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**Title :** Acute Lymphoblastic Leukemic cells modulate bone marrow angiogenesis : role of endothelin-1 and hypoxia.

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**Keywords :** Acute Lymphoblastic Leukemia, bone marrow, angiogenesis, hypoxia, VEGF<sub>A</sub>, endothelin-1

**Abstract :**

Increased microvessel density has been described in the bone marrow of patients with Acute Lymphoblastic Leukemia (ALL). The bone marrow is an hypoxic microenvironment, and hypoxia is an important event in triggering angiogenesis. However, the crosstalk between ALL cells and endothelial cells has not been well explored, and usual *in vitro* assays use cells maintained under 21% O<sub>2</sub>, which did not mimic bone marrow environment. In this study, the angiogenic activity of factors secreted by the human B-ALL Nalm-6 cell line was tested by using conditioned serum-free medium, that was applied on the human bone marrow endothelial cells HBME-1. Under 21% O<sub>2</sub>, factors secreted by Nalm-6 cells induced an angiogenic response on HBME-1 cells *in vitro*. This angiogenic response was not dependent on VEGF secretion but involved, at least in part, the endothelin-endothelin receptor axis. The influence of hypoxia was then studied by culturing both cell lines under 5% O<sub>2</sub>, an oxygen tension that fits the bone marrow microenvironment. Hypoxia stimulated the secretion of VEGF<sub>A</sub> by both Nalm-6 and HBME-1 cells, but the angiogenic response to leukemic conditioned medium was altered by chronic hypoxia, which affects the ability of endothelial cells to respond to endothelin-1. Thus, we concluded that leukemic secretion products did not induce angiogenesis at oxygen conditions met *in vivo*. This work highlights the importance of the oxygen rate in the modulation of cell interactions within the bone marrow, not yet well explored in ALL.

## Introduction

It has been well established that angiogenesis, e.g. the formation of new blood vessels from pre-existing vessels, plays a critical role in solid tumor development and metastasis<sup>1</sup>. The requirement of angiogenesis for growth and development of hematologic malignancies, such as acute lymphoblastic leukemia (ALL), has been also studied. Indeed, increased vascular density has been reported in the bone marrow of ALL patients, associated with the disruption of normal vascular architecture<sup>2</sup>. Vascular endothelial growth factor A (VEGF<sub>A</sub>) and basic fibroblast growth factor (bFGF) are the strongest inducers of medullar angiogenesis and are synthesized in various types of cells<sup>3,4</sup>. Several clinical studies have explored angiogenic factors levels in plasma and urine from patients with ALL. While Aguayo *et al*<sup>5</sup> noted no modification in VEGF<sub>A</sub> plasma levels in ALL patients compared to healthy controls, a higher plasma VEGF<sub>A</sub> level at relapse compared to those measured in plasma at diagnosis was observed by Schneider *et al*<sup>6</sup>, although without prognostic value. Higher bFGF plasma levels have been also reported in ALL at diagnostic<sup>5</sup>. Using more invasive methods, Veiga *et al*<sup>7</sup> described a higher bFGF level but no variation in VEGF<sub>A</sub> concentration in the bone marrow of ALL patients. *In vitro*, acute leukemic cells from patients secrete VEGF<sub>A</sub> but not bFGF, suggesting that this last factor derived from other cell types present in the medullar microenvironment<sup>8</sup>. Thus, many evidences suggest that angiogenesis is involved in ALL development, but the role of leukemic cells in the development of local angiogenesis is unclear.

Both *in vitro* and *in vivo* studies demonstrate that hypoxia is an important feature of healthy bone marrow microenvironment. Low oxygen tension is necessary for stem cell maintenance and hematopoiesis in bone marrow, and these effects are mediated by the hypoxia-dependent transcription factor, HIF-1 $\alpha$  (hypoxia inducible factor-1 alpha)<sup>9</sup>. However, the inaccessibility

of human bone marrow to direct noninvasive oxygen measurements makes difficulties to obtain precise data about the oxygen distribution in this organ. Harisson *et al*<sup>10</sup> measured a mean of 54.9 mmHg or 7.2% O<sub>2</sub> in the bone marrow of healthy volunteers. Mice studies using hypoxic markers brought out the existence of a 1% to 5% oxygen gradient from endosteum to capillaries in the bone marrow<sup>11</sup>. It is now assumed that gradients of O<sub>2</sub> from below 1% in endosteal niche to 6% in capillary region exist in the healthy human bone marrow. In ALL, Benito *et al* recently demonstrated the expansion of hypoxia in the bone marrow of immunodeficient mice engrafted with leukemic cells, promoting chimioresistance<sup>12</sup>. However, data reported on patients with acute myeloid leukemia suggested that the oxygen tension remained stable compared to what was previously described in healthy patients<sup>13</sup>.

It is still unknown whether bone marrow hypoxia contributes to local angiogenesis that supports ALL development. Interestingly, injection of the ALL cell line Nalm-6 into mice resulted in a homing of leukemic blasts around bone marrow capillaries, suggesting that a cross-talk may exist between these cell types via chemoattractant cytokines<sup>14</sup>. However, the influence of ALL cells on local capillary development was not studied.

The aim of this study was to investigate whether ALL blasts contribute to local angiogenesis by evaluating (1) the ability of factors secreted by leukemic cells to promote angiogenic features in human bone marrow endothelial cells, and (2) the influence of local hypoxia on ALL blast secretions and its consequences on angiogenesis. Our results demonstrated that leukemic conditioned medium induced an angiogenic response which involves endothelin-1. Chronic hypoxia increased angiogenic factors secretion, in particular that of VEGFA, but altered angiogenic response in bone marrow endothelial cells. Taken together, these results provide evidence that low oxygen rate alters response of bone marrow endothelial cells to angiogenic factors and open new perspectives concerning the role of endothelin-1 in ALL physiopathology.

## Materials and methods

### Cell lines and culture

The human pre-B lymphoblastic Nalm-6 cell line was purchased at the beginning of the present study from DSMZ (Braunschweig, Germany) and was maintained in RPMI 1640 (Eurobio) supplemented with 10% Fetal Calf Serum (FCS), 2mM L-glutamine and 0.1% penicillin-streptomycin. Six months after receipt, flow cytometry analysis was performed at the Department of Hematology & Cytometry, Rouen University Hospital, with an EPICS XL (Beckman-Coulter), and confirmed that these cells were still CD3-, CD4-, CD5-, CD8-, CD10+, CD13-, CD19+, CD24+, CD34-, and were positive for cytoplasmic IgM but not IgG1. They were also regularly checked for mycoplasma contamination by DNA labelling using DAPI and fluorescence microscopy.

The Human Bone Marrow Endothelial (HBME-1) cell line, derived from human fenestrated sinusoidal bone marrow endothelium, was kindly given in 2011 by Dr Kenneth Pienta (University of Michigan, Ann Arbor Comprehensive Cancer Center, Michigan<sup>15</sup>). This cell line was expanded in HDMEM medium (Eurobio) containing 10% FCS, 4mM L-glutamine and 0.1% penicillin-streptomycin to allow freezing down of aliquots of cells between passages 12 and 20. Aliquots were kept in liquid nitrogen. After thawing, cells were tested for VEGF-R1, VEGF-R2, VEGF-R3, angiopoietin receptors (Tie1 and Tie2), ET<sub>A</sub>R and ET<sub>B</sub>R mRNA expression by PCR analysis as previously described<sup>16</sup>, and tested for serum-induced capillary formation on Matrigel®. They were also regularly checked for mycoplasma contamination by DNA labelling using DAPI and fluorescence microscopy. HBME-1 cells were used between passages 22 and 40 to avoid phenotypic alterations.

Cell lines were grown in standard laboratory conditions comprised 21% O<sub>2</sub>, 5% CO<sub>2</sub> and a humidified atmosphere at 37°C. For experiments in hypoxia, cells were incubated in a work chamber under 5% O<sub>2</sub>, 5% CO<sub>2</sub> and a humidified atmosphere at 37°C as previously described<sup>16</sup>. Stability of hypoxic condition was regularly assessed by measuring pH, pCO<sub>2</sub> and pO<sub>2</sub> in culture media, and the metabolic shift induced by hypoxia was assessed by measuring glucose intake and lactate production in culture media (Supplementary Figure 1).

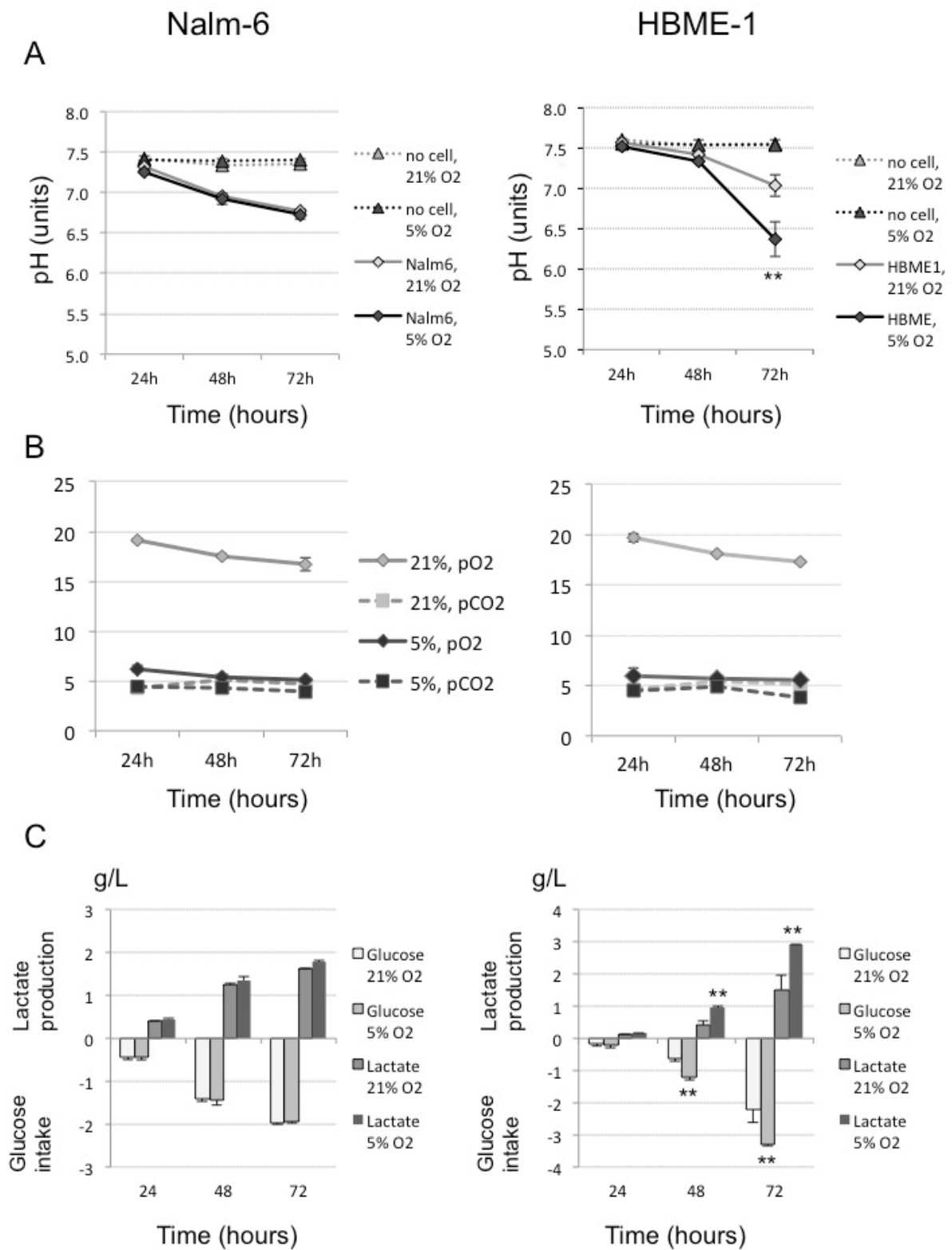
#### Leukemic conditioned medium (CM)

Nalm-6 cells (10<sup>6</sup> cells /ml) were incubated for 24 hours in serum-free RPMI supplemented with insulin, transferrin and selenium (ITS 100X, Gibco) and under 21% O<sub>2</sub> or 5% O<sub>2</sub>. Culture supernatants were harvested by cell centrifugation (10 minutes, 1500 rpm) and immediately mixed as a 2:1 vol/vol ratio with fresh serum-free HDMEM to obtain conditioned media (CM). CM21 was prepared from Nalm-6 cultures under 21% O<sub>2</sub>, while CM5 was from Nalm-6 cultured under 5% O<sub>2</sub>. A 2:1 vol/vol mix of serum-free RPMI:HDMEM was used as control.

#### *In vitro* angiogenesis assays

*In vitro* assays were performed with HBME-1 previously incubated with or without inhibitors for 30 minutes: endothelin receptor A inhibitor ABT-627 and endothelin receptor B inhibitor A-192621 kindly given by Jerry Wessale (Abott Laboratories), and the human neutralizing VEGF<sub>A</sub> antibody MAB293 (2 and 4 µg/ml, R&D systems). DMSO (0.01%) and PBS were used as vehicle control for endothelin receptor inhibitors and MAB293, respectively.

Supplementary figure 1





**Fig. S1 : Metabolic characteristics of Nalm-6 and HBME-1 cells cultured under 5% or 21% O<sub>2</sub>.**

Nalm-6 and HBME-1 cells were seeded in fresh serum-free medium, and pH (A), pO<sub>2</sub> and pCO<sub>2</sub> (B), glucose intake and lactate production (C) were measured in the culture medium after 24, 48 and 72 hours in culture. As many cancer cells, Nalm-6 cells have impaired mitochondria and produce energy via anaerobic glycolysis even in the presence of 21% O<sub>2</sub> (which is known as the Warburg effect). On the other hand, HBME-1 shifted from aerobic metabolism, with low lactate production under 21% O<sub>2</sub>, to an anaerobic metabolism under hypoxic conditions, characterized by an increase in glucose intake and lactate production, and a more rapid decrease in pH measured in the culture medium. Data are mean ± SEM of 4 independent assays. \*\*: p<0.01 vs 21% O<sub>2</sub>.

*Proliferation assay.* Cell proliferation was evaluated with a colorimetric assay (CellTiter 96®, Promega), which measures the increased activity of cellular mitochondrial dehydrogenases that can cleave the tetrazolium dye MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) into formazan. Briefly, HBME-1 cells were seeded onto 96-well plates (5000 cells/well) in HDMEM containing 10% FCS and let for 6 hours to adhere. Then, HBME-1 cells were incubated with CM21, CM5 or control medium for 24 to 72 hours. At the end of the treatment period, 20 µl of MTS were added and cells were further incubated at 37°C for 1h30 before reading optical density at 490 nm.

*Cell cycle analysis.* HBME-1 were cultured in serum-free medium with CM21, CM5 or control medium under 21% or 5% O<sub>2</sub>. After 24 hours, cells were scraped under 4°C, fixed in cold absolute ethanol (VWR), washed in PBS containing 0.5% FBS, and then incubated with a 50 µg/ml propidium iodide solution (Sigma Aldrich) containing RNaseA (100 µg/ml) protected from light. After 20 min of incubation, cells were diluted in PBS and subjected to analysis by fluorescence-activated pulse cytophotometry (FACS) on a BD FACSCalibur cytometer (BD Biosciences). The percentage of cells in each phase was analyzed using FlowJo Mac 9.5.2® software.

*Capillary formation.* HBME-1 cells were maintained in serum-free HDMEM for 16 hours before each experiment. Quiescent cells were then seeded on 96 well-plates coated with polymerized growth factor reduced Matrigel<sup>®</sup> mixed as a 2:3 vol/vol ratio with cold sterilized PBS ( $3.5 \times 10^4$  cells/well) and cultured under 21% O<sub>2</sub> or 5% O<sub>2</sub> in the presence of CM21, CM5 or control medium. After a 6-hours incubation period, capillary network was scored on random fields under phase contrast microscopy by measuring the tube length and the number of junctions between three or more capillary-like structures. Results were normalized to control conditions.

*Wound healing assay.* Migration assay was performed by wounding a layer of confluent, growth-arrested cells as previously described<sup>17</sup>. Briefly, HBME-1 cells were seeded on 24 well-plates ( $10^5$  cells/well) in HDMEM containing 10% FCS, let to adhere for 6 hours and to form a confluent layer, and then maintained in HDMEM supplemented with 0.1% FCS for 16 hours. Wounds were made by scraping the quiescent cell layer with a pipette tip, and 4 fields per well were digitized under phase-contrast microscopy immediately after wounding (time 0). Then medium was changed for CM21, CM5 or control medium, and images from the same fields were digitized after 24 hours under 21% or 5% O<sub>2</sub> (time 24). Cell migration was determined by measuring the difference between wound width at time 24 – time 0.

*Transwell assays.* Migration assay was assessed by standard methodology using transwell inserts with 8.0 µm pore size (Becton Dickinson Labware). Briefly,  $5 \times 10^5$  HBME-1 cells in 200 µl were added to the top chamber and inserts were placed in well containing 800µl of CM21, CM5 or control medium. After 24h under 21% or 5% O<sub>2</sub>, inserts were removed and fixed by crystal violet. Cells migrating to the lower side of the membrane were counted using inverted phase contrast microscopy.

### RT-PCR

Total RNA was extracted from cells using TRIZOL<sup>®</sup> Reagent (Invitrogen) and submitted to reverse transcription and PCR analysis using gene specific primers (Eurogentec) as previously described (16). Beta-actin was used as internal control.

### Western Blot

Total protein were extracted by lysing the cells in cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0,5% EDTA, 1% NP40, and 0.1% SDS), in the presence of protease inhibitors mix (Complete Mini, Roche) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Fisher Scientific). Equal protein amounts were then separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (GE Healthcare) probed with appropriate antibodies. Antibodies used included the polyclonal rabbit anti-human VEGF-R1 (1/1000, Cell Signaling), the polyclonal rabbit anti-human VEGF-R2 (1/1000, Sigma Aldrich), the polyclonal rabbit anti-human FLT-4 (1/1000, Novus Biologicals), the polyclonal rabbit anti-human ET<sub>A</sub>R (1/1000, Abgent), the polyclonal rabbit anti-human ET<sub>B</sub>R (1/500, Sigma Aldrich) and the monoclonal anti-human beta-actin (AC-15 clone, Sigma Aldrich). Rabbit anti-mouse and goat anti-rabbit horseradish peroxidase-coupled secondary antibodies were purchased from Cell Signalling Technology (Ozyme).

Specific proteins were visualized using a chemiluminescence kit (Roche Diagnostics) and analyzed using ImageJ 1.42q software. Beta-actin was used as internal control.

### VEGF-Rs expression analysis by flow cytometry

HBME-1 cells were cultured in different conditions for 24 hours. Cells were harvested using 0.5% EDTA, fixed with 4% paraformaldehyde and half of the cell population was permeabilized with 0.3% Triton. Cells were then stained with the phycoerythrin labeled antihuman VEGF-R1 (clone 49560), VEGF-R2 (clone 89106), VEGF-R3 (clone 54733) from Sampler Pack (R&D Systems) or with the mouse IgG1 $\kappa$  as a control (clone MOPC, Biologend). Analysis was performed by flow cytometry (BD FACSCalibur BD Biosciences) using FlowJo Mac 9.5.2<sup>®</sup> software.

### Measure of vascular endothelial growth factor receptor 2 (VEGF-R2) and phospho-VEGF-R2 in cell lysates:

HBME-1 cells were cultured in different conditions for 24 hours. Then medium was changed for fresh serum-free HDMEM supplemented or not with 100 ng/ml recombinant human VEGF<sub>165</sub> (R&D Systems) to induce tyrosine phosphorylation of VEGF-R2. After 7 minutes, cells were rapidly rinsed with Tris Buffered Solution and solubilized in lysis buffer (NP40 1%, Tris 20mM, NaCl 137mM, glycerol 10%, EDTA 2mM, protease and phosphatase inhibitors, pH 8). After centrifugation (2000 g for 5 minutes), supernatants were collected and total protein content were assayed using micro BCA assay kit (Pierce). Samples were stored at -70°C until use. Enzyme-linked immunosorbent assay (ELISA) of total VEGF-R2 and phospho-VEGF-R2 were performed on 100  $\mu$ g of lysate using the Human Total VEGF-R2/KDR DuoSet and the Human Phospho-VEGF-R2/KDR DuoSet kits from R&D Systems.

### Measurement of angiogenic factors

The levels of VEGF<sub>A</sub>, angiopoietin-2, angiopoietin-1 and endothelin-1 in conditioned media were measured by competitive ELISA (R&D systems) following manufacturer's instructions.

### Statistical analysis

Data are presented as mean  $\pm$  SEM. Significance was determined using the Student's *t* test. For cell cycle analysis, distribution of cell populations were compared using Fischer's exact test. All statistical tests were performed using R free software, and a p-value  $< 0.05$  was considered significant.

## Results

### **Nalm-6 pre-B cells secreted a specific pattern of angiogenic factors that was modified under reduced oxygen tension.**

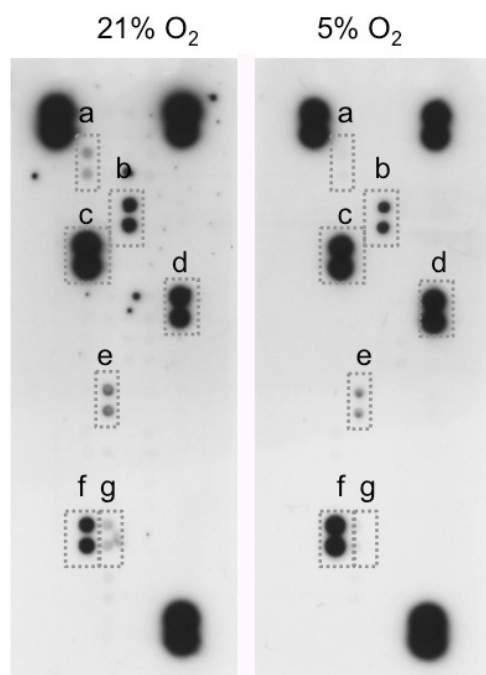
In a previous study<sup>8</sup>, we studied the pro- and anti-angiogenic factors secreted by blasts from ALL patients. The secretion pattern of Nalm-6 cells was very similar, and included VEGF<sub>A</sub>, angiogenin, Heparin-Binding EGF-like Growth Factor (HB-EGF), and Platelet Factor 4 (Supplementary Figure 2), as well as endothelin-1 (ET-1). No expression of basic Fibroblast Growth Factor was observed, neither at the protein nor at the mRNA level. We extended and confirmed these results by a precise measurement of the expression of VEGF isoforms, angiopoietins and ET-1 by Nalm-6 cells cultured in serum-free conditions, using RT-PCR and ELISA analysis. As shown in Figure 1A, Nalm-6 cells preferentially expressed the 121 and 165 isoforms of VEGF<sub>A</sub>, whose expression did not vary when cells have been cultured for up to 2 days under 21% O<sub>2</sub> and in the absence of serum. ELISA measurements showed that the extracellular concentration of VEGF<sub>A</sub> was  $54.65 \pm 5.59$  pg/ml after 24 hours of culture, and reached  $79.79 \pm 3.69$  pg/ml after 48 hours under 21% O<sub>2</sub> (Figure 1B). Nalm-6 also expressed VEGF<sub>B</sub> mRNA (Figure 1C), whereas no mRNA was detected for VEGF<sub>C</sub> or VEGF<sub>D</sub>. Nalm-6 did not express angiopoietin-1 but angiopoietin-2 was expressed and secreted by Nalm-6 cells, and its extracellular concentration was  $427.96 \pm 42.10$  pg/ml after 24 hours of culture. At last, ET-1 expression was detected in Nalm-6 cells at the mRNA level (Figure 1D), but this protein level could not be quantitated in the culture medium using ELISA kit, suggesting that the extracellular concentration was lower than 4.13 pg/ml.

Culture under 5% O<sub>2</sub> significantly stimulated VEGF<sub>A</sub> mRNA expression and protein secretion after 24 and 48 hours when compared to normoxia (Figures 1A & 1B). Both VEGF<sub>A</sub> isoforms (VEGF 165 and VEGF 121) expression were stimulated by hypoxia (Figure 1A). Hypoxia did not affect neither VEGF<sub>B</sub> mRNA expression (Figure 1C), nor angiopoietin-2 expression at the

mRNA or protein level (data not shown), and did not change that of ET-1 mRNA (Figure 1C) in Nalm-6 cells.

Thus, these results showed that Nalm-6 cells expression pattern of angiogenic factors was not altered by culture in serum-free medium. Culture under 5% O<sub>2</sub> specifically stimulated VEGF<sub>A</sub> production by Nalm-6, but not that of others angiogenic factors studied.

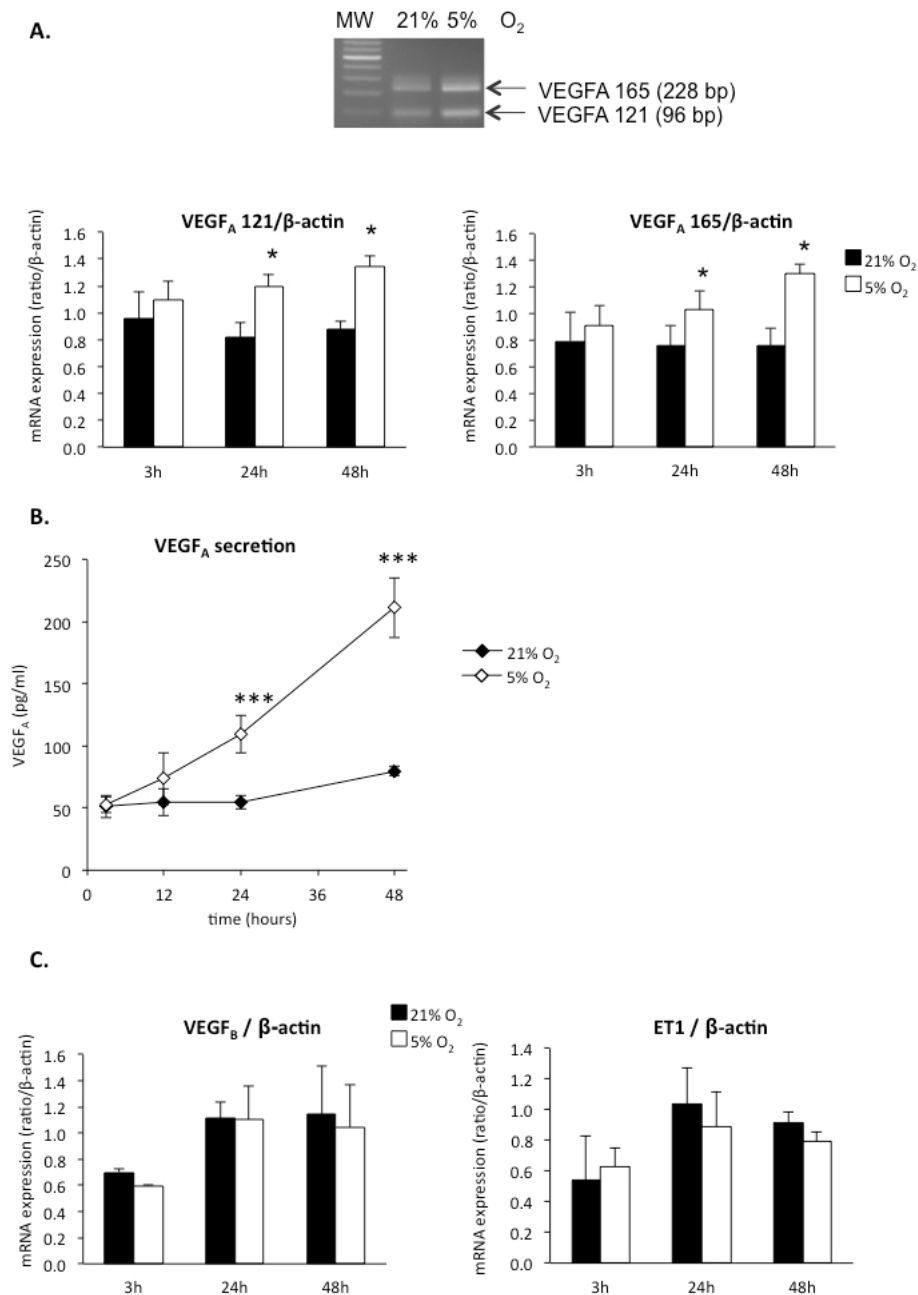
Supplementary figure 2



**Fig. S2 : Pro-and anti-angiogenic factors secreted by leukemic cells under normoxic or hypoxic conditions.**

Proteome Array® membranes, spotted in duplicates with specific capture antibodies, were incubated with culture supernatants from Nalm-6 cells (10<sup>6</sup> /ml, 24h in culture under 21 or 5% O<sub>2</sub>) and protein amounts were revealed by biotinylated detection antibodies. a : PAI-1 (serpin E1), b : Heparin-Binding EGF-like Growth Factor (HB-EGF), c : Tissue Inhibitor of MetalloPeptidases-1 (TIMP-1), d : Angiogenin, e : Platelet-derived Growth Factor-AA (PDGF-AA), f : VEGF<sub>A</sub>, g : Platelet-factor 4 (PF4). Endothelin was not detected using these membranes. Data are representative of 3 independent assays.

Figure 1



**Figure 1.** Expression and secretion of angiogenic cytokines by Nalm-6 cells cultured under 21% or 5% O<sub>2</sub>. A : Relative expression of VEGF<sub>A</sub> isoforms (VEGF121 and VEGF 165) was normalized against beta-actin mRNA expression level. The image on top shows the main VEGF<sub>A</sub> isoforms expressed by Nalm-6 cells maintained for 2 days under 21% or 5% O<sub>2</sub>. MW: molecular weight marker. For quantitative analysis, Nalm-6 cells were maintained in serum-free medium for 3-48 hours under 21% or 5% O<sub>2</sub> before RNA extraction and analysis. Results are presented as the means  $\pm$  SEM of ratios obtained from 3 independent assays. \* :  $p < 0.05$  vs 21% O<sub>2</sub>. B : VEGF<sub>A</sub> secretion in the culture medium as a function of time in

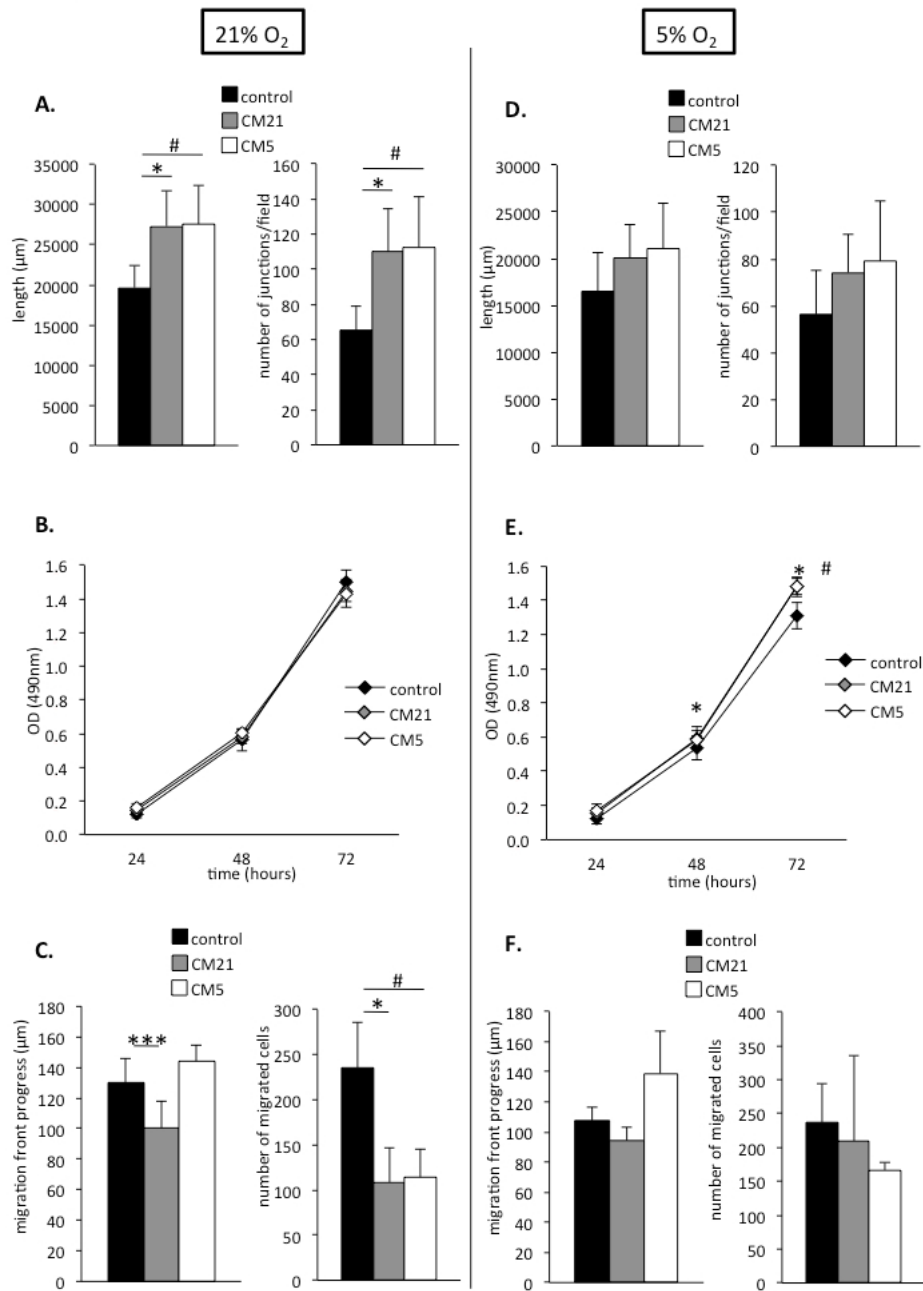


culture. Results are means  $\pm$  SEM of 5 independent assays. \*\*\* :  $p < 0.001$  vs 21% O<sub>2</sub>. C : VEGF<sub>B</sub> and endothelin-1 (ET1) mRNAs expression normalized against beta-actin mRNA expression level.

### **Angiogenic response to leukemic conditioned medium was altered under hypoxia.**

To investigate the influence of factors secreted by leukemic cells on medullar angiogenesis, conditioned media from Nalm-6 cultures performed under 21% O<sub>2</sub> (CM21) or 5% O<sub>2</sub> (CM5) were harvested, mixed with fresh serum-free HDMEM (2:1 vol/vol ratio), and used immediately for angiogenic assays. These assays were performed using the human bone marrow endothelial cell line HBME-1, which was characterized by VEGF-R3<sup>+</sup> Tie2<sup>-</sup> expression, a specific phenotype similar to that described in bone marrow sinusoids<sup>16</sup>. We first evaluated the influence of conditioned media on angiogenic activity of HBME-1 maintained under 21% O<sub>2</sub>. As shown in Figure 2A, both CM21 and CM5 significantly increased the length and number of junctions formed by endothelial cells after 6 hours, when compared to control medium. The conditioned media had no effect on HBME-1 proliferation after 24 hours (Figure 2B), but significantly reduced endothelial migration measured in wound-healing or Transwell assays (Figure 2C). Taken together, these data showed that Nalm-6 cells, cultured for 24 hours in serum-free conditions and under 21% O<sub>2</sub>, produce angiogenic factors stimulating capillary formation, but not migration, of HBME-1 cells maintained in serum-free medium and under 21% O<sub>2</sub>.

Figure 2



**Figure 2. Angiogenic response of bone marrow endothelial HBME-1 cells to leukemic conditioned medium (CM) was altered under hypoxia.** The HBME-1 cells were cultured in a serum-free medium under 21% O<sub>2</sub> (A, B, C) or under 5% O<sub>2</sub> (D, E, F) in control conditions or stimulated with either CM21 or CM5. Angiogenic response was estimated by measuring length and number of junctions after 6 hours (A, D). Cell proliferation was measured using MTS assay after 24, 48 and 72 hours (B, E). Cell migration (C, F) was measured using wound healing (left) or Transwell (right) assays after 24 hours. Results are means ± SEM of 5 independent assays. \*: p<0.05, \*\*\* p<0.001 CM21 vs control ; # p<0.05 CM5 vs control.

Angiogenic features of HBME-1 cultured under 5% O<sub>2</sub>, an oxygen tension which is similar to that found in the bone marrow vascular niche, were then studied in response to CM21 or CM5. Surprisingly, in these conditions, no significant change in capillary-like structures formation (Figure 2D) or cell migration (Figure 2F) was observed in response to factors secreted by leukemic cells. However, a significant stimulating effect on HBME-1 proliferation was observed after 72 hours of contact with CM5 or CM21 (Figure 2E). Thus, despite quantitative or qualitative changes in Nalm-6 cytokines secretion induced by hypoxia, no significant differences in cell migration or capillary-like structures formation were observed in response to CM5 vs CM21. These results suggest an impaired angiogenic response of HBME-1 cells to leukemic conditioned medium under 5% O<sub>2</sub>.

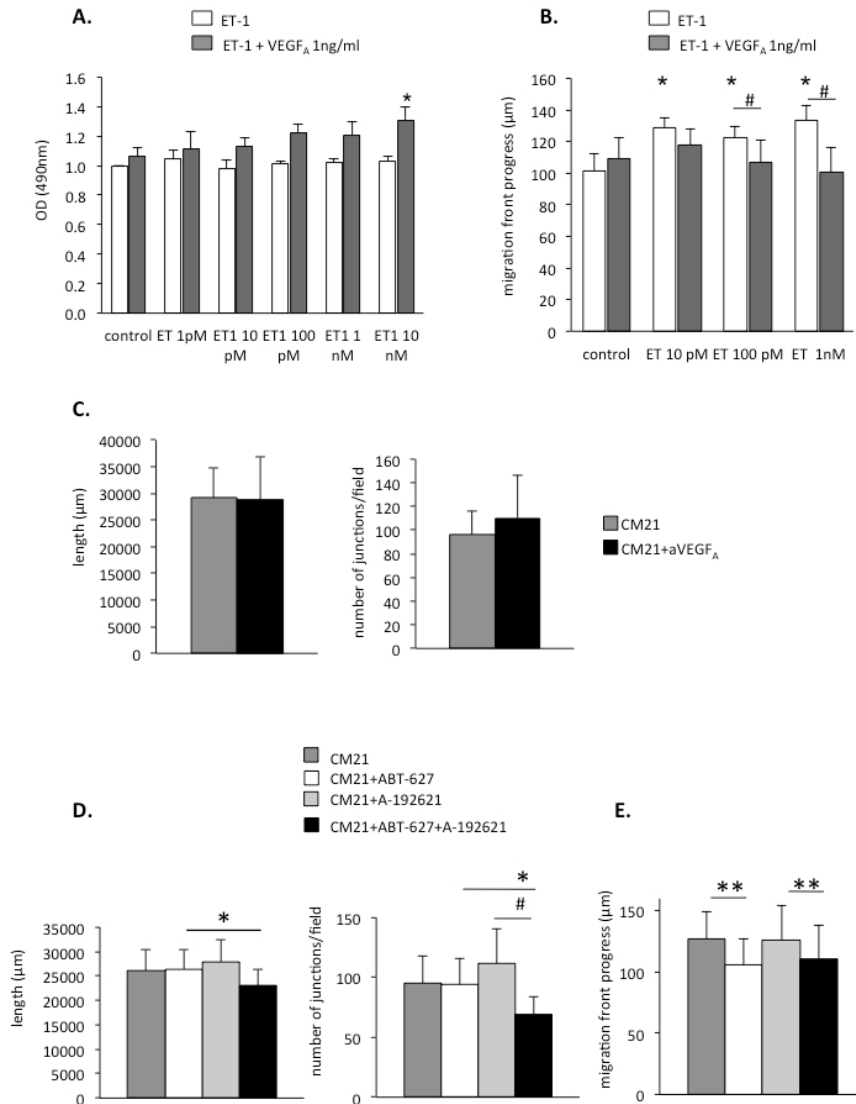
**Endothelin-1, but not VEGF<sub>A</sub>, was involved in angiogenic response of HBME-1 cultured under 21% O<sub>2</sub>.**

Previous results from our laboratory showed that HBME-1 express both VEGF receptors type 1, 2 and 3, as well as endothelin receptors A and B, but not angiopoietin receptors<sup>16</sup>. To understand the differences observed on angiogenic response depending on oxygenation, we focused on the role of VEGF<sub>A</sub> and ET-1 in the pro-angiogenic effects of leukemic conditioned medium. HBME-1 cells cultured under 21% O<sub>2</sub> were treated by 1 ng/ml exogenous human VEGF<sub>A</sub> and/or various doses of human recombinant ET-1. No stimulation of cell proliferation, cell migration nor capillary formation was observed after 24 hours in response to VEGF<sub>A</sub> alone (Figures 3A and 3B). Concentrations of ET-1 from 1pM to 10nM had no significant effect on HBME-1 cell density (Figure 3A). However, a combination of ET-1 and VEGF<sub>A</sub> increased cell density after 24 hours, an effect that was significant for higher concentrations (10nM of ET-1+1ng/ml VEGF<sub>A</sub>), suggesting a potential synergistic effect of

both cytokines on HBME-1 proliferation (Figure 3A). At last, exogenous ET-1 exerted a stimulatory effect on HBME-1 migratory activity, which was prevented by co-incubation with 1ng/ml VEGF<sub>A</sub> (Figure 3B)

To confirm these results, angiogenesis assays were performed with CM21 on HBME-1 cultured under 21% O<sub>2</sub> and pretreated with the human neutralizing VEGF<sub>A</sub> antibody (aVEGF<sub>A</sub>) used at 2 or 4μg/ml. VEGF<sub>A</sub> antibody did not significantly prevent CM21-induced tube formation on Matrigel<sup>®</sup> (Figure 3C) and had no effect on endothelial cell proliferation and migration activities (Supplementary figure 3). To explore the role of endothelin receptors, angiogenesis assays were performed on HBME-1 cultured under 21% O<sub>2</sub> and pretreated for 30 minutes with an inhibitor of ET<sub>A</sub>R (ABT-627) or an inhibitor of ET<sub>B</sub>R (A-192621) used at 100nM. No modification of spontaneous or CM21-induced proliferation was observed after 24 hours in the presence of endothelin receptor inhibitors, alone or in combination (Supplementary figure 3). However, the association of both inhibitors resulted in a significant decrease, when compared to each inhibitor alone, of the length and the number of junctions of capillaries formed by HBME-1 after 6 hours in the presence of CM21 (Figure 3D), suggesting the involvement of both receptors in this process. At last, as shown in Figure 3E, endothelial migration in the presence of CM21 was significantly reduced for cells pretreated with ET<sub>A</sub>R inhibitor when compared to control, and with both inhibitors when compared to ET<sub>B</sub>R inhibitor alone.

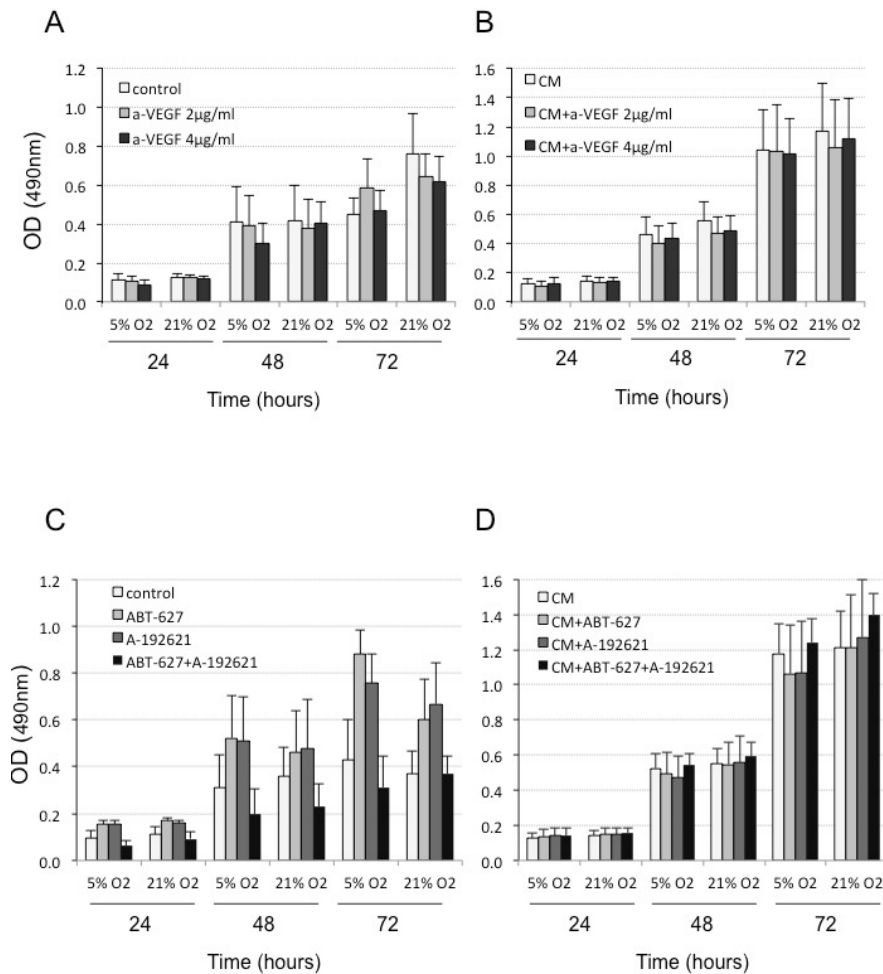
Figure 3



**Figure 3. Endothelin-1 but not VEGF<sub>A</sub> was involved in angiogenic response of HBME-1 cells to CM21.** **A, B** : Effects of various concentrations of human recombinant endothelin-1 (ET-1) alone (white columns) or in combination with 1 ng/ml VEGF<sub>A</sub> (grey columns) on cell density (A) or migration activity (B) of HBME-1 cultured for 24 hours in serum-free conditions and under 21% O<sub>2</sub>. Control : vehicle (PBS). Results are means ± SEM of 6 independent assays. \*: p<0.05 vs untreated cells ; #: p<0.05 vs ET-1 alone. **C** : Pretreatment with a neutralizing VEGFA antibody (aVEGF<sub>A</sub>, 4 µg/ml, black columns) did not modify CM21-induced capillary network formation, as evidenced by capillary length (left panel) and number of junctions (right panel). Results are means ± SEM of 3 independent assays. Similar results were obtained with aVEGF<sub>A</sub> used at 2 µg/ml. **D** : Blockade of ET<sub>A</sub>R and/or ET<sub>B</sub>R by 100 nM ABT-627 and A-192621, respectively, resulted in an inhibition of CM21-induced

capillary formation. Results are means  $\pm$  SEM of 3 independent assays. \* :  $p < 0.05$  vs ABT-627 alone ; # :  $p < 0.05$  vs A-192621 alone. **E:** Blockade of  $ET_A$ R by 100 nM ABT-627 inhibited migration activity of HBME-1 cells as measured in a wound assay. Results are means  $\pm$  SEM of 3 independent assays. \*\* :  $p < 0.01$  vs condition without ABT-627.

Supplementary figure 3



**Fig. S3 : HBME-1 cell density in the presence of inhibitors**

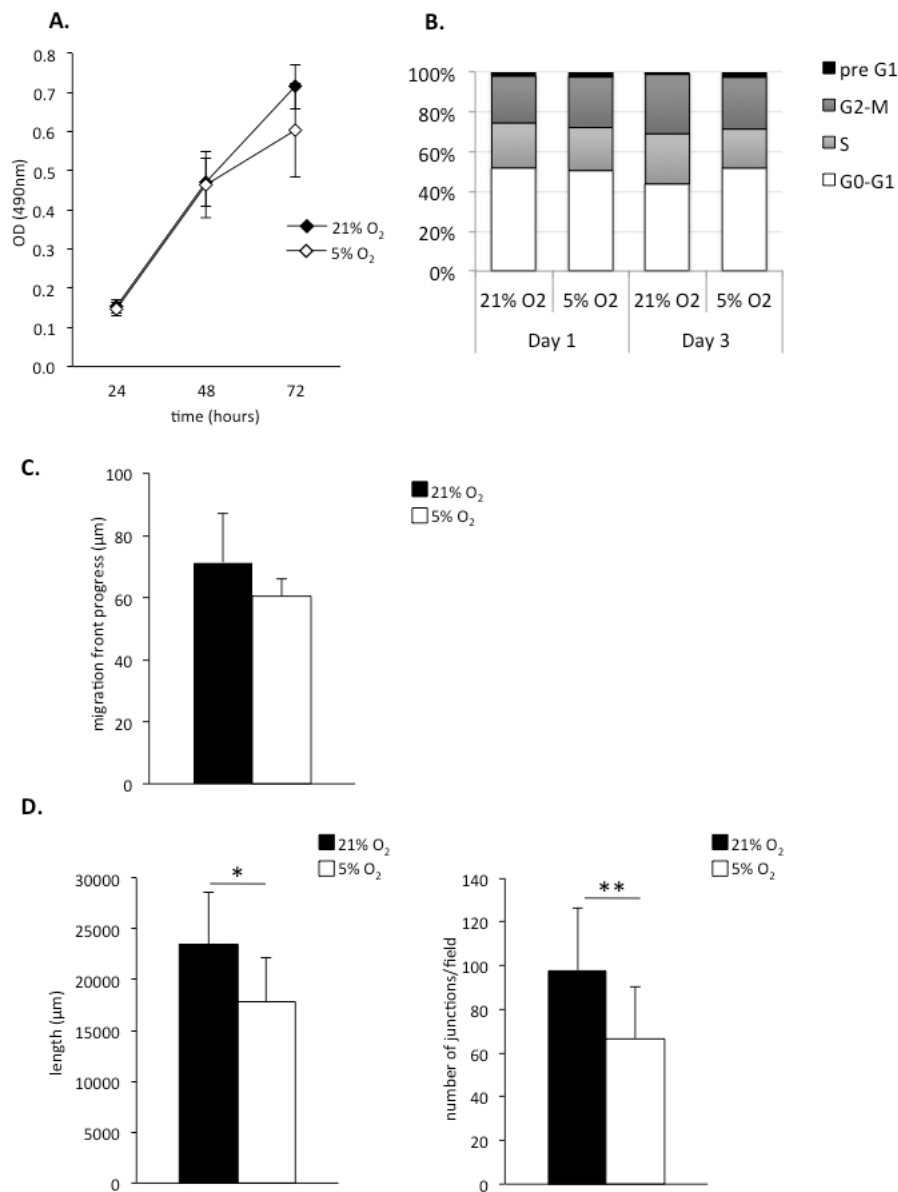
HBME-1 cells were incubated in serum-free medium with anti-VEGF<sub>A</sub> antibody (A) or endothelin receptors antagonists (B) for 24 to 72 hours, and under 21% or 5% O<sub>2</sub>. The presence of anti-VEGF<sub>A</sub> antibody (a-VEGF<sub>A</sub>, 2 or 4 ng/ml),  $ET_A$ R antagonist (ABT-627, 100 nM) or  $ET_B$ R antagonist (A-192621, 100 nM) did not significantly affect viable cell density.

These results indicate that endothelin receptors control, at least in part, the angiogenic activity of oxygenated HBME-1 cells by different mechanisms, involving both ET<sub>A</sub>R and ET<sub>B</sub>R in capillary structure formation, while ET<sub>A</sub>R may control endothelial migration. Moreover, VEGF<sub>A</sub> may modulate the effects of ET-1 on HBME-1 cells, as shown in Figure 3B. Thus, among factors secreted by leukemic cells, VEGF<sub>A</sub> did not appear to be involved in the stimulation of angiogenic activity in HBME-1 cells observed in classic culture conditions (21% O<sub>2</sub>).

#### **Spontaneous angiogenic response of HBME-1 was impaired under hypoxia.**

Then, we explored whether hypoxia altered angiogenic response of HBME-1 cells. Neither spontaneous proliferation (Figure 4A), cell cycle repartition (Figure 4B) nor migration (Figure 4C) of HBME-1 cells were altered by hypoxia, whereas spontaneous tube formation of HBME-1 cells on Matrigel<sup>®</sup> was significantly decreased (Figure 4D). Since HBME-1 cells produced VEGF<sub>A</sub> and ET-1, their secretion was measured using ELISA after 24 hours under 21% or 5% O<sub>2</sub>. Hypoxia induced a significant increase in VEGF<sub>A</sub> secretion by HBME-1 cells (21% O<sub>2</sub> : 241 ± 26 pg/ml/million cells ; 5% O<sub>2</sub> : 744 ± 57 pg/ml/million cells, p<0.001) but had no effect on ET-1 secretion (21% O<sub>2</sub> : 7.84 ± 1.56 pg/ml/million cells ; 5% O<sub>2</sub> : 8.68 ± 0.71 pg/ml/million cells).

Figure 4



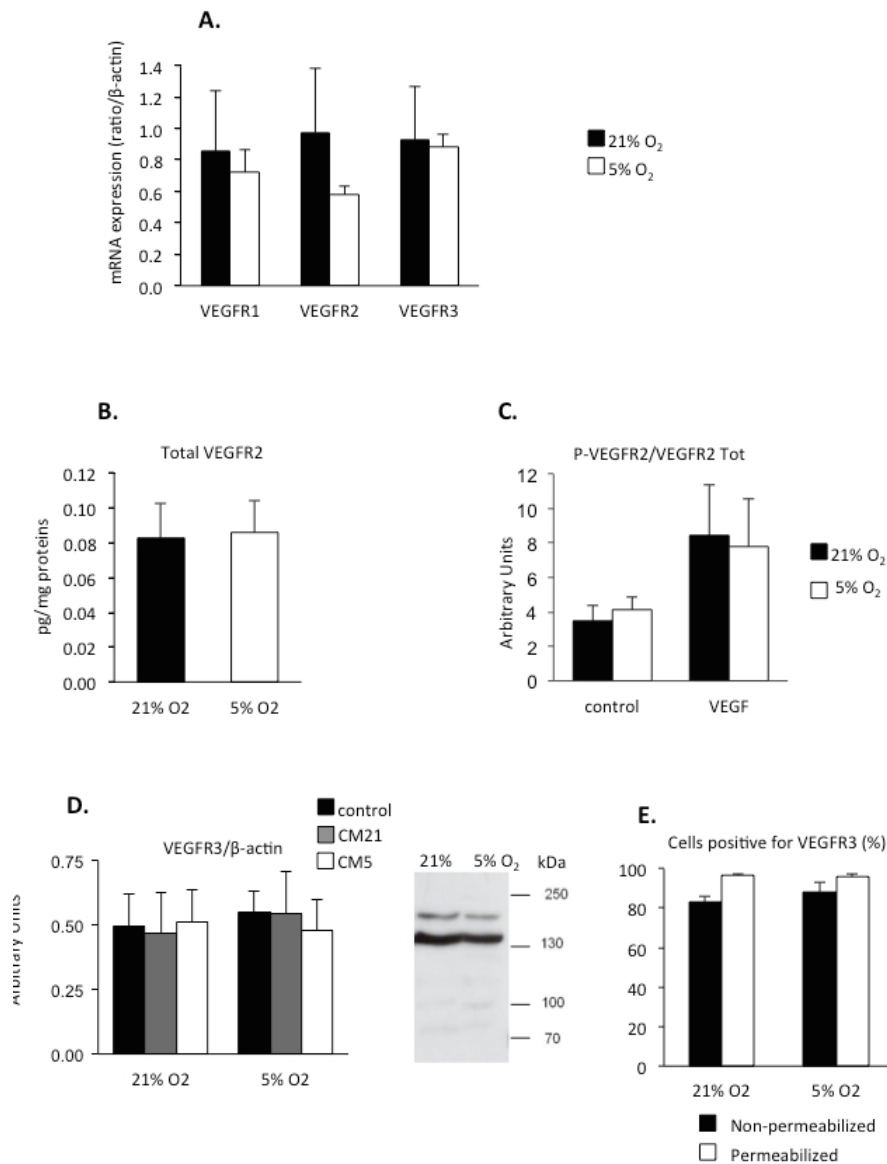
**Figure 4. Spontaneous angiogenic response of HBME-1 cells was altered under hypoxic conditions.** HBME-1 were cultured in serum-free conditions either under 21% or 5% O<sub>2</sub>. Cell density (A) and cell cycle repartition (B) were measured after 24-72 hours in culture. Cell migration (C) and spontaneous capillary network formation (D) were measured after 24 hours. Results are means  $\pm$  SEM of at least 3 independent assays. \*:  $p < 0.05$ , \*\*  $p < 0.01$  vs culture under 21% O<sub>2</sub>.



In a previous report, we showed that hypoxia decreased VEGF-R1, VEGF-R2, and ET<sub>A</sub>R mRNA expression in HBME-1 cells when cultured in the presence of foetal calf serum<sup>16</sup>. Since all our study of angiogenic activity was performed in serum-free medium, we explored the effects of hypoxia on the VEGF/VEGF-R and the ET-1/ET-R axis in HBME-1 cells in matching conditions.

In serum-free conditions, hypoxia did not significantly change the relative expression of VEGF-R1, -R2 nor -R3 mRNAs (Figure 5A). We could not detect the expression of VEGF-R1 protein neither by western blot nor by flow cytometry analysis, but mRNA results were confirmed at the protein level for VEGF-R2 (Figure 5B) and VEGF-R3 (Figure 5D). Using ELISA quantification, we showed that total VEGF-R2 expression did not change under hypoxia (Figure 5B). Moreover, the auto-phosphorylation of VEGF-R2 on tyrosine residues triggered by exogenous VEGF<sub>A</sub> was not modified by oxygen condition (Figure 5C), suggesting that hypoxia did not alter its kinase activity. Western-blot analysis of VEGF-R3 evidenced a preferential expression of the short isoform (145 kDa), but not modification under hypoxia or under the influence of leukemic conditioned medium was observed (Figure 5D). This was confirmed by flow cytometry analysis, which did not evidence significant changes in cell-surface or total VEGF-R3 expression under hypoxic condition (Figure 5E). At last, treatment of HBME-1 cells maintained under 5% O<sub>2</sub> with exogenous human VEGF<sub>A</sub> did not induce significant changes in cell proliferation, tube formation or cell migration activity (data not shown), as already observed with cells cultured under 21% O<sub>2</sub>.

Figure 5



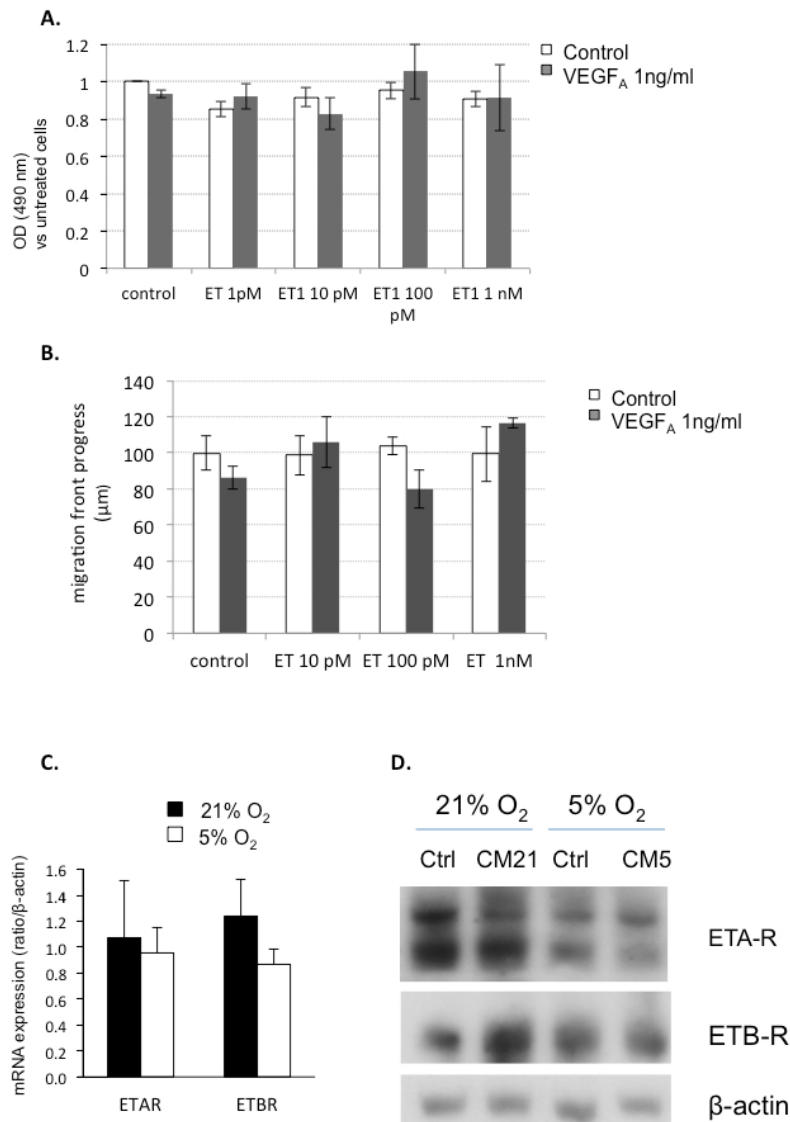
**Figure 5. Effect of hypoxia on the VEGF/VEGF-R axis in HBME-1 cells.**

**A :** Relative expression of VEGF receptors mRNA in HBME-1 cells cultured for 24 hours under 21% or 5% O<sub>2</sub>. Results were obtained from 5 independent experiments and were normalized on beta-actin mRNA expression. **B :** VEGF-R2 protein expression measured using ELISA in total cell extracts (n=6 independent cultures) **C :** Measurement of phosphorylated/total VEGF-R2 ratio using ELISA (n=4 independent cultures). **D :** Western-blot analysis of VEGF-R3 expression (normalized on beta-actin) in HBME-1 cultured for 24 hours under 21% or 5% O<sub>2</sub>, and in control medium or in the presence of leukemic conditioned

media (CM21 and CM5). Data are mean  $\pm$  SEM of 3 independent assays. A representative image on the right shows that HBME-1 express the long (153 kDa) and short (145 kDa) isoforms of VEGF-R3, but not its soluble (95 kDa) isoform. E : Flow cytometry analysis of cell-surface (white columns) or total (gray columns) VEGF-R3 expression in HBME-1 cells cultured for 24 hours under 21% or 5% O<sub>2</sub>. Data are mean  $\pm$  SEM of 3 independent assays.

When HBME-1 cells cultured under 5% O<sub>2</sub> were treated for 24 hours with exogenous ET-1 (0.1-1 nM), alone or in combination with 1ng/ml VEGF<sub>A</sub>, no significant change in cell density (Figure 6A) or in migratory activity (Figure 6B) was observed. Thus, hypoxia appears to hamper the responsiveness of HBME-1 to exogenous ET-1, which may be due to a down-regulation of endothelin receptors. To test this hypothesis, we checked the expression of ET<sub>A</sub>R and ET<sub>B</sub>R in HBME-1 cells maintained for 24 hours under 5% O<sub>2</sub>. Hypoxic conditions did not significantly alter ET<sub>A</sub>R nor ET<sub>B</sub>R mRNA (Figure 6C) and protein expression (Figure 6D) in HBME-1 cells. Treatment with leukemic conditioned media did not induce changes in ET-R expression, at least during 24 hours (Figure 6D). Taken together with the results presented in Figure 2, this result suggested a loss of sensitivity to angiogenic factors in HBME-1 cells maintained under 5% O<sub>2</sub>.

Figure 6



**Figure 6. Effect of hypoxia on the ET1/ETR axis in HBME-1 cells.**

**A, B :** Effects of various concentrations of human recombinant endothelin-1 (ET-1) alone or in combination with 1ng/ml VEGF<sub>A</sub> on cell density (A) or migration activity (B) of HBME-1 cells cultured for 24 hours in serum-free conditions and under 5% O<sub>2</sub>. Results are means ± SEM of 5 independent assays. **C :** Relative expression of ETA and ETB receptors mRNAs in HBME-1 cells maintained for 24 hours in serum-free conditions and under 21 or 5% O<sub>2</sub>. Results were normalized against beta-actin mRNA expression level and are the result of 3 independent experiments. **D :** Western-blot analysis of ETA and ETB receptors expression in HBME-1 cells cultured for 24 hours in serum-free conditions and under 21 or 5% O<sub>2</sub>, and in the presence or absence of leukemic conditioned medium (CM21 and CM5).

## DISCUSSION

The objective of this work was to study whether leukemic lymphoblasts can exert a paracrine effect to induce an angiogenic response in the bone marrow. Bone marrow has a very complex architecture, and its vasculature is composed of sinusoidal vessels which differ from blood vessels of other organs. In particular, sinusoidal endothelium share some of the characteristics of lymphatic vasculature, such as the expression of VEGF-R2 and VEGF-R3<sup>4,18</sup> and the absence of expression of the Tie-1 and Tie-2 receptors<sup>19, 20</sup>. For this reason, we used an endothelial cell line established from normal human bone marrow, the HBME-1 line<sup>15</sup>. In a previous study, we characterized its expression profile of angiogenic factors and their receptors, and showed that HBME-1 cells are VEGF-R2<sup>+</sup> VEGF-R3<sup>+</sup> Tie2<sup>-</sup>, as their *in vivo* counterparts<sup>16</sup>. Moreover, most *in vitro* angiogenesis studies reported were performed in classical culture conditions, e.g. under 21% O<sub>2</sub>, and did not take into account the hypoxic conditions which are characteristics of the bone marrow microenvironment (about 5% O<sub>2</sub> in the vascular domains). Thus, both leukemic and endothelial cells were maintained for at least 24 hours under 21% or 5% O<sub>2</sub> to study the influence of chronic hypoxic conditions on angiogenic response to cytokines secreted by leukemic cells.

Our results demonstrated that leukemic supernatant induced angiogenic response of bone marrow endothelial cells under 21% O<sub>2</sub>. Many angiogenic growth factors have been described in the bone marrow, including VEGFs, bFGF, TNF $\alpha$ , TGF $\beta$  and HGF, that can be secreted by either leukemic cells or other cell types present in the bone marrow, such as fibroblasts, immune cells, osteoclasts, thrombocytes and megakaryocytes<sup>3</sup>. The present study evidenced that, in a serum-free medium, the human Nalm-6 pre-B cell line express VEGF<sub>A</sub>, VEGF<sub>B</sub>, angiopoietin-2 and endothelin-1 (ET-1), but does not express bFGF, a secretion pattern which

is similar to that observed in leukemic cells isolated from ALL patients<sup>8</sup>. Among these factors, VEGF<sub>A</sub> was the only one whose expression and secretion were enhanced by hypoxic conditions, which was consistent with the direct binding of the hypoxia-inducible factor-1 (HIF-1) on VEGF<sub>A</sub> promoter that promotes gene transcription<sup>21</sup>. However, our results showed that leukemic secretion of VEGF<sub>A</sub> was not sufficient to induce angiogenic response in bone marrow endothelial cells, since blockade of VEGF<sub>A</sub> with a specific antibody did not prevent capillary organization induced by leukemic supernatants, and exogenous VEGF<sub>A</sub> did not increase HBME-1 proliferation nor migration activities. It must be emphasized that the spontaneous secretion of VEGF<sub>A</sub> by endothelial cells is much greater than that due to leukemic cells (about 250 pg/ml in endothelial CM vs 55 pg/ml in leukemic CM). Therefore, it is not surprising that VEGF<sub>A</sub> secreted by leukemic cells did not play a crucial role in endothelial stimulation. Nevertheless, our results suggest that under classic culture conditions (21% O<sub>2</sub>), VEGF<sub>A</sub> could act synergistically with other factors such as ET-1 on the proliferation of HBME-1, by mechanisms that remain unknown. Cooperation between the ET<sub>A</sub>R and ET<sub>B</sub>R receptors seems to be necessary to promote capillary formation by endothelial cells. However, the effects of ET-1 were abolished by chronic hypoxia. In a previous study<sup>16</sup>, we evidenced a decrease in ET<sub>A</sub>R mRNA under 5% O<sub>2</sub>, while that of ET<sub>B</sub>R and endothelin-converting enzyme remained unchanged. However, these results were obtained in the presence of 10% SVF ; in serum-free medium, which is a condition necessary for angiogenesis studies that was used in this study, we did not evidence any significant change in ET-1 receptors induced by hypoxia, at least for 24 hours.

Other factors identified in the supernatant of leukemic cells were not explored in this study. In particular, we evidenced the secretion of HB-EGF (heparin-binding EGF-like growth factor) by leukemic cells. HB-EGF is a ligand of EGF receptors that cooperates with VEGF<sub>A</sub> to

promote angiogenesis<sup>22</sup>. Moreover, stimulation of the EGFR pathway is one mechanism by which the bone marrow microenvironment may contribute to the growth and survival of B-cell acute leukemia<sup>23</sup>. Thus, it would be interesting to study the expression of HB-EGF receptors in these cell lines, and their involvement in the survival and proliferation of both cell types, in hypoxic conditions.

This *in vitro* study focused on paracrine factors secreted by leukemic cells, but did not explore the role of direct interactions between leukemic and endothelial cells, which could also participate in the regulation of angiogenesis. In the bone marrow, specific areas of the endothelium express high levels of cell adhesion molecules, that regulate hematopoietic stem cells trafficking, but also allow the entrance of circulating tumor cells<sup>14,24</sup>. A recent study demonstrated that acute myeloid leukemia cells mediate angiogenesis by secreting soluble factors as well as by direct cellular interactions with endothelial cells<sup>25</sup>. However, this *in vitro* study used human umbilical vein endothelial cells, which may differ from bone marrow sinusoidal cells, and was performed under 21% O<sub>2</sub>, eg in oxygen conditions that did not fit the bone marrow microenvironment.

In conclusion, our results emphasize the importance of using low oxygen levels to study the medullar endothelial phenotype. Since the pro-angiogenic effects of ET-1 or leukemic conditioned medium were abolished by chronic hypoxia, we cannot conclude that leukemic lymphoblasts play a major role in the induction of local angiogenesis. Thus, models using bone marrow sinusoidal cells maintained under reduced oxygen tension should be used to explore the interactions between endothelial and malignant cells in the bone marrow microenvironment.

**Abbreviations :** ALL, Acute Lymphoblastic Leukemia ; CM, conditioned medium ; ET-1, endothelin-1 ; VEGF, vascular endothelial growth factor.

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**Author's contributions :**

ID, CC and PM designed the research study and analysed the data. FG, KB and AP performed the research. ID, FG and CC wrote the paper.

**Conflict of interest :** none.



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