygen tension is low even in the highly vascular regions ranging between 1.5% and 4.2% O_2 (21). In case of hematologic malignancies, including multiple myeloma and CML, hypoxia was shown to promote progression and dissemination of the disease (12, 13, 15, 16). Lymphomas were found to show hypoxic features, based on the expression of main endogenous hypoxic marker, HIF1α. Constitutive stabilization of HIF1α was found in the majority of patients with diffuse large B-cell lymphoma and follicular lymphoma (22), as well as in mantle cell lymphoma (18).

Targeting hypoxia in solid tumors is one of the current approaches to prevent cancer angiogenesis, metastasis, and

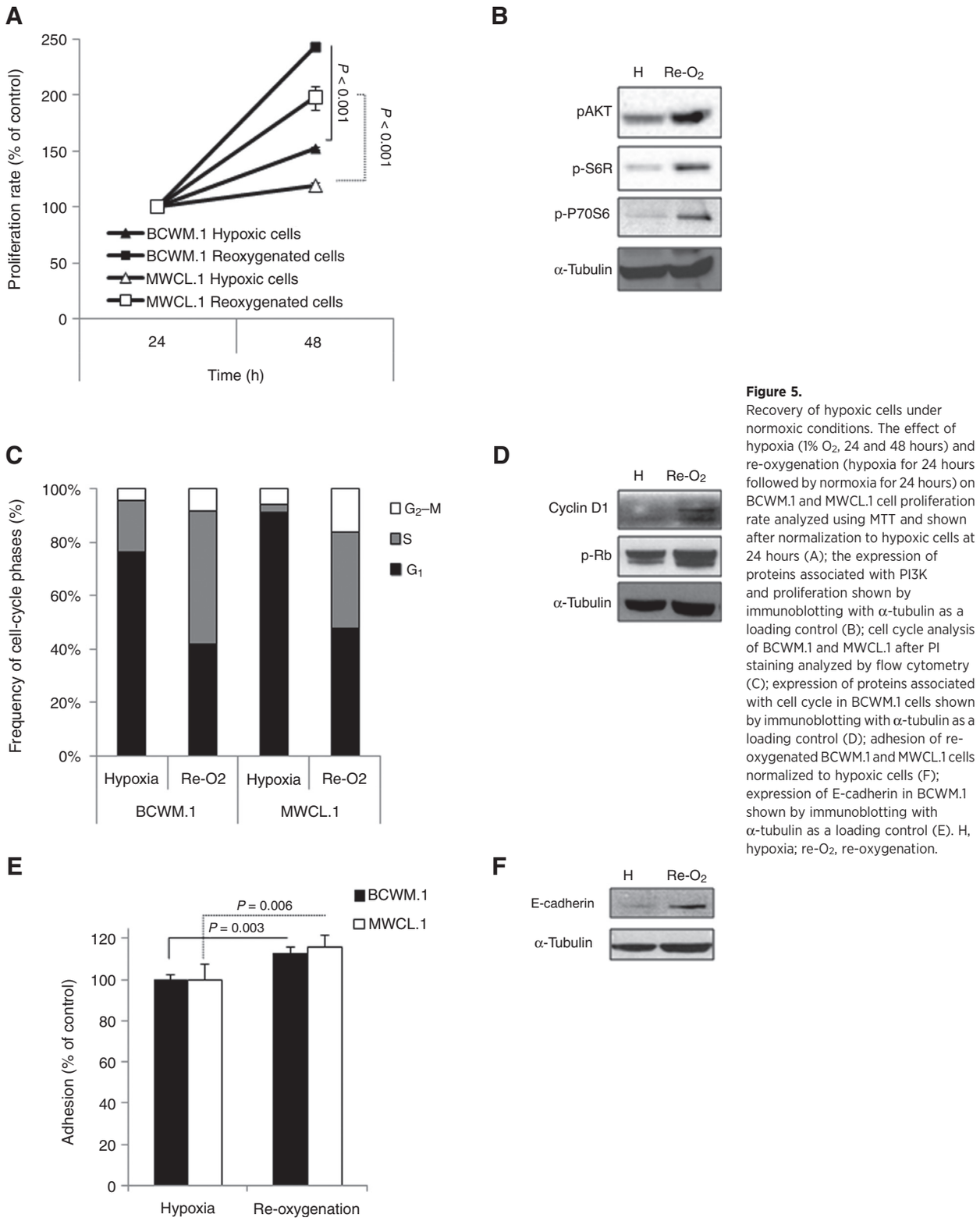

Figure 5.
Recovery of hypoxic cells under normoxic conditions. The effect of hypoxia (1% O_2, 24 and 48 hours) and re-oxygenation (hypoxia for 24 hours followed by normoxia for 24 hours) on BCWM.1 and MWCL.1 cell proliferation rate analyzed using MTT and shown after normalization to hypoxic cells at 24 hours (A); the expression of proteins associated with PI3K and proliferation shown by immunoblotting with α-tubulin as a loading control (B); cell cycle analysis of BCWM.1 and MWCL.1 after PI staining analyzed by flow cytometry (C); expression of proteins associated with cell cycle in BCWM.1 cells shown by immunoblotting with α-tubulin as a loading control (D); adhesion of re-oxygenated BCWM.1 and MWCL.1 cells normalized to hypoxic cells (F); expression of E-cadherin in BCWM.1 shown by immunoblotting with α-tubulin as a loading control (E). H, hypoxia; re-O_2, re-oxygenation.

drug resistance, and it became also an emerging strategy in hematologic malignancies. Antitumor activity of the hypoxia-activated pro-drug (TH-302) was demonstrated in combination with bortezomib which improved survival of mice with myeloma (14). TH-302 also delayed onset and progression of acute myeloid leukemia (AML) xenograft model (23). Moreover,

blocking HIF1α signaling, which is constitutively expressed in mouse lymphoma and human AML stem cells, preferentially eliminated cancer stem cells abrogating colony-forming unit activity and tumor initiation (24). Because hypoxia is one of the features of Waldenström macroglobulinemia progression and spread, it would be a promising target to improve patient survival.

In this study, we focused on the role of hypoxia in the dissemination and progression of Waldenström macroglobulinemia. We examined the hypoxic state of tumor cells *in vivo* in the xenograft mouse model of Waldenström macroglobulinemia. Tumor progression induced hypoxic conditions in Waldenström macroglobulinemia cells and to a lower extent in the bone marrow microenvironment; the higher the tumor involvement in the bone marrow, the higher the hypoxic conditions observed. These results were consistent with the development of hypoxic conditions in the tumor cells and the bone marrow microenvironment in multiple myeloma and CML animal models (12, 13). In addition, we tested the correlation between the hypoxic conditions in the bone marrow and the number of circulating Waldenström macroglobulinemia cells. A direct correlation was found between the hypoxic conditions in the bone marrow and the number of circulating Waldenström macroglobulinemia cells. Similar results were observed in myeloma; however, no such correlation was found in CML; we hypothesize that the reason for these observations is that both multiple myeloma and Waldenström macroglobulinemia are more dependent on the bone marrow for their progression, whereas CML is less bone marrow–dependent due to its circulating nature (8–10).

To mimic the effect of hypoxia at the primary tumor bed *in vitro*, we used 1% O_2, which is believed to be the acceptable level in tumor-induced hypoxia (25). We examined the effect of hypoxia on the cellular functions of Waldenström macroglobulinemia cells *in vitro* including proliferation, cell cycle, survival, apoptosis, cell adhesion, and cell migration. It was confirmed that 1% O_2 *in vitro* induced hypoxic response in the Waldenström macroglobulinemia cells including high expression of HIF1α and HIF2α. This is in agreement with our previous findings showing that these *in vitro* conditions induced hypoxic response in multiple myeloma and CML cells (12, 13).

It is well established in solid tumors that hypoxia regulates tumor cell proliferation; highly proliferative cancer cells are neighboring blood vessels, compared with the quiescent tumor cells in the hypoxic core (26). Therefore, we tested the effect of hypoxia on proliferation of Waldenström macroglobulinemia cells. Hypoxia inhibited the proliferation and decreased proliferative cell signaling pathways such as PI3K in Waldenström macroglobulinemia cells. This also led to induction of G_1 cell cycle arrest shown in DNA staining with PI as well as inhibition of cell cycle signaling. Despite the inhibition of proliferation, no apoptosis was observed because of hypoxia shown by Annexin/PI assay, and no caspase cleavage or changes in the expression of other proapoptotic proteins was observed. These are the first results to show the effect of hypoxia on proliferation and cell cycle in hematologic malignancies; further experiments to examine the effect of hypoxia on proliferation of other hematologic malignancies including multiple myeloma and CML are warranted.

We have previously shown that hypoxia promotes egress of multiple myeloma and CML cells from the bone marrow through activation of an EMT-like mechanism in which these cells down-regulated E-cadherin in the initial stages of egress (12, 13). Hypoxic Waldenström macroglobulinemia cells showed a similar pattern of behavior of decreased E-cadherin which resulted in a decreased adhesion of Waldenström macroglobulinemia to stromal cells *in vitro*, which was further confirmed to be E-cadherin–mediated. These results are in accord with the findings that the number of circulating Waldenström macroglobulinemia cells was correlated to the hypoxia in the bone marrow, in which hypoxic cells are less adhesive to the microenvironment and egress more to the circulation.

The next step of cell dissemination, after egress to circulation, is homing to new bone marrow niches. It was found that hypoxic Waldenström macroglobulinemia cells have the capability to extravasate from the circulation faster than normoxic ones and that the hypoxic cells ended up homing more efficiently into new bone marrow niches. These results are associated with increased CXCR4 expression in hypoxic Waldenström macroglobulinemia cells which facilitates faster chemotaxis of Waldenström macroglobulinemia cells toward the chemoattractant SDF-1. In addition, hypoxia did not change the SDF-1–induced adhesion of Waldenström macroglobulinemia cells to fibronectin (mediated by integrin VLA-4), as the first step of the process of homing into new bone marrow niches (3). Therefore, the hypoxic cells seem to have improved machinery for homing to new bone marrow niches including enhanced chemotaxis and integrin-mediated adhesion.

The last step of Waldenström macroglobulinemia dissemination is recovery of cells in the new "normoxic" bone marrow site, which implies exposure to normal oxygenation (re-oxygenation). Therefore, we tested the behavior of hypoxic cells after re-exposure to normoxic conditions. It was found that the proliferation after re-oxygenation of hypoxic cells induced exit of the G_1 arrest and boosted cell proliferation rate. Moreover, the cells restored their E-cadherin expression, which facilitated Waldenström macroglobulinemia cell adhesion to stroma.

In summary, hypoxia plays a critical role in Waldenström macroglobulinemia progression, dissemination, and cell trafficking. This study demonstrated for the first time that hypoxia decreases proliferation in Waldenström macroglobulinemia cells and induces more quiescent cell phenotype. In parallel, hypoxia induces loose adhesion to the primary tumor site to facilitate egress, enhances the ability of Waldenström macroglobulinemia cells to home from the circulation to new bone marrow sites and that hypoxic Waldenström macroglobulinemia cells recover and promote a proliferative form of the disease in the new bone marrow site. Further studies are warranted to test targeting hypoxic response in Waldenström macroglobulinemia cells, through inhibition of HIFs or their downstream targets, as a novel strategy to prevent dissemination of Waldenström macroglobulinemia. Furthermore, studies are warranted to test the effect of the hypoxia-induced G_1 arrest on Waldenström macroglobulinemia cell sensitivity to chemotherapy and targeting hypoxic responses in Waldenström macroglobulinemia cells to overcome drug resistance in Waldenström macroglobulinemia.

Disclosure of Potential Conflicts of Interest

A.K. Azab reports receiving commercial research grant from Selexys, Verastem, Karyopharm and Cellworks. No potential conflict of interest were disclosed by other authors.

Authors' Contributions

Conception and design: I.M. Ghobrial, A.K. Azab
Development of methodology: B. Muz, I.M. Ghobrial, A.K. Azab
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Muz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Muz, P. de la Puente, I.M. Ghobrial, A.K. Azab
Writing, review, and/or revision of the manuscript: B. Muz, P. de la Puente, F. Azab, I.M. Ghobrial, A.K. Azab
Study supervision: A.K. Azab

Grant Support

This study was supported by a grant from the International Waldenström's Macroglobulinemia Foundation.

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Received March 19, 2014; revised August 27, 2014; accepted September 8, 2014; published OnlineFirst September 17, 2014.

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