

Differential hypoxic regulation of the microRNA-146a/CXCR4 pathway in normal and leukemic monocytic cells: impact on response to chemotherapy

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Supplementary Materials and Methods

Cell growth, apoptosis and cell cycle analysis

Cell proliferation, viability of the cells, apoptosis and cell cycle analysis were evaluated by standard procedures (Annexin V-FITC apoptosis kit, 7-aminoactinomycin D and Cycletest Plus DNA detection kits, BD Pharmingen, San Jose, CA, USA).

Flow cytometry (FC) analysis

Analysis of total and membrane CXCR4 protein and Mo membrane markers CD11b and CD14 expression by FC was performed as previously described.³⁹

Western blot analysis

Western blot analysis were performed by using anti-HIF1 α - and anti-HIF2 α - polyclonal antibodies (pAb) (AF1935 and AF2886, R&D Systems, Minneapolis, MN, USA), normalized with anti-Nucleolin antibody (Oncogene Research Products, Boston, MA, USA), according standard methods.³⁹

RNA extraction and quantitative real-time PCR

Total RNAs were extracted using Trizol reagent and reverse transcribed by Moloney murine Leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random primers.

CXCR4, HIF-1 α and HIF-2 α mRNAs were detected by quantitative real-time PCR analysis (qRT-PCR) and normalized with the internal control β -Actin (ACTB), using commercial ready-to-use primers/probe mixes (Online Supplementary Table S1) for CXCR4 (Hs00607978_s1), HIF-1 α (Hs00153153_m1), HIF-2 α (or EPAS1 Hs01026149_m1) and ACTB (20X, 4310881E) (Applied

Biosystems, Foster City, CA, USA) according to the manufacturer's procedure and TaqMan technology.³⁹

qRT-PCR specific for miR-146a was performed and normalized using TaqMan^H MiRNA Assays and protocol (miR-146a assay ID 000468 and RNU6B primer kit ID 001093, Applied Biosystems, Foster City, CA, USA) as previously described.³⁹

Promoter assays

The miR-146a promoter region (Prom-146a) was previously identified and pGL3Basic/Prom-146a vector and activity were characterized.²⁹ By mutagenesis of the putative HIF-binding site (or Hypoxia Response Element, *HRE*, site) into the pGL3Basic/Prom-146a vector using the QuickChange Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA), we prepared a mutated HRE-binding site pGL3.1Basic/Prom-146a vector (pGL3Basic/Mut.Prom-146a). Human HIF-1 α -, HIF-2 α - full length cDNAs were cloned into a pcDNA3.1(+) expression vector (pcDNA3.1/HIF-1 α and pcDNA3.1/HIF-2 α vectors from GenScript, Piscataway, NJ, USA). Luciferase assays were performed in 293T cells in normoxia with a *Renilla* luciferase vector (10 ng), together with pGL3Basic/Prom-146a or pGL3Basic/Mut.Prom-146a vector (500 ng) and, where indicated, with pcDNA3.1/HIF-1 α (HIF-1 α) or pcDNA3.1/HIF-2 α (HIF-2 α) vectors, as described.²⁹ Enforced expression of HIFs- α protein by pcDNA3.1/HIF-1 α or pcDNA3.1/HIF-2 α vector was controlled in 293T cells by western blot analysis.

Chromatin immunoprecipitation (ChIP) assays

2×10^6 of cells were crosslinked to DNA and sonicated before using the ChIP assay kit (Upstate, Charlottesville, VA, USA) according to the manufacturer's procedure, and protein-DNA complexes were immunoprecipitated with the anti-HIF1 α - or anti-HIF2 α - ChIPgrade polyclonal antibodies (pAbs) (ab2185; ab199; Abcam, Cambridge, UK) or with the unrelated anti-c-abl monoclonal antibody (mAb) (Oncogene Research Products, Boston, MA, USA) used as a negative control, or protein-A sepharose only. A genomic region of 163 bp containing the HRE sequence, RCgTg,²⁰ in the miR-146a promoter²⁹ was amplified in the immunoprecipitates by PCR using specific primers

flanking the HIF-binding motif, putative HRE site in the Prom-miR146a region, (sense: 5'-GACAGGGTCTCTCTCTGTG-3'; antisense:5'-CTAGCCTGGGCAACATGGA-3') and PCR conditions: 94°C/1 min; 40 cycles of (95°C/30s; 61°C/30s; 72°C/40s); 72°C/1 min. PCR products were Southern blotted and hybridized with an internal 32P-labeled oligonucleotide miR-146a* probe 5'-GACTGGAGTGCAGTGGTGCAATCATAGC-3'. As control, a flanking 140 bp genomic region of the Prom-miR146a region, without any HRE site, was amplified by PCR and analyzed using primers, internal probe and PCR conditions as described.²⁹ Non relevant cellular DNA sequences were detected by amplification of a GAPDH coding region using primers and PCR conditions as described.²⁹

Analysis of HIF- α binding to the HRE previously found in the promoter of CXCR4 (Prom-CXCR4) was performed using the primers flanking the HRE site, as described,⁴⁴ in the same protein-DNA complexes prepared for U937 and Mo cells.

Given the limited number of cells obtained in our cultures, we used Mo cells harvested at day 12 from cultures grown under mild hypoxia, but at day 18 from cultures grown under severe hypoxia, taking into account the delay of cell differentiation observed at 1% O₂.

Transfection of miR-146a or antagomiR-146a.

miR-146a mimics or antagomiR-146a oligonucleotides, non-targeting RNA or antagomiR oligonucleotides of control and FITC-conjugated non-targeting oligonucleotide (Dharmacon, Lafayette, CO, USA) were used to transfect cells using Lipofectamine 3000 and as described.³⁹

Enforced expression of CXCR4 in HL-60 and K562 cells

By using the previously prepared full-length CXCR4 coding sequence and its 3'UTR region (CXCR4-3'UTR)²⁹ that we subcloned in pBABEpuro retroviral vector, we infected HL-60 and K562 cells with (i) pBABEpuro empty vector (E); (ii) pBABEpuro-CXCR4-3'UTR expressing vector (CXCR4), as described²⁹. Infected HL-60 and -K562 cells were grown in RPMI supplemented with 10% FCS and puromycin (2,5 μ g/ml) for selection. Stable transduced cell lines, puromycin resistant, HL-60(CXCR4), K562(CXCR4) and HL-60(E), K562(E) were then

maintained in RPMI medium complemented with FCS 10%. CXCR4 overexpression was controlled by FC analysis in HL-60(CXCR4) and K562(CXCR4) cells, as compared to HL-60(E) and K562(E) cells.

SDF-1 α and drug treatment of cells

Leukemic cell lines and AML-M5 cells were cultured in hypoxia, mild or severe, as compared to normoxia at 50 000 cells/ml and treated with SDF-1 α (100 ng/ml) (Sigma-Aldrich), added every 2 days of culture, then with or without cytosine arabinoside, Ara-C (Sigma-Aldrich), at 1 μ M for 48 h, compared with cultures with Ara-C. The murine stromal cell line MS5 was grown in minimum essential medium. For chemotherapy sensitivity experiments these cells were grown in 12-wells cell culture plates (Costar, Corning, NY, USA) until the formation of a cell layer; then, culture supernatant was removed and replaced with leukemic cells in 1ml of tissue culture medium as described.³⁹

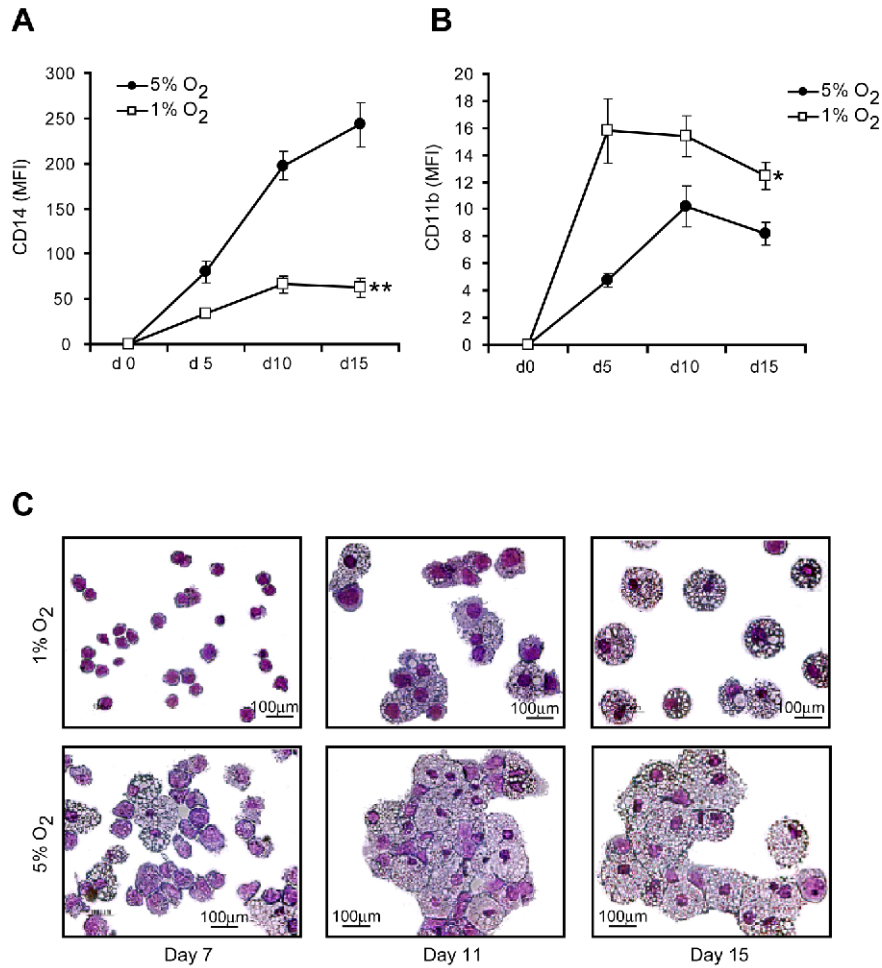
K562(CXCR4) cells were also treated with Imatinib (1 μ M) alone or in combination with SDF-1 α (100 ng/ml), as compared with untreated (C) control cells, under both normoxic (21% O₂) and hypoxic (1% O₂) conditions. Percentage of cell death was then evaluated after 24-48 hours of treatment, in these cultures.

Statistical Analysis

Statistical significance of differences observed between different experimental groups was determined using a Student's t test. The minimal level of significance was a P value below 0.05.

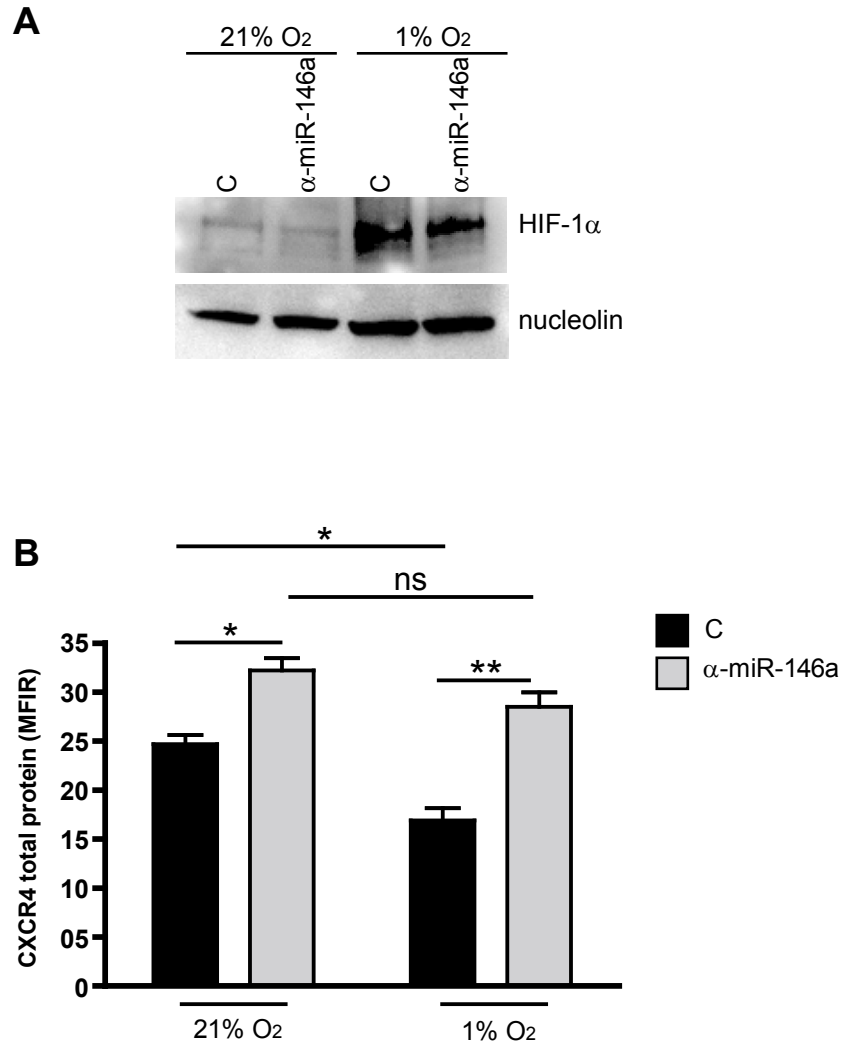
Supplementary Figures and Legends

Mo



Supplementary Figure S1. Severe hypoxia delays Mo differentiation of HPCs, as compared to mild hypoxia. (A, B) Quantitative analysis of specific Mo markers CD14 and CD11b expression by MFI values shows that severe hypoxia (1% O₂) delays CD14 expression, but enhances CD11b expression levels in Mo cells during Mo proliferation and differentiation of CD34+ (d0) HPCs, as compared to mild hypoxia (5% O₂). (A, B) Mean ± SEM values from three independent experiments are shown. *, **: p<0.05, p<0.01, respectively. C: Morphological analysis of Mo cells at days 7, 11 and 15 shows the delay in Mo differentiation and maturation of Mo cells cultured in severe hypoxia (panels 1% O₂), as compared to mild hypoxia (panels 5% O₂).

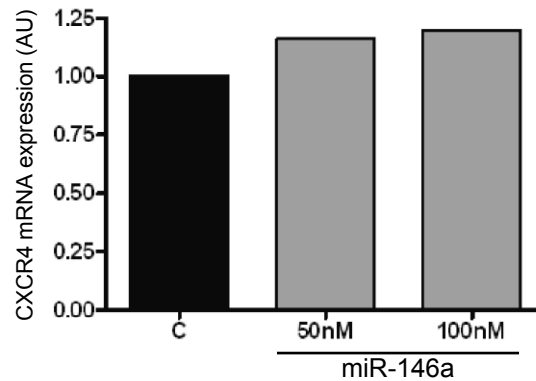
U937+ α -miR-146a



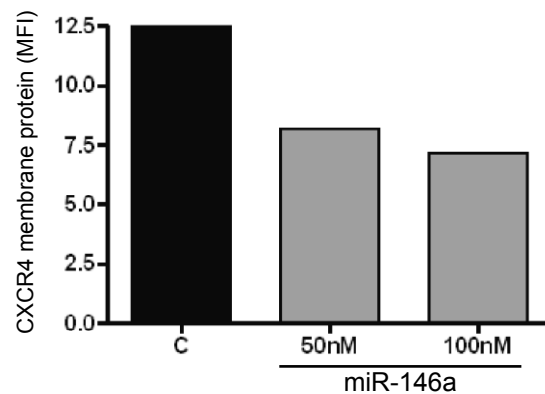
Supplementary Figure S2. AntagomiR-146a that impairs CXCR4 regulation by miR-146a targeting, upregulates CXCR4 protein level in U937 cells grown under normoxic (21% O₂) or hypoxic (1% O₂) conditions. (A) Western blot analysis shows that severe hypoxia (1% O₂) increases HIF-1 α nuclear protein level in U937 cells transfected with antagomiR-146a (α -miR-146a) or control antagomiR (C) oligonucleotides, as compared to normoxia (21% O₂). Nucleolin expression is shown as an internal control of nuclear proteins; one representative experiment of three is shown. (B) Flow cytometry analysis shows that CXCR4 total protein level increases in U937(α -miR-146a) cells, as compared to U937(C) control cells, under both normoxic and hypoxic conditions of culture. Mean \pm SEM values from three independent experiments are shown. *, **: p < 0.05, p < 0.01, respectively; ns: no significant.

AML-M5

A

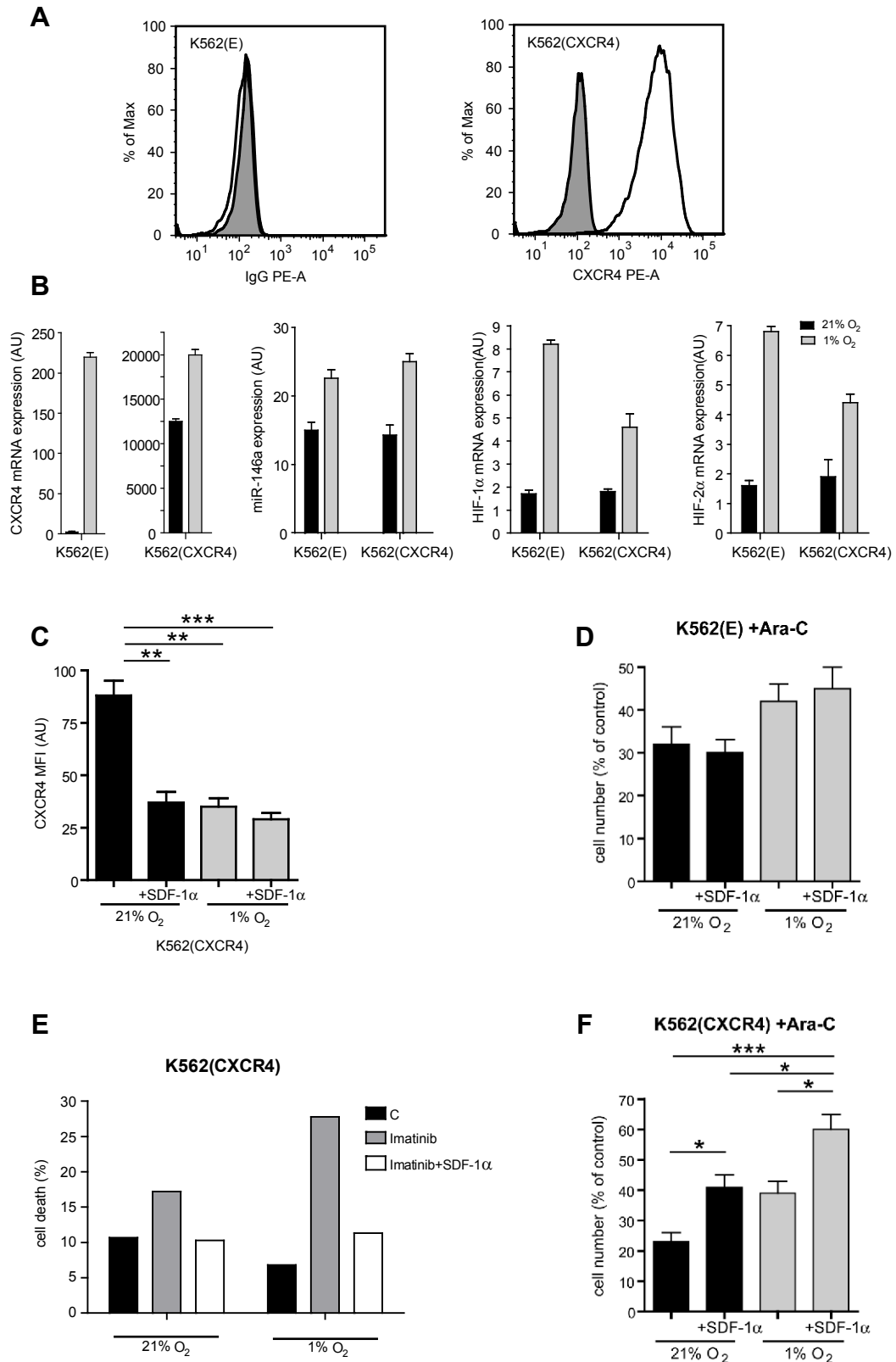


B



Supplementary Figure S3. miR-146a enforced expression in blast leukemic cells by blocking mRNA translation, decreases CXCR4 protein expression level in primary AML-M5. (A) Enforced expression of miR-146a mimics in blast leukemic cells obtained from primary AML-M5, performed by transfecting two concentrations (50 and 100 nM) of miR-146a as compared to control oligonucleotide (C), is without effect on CXCR4 mRNA expression as shown by qRT-PCR analysis. (B) Enforced expression of miR-146a mimics in AML-M5 cells decreases CXCR4 membrane protein expression level compared to control oligonucleotide (C), as shown by Flow cytometry analysis. One representative experiment is shown.

K562(CXCR4)



Supplementary Figure S4. Severe hypoxia activates the hypoxic-mediated control of CXCR4 mRNA and miR-146a expression and improves leukemic cell survival in combination with SDF-1 α in K562(CXCR4) cells treated with Ara-C. (A) CXCR4 protein overexpression in K562(CXCR4) cells is shown by Flow cytometry (FC) analysis, as compared to K562(E) control cells. (B) qRT-PCR shows that severe hypoxia (1% O₂) activates HIF-1 α and HIF-2 α mRNA expression (right panels), upregulates CXCR4 mRNA and miR-146a expression (left panels) in K562(E) and K562(CXCR4) cells, as compared to normoxia (21% O₂). (C) FC analysis shows that severe hypoxia significantly decreases CXCR4 protein level in K562(CXCR4) cells, as compared to normoxia; in K562(CXCR4) cells SDF-1 α addition significantly decreases CXCR4 membrane expression in normoxic, but not in hypoxic conditions. (D) SDF-1 α treatment did not significantly modify the survival of K562(E) cells treated with Ara-C, in normoxic or in hypoxic conditions. (E) SDF-1 α addition exerts a protective effect on Imatinib-mediated induction of cell death. (F) SDF-1 α induces a significant increase of the cell survival of K562(CXCR4)+Ara-C+SDF-1 α cells (+SDF-1 α), as compared with untreated cells in both normoxia and hypoxia. (B-C-D-F) Mean \pm SEM values from three independent experiments are shown. *, **, ***: p < 0.05, p < 0.01, p < 0.001, respectively. (A-E) One representative experiment out of three is shown.