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Patricia A Cronin (PatCronin@rcsi.ie)
Jiang H Wang (PatCronin@rcsi.ie)
H PAUL Redmond (PatCronin@rcsi.ie)

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**Hypoxia increases the metastatic ability of breast cancer cells via
upregulation of CXCR4**

Patricia A. Cronin¹, Jiang H. Wang¹, H. Paul Redmond²

Department of Academic Surgery, University College Cork,
Cork University Hospital, Cork, Ireland

¹These authors contributed equally to this work

Email Addresses:

PAC: PatCronin@rcsi.ie

JHW: jh.wang@ucc.ie

HPR: henry.redmond@hse.ie

²Corresponding author: Professor H. Paul Redmond, Department of Academic Surgery,
University College Cork, Cork University Hospital, Cork, Ireland. Telephone: +353 21
4922371; Fax: +353 21 4922811

Abstract

Background: Chemokine SDF1 α and its unique receptor CXCR4 have been implicated in organ-specific metastases of many cancers including breast cancer. Hypoxia is a common feature of solid tumors and is associated with their malignant phenotype. We hypothesized that hypoxia would upregulate CXCR4 expression and lead to increased chemotactic responsiveness to its specific ligand SDF1 α .

Methods: Three breast cancer cell lines MDA-MB-231, MCF7 and 4T1 were subjected to 48 hrs of hypoxia or normoxia. Cell surface receptor expression was evaluated using flow cytometry. An extracellular matrix invasion assay and microporous migration assay was used to assess chemotactic response and metastatic ability.

Results: CXCR4 surface expression was significantly increased in the two human breast cancer cell lines, MDA-MB-231 and MCF7, following exposure to hypoxia. This upregulation of CXCR4 cell surface expression corresponded to a significant increase in migration and invasion in response to SDF1- α *in vitro*. The increase in metastatic potential of both the normoxic and the hypoxic treated breast cancer cell lines was attenuated by neutralization of CXCR4 with a CXCR4 neutralizing mAb, MAB172 or a CXCR4 antagonist, AMD3100, showing the relationship between CXCR4 overexpression and increased chemotactic responsiveness.

Conclusions: CXCR4 expression can be modulated by the tissue microenvironment such as hypoxia. Upregulation of CXCR4 is associated with increased migratory and invasive potential and this effect can be abrogated by CXCR4 inhibition. Chemokine receptor CXCR4 is a potential therapeutic target in the adjuvant treatment of breast cancer.

Background

Breast cancer is the most common malignancy in women, characterized by a distinct pattern of metastasis involving regional lymph nodes, bone marrow, lung and liver.

About 1 million cases of breast cancer are detected each year in the world [1]. Although early stage breast cancers are not life threatening, development of metastatic breast cancer is responsible for the majority of cancer-related death. Metastasis is the result of several sequential steps and represents a highly organized, non-random and organ-selective process [2]. A wide number of molecules such as cytokines, chemokines and their receptors, and growth factors have been implicated to be responsible for the metastatic property of breast cancer cells [3-9]. However, the precise cellular and molecular mechanisms that determine primary tumour growth and the directional migration and invasion of tumour cells into the secondary organs have yet to be established.

Hypoxia is the result of an imbalance between oxygen delivery and oxygen consumption resulting in the reduction of oxygen tension below the normal level for a specific tissue [10]. Oxygen tensions have been measured in several cancer types, using eppendorf histography electrodes showing a range of values between 0 and 20 mmHg in tumour tissues, which were significantly lower than those of the adjacent tissue (24– 66 mmHg) [11-13]. In breast cancers of stages T1b-T4, measurement of oxygen tension revealed a median pO₂ of 28 mmHg compared with 65 mmHg in normal breast tissue [14].

Hypoxia in solid tumours like breast cancer is felt to be due to the tumour outgrowing the existing vasculature. Under these hypoxic conditions, numerous cellular mechanisms are

compromised and an adaptive response occurs which allows cancer cells to adapt to this hostile environment. This renders them more resistant and with improved ability to survive and even proliferate, promoting tumour development [15].

Hypoxia-inducible factor (HIF) is a transcription factor that responds to changes in available oxygen in the cellular environment [16]. HIF consists of two subunits, a α subunit whose level increases during hypoxia and a β subunit that is constitutively expressed [17]. Although HIF-1 α expression may also be influenced by other pathways, a significant correlation between oxygen tension and HIF-1 α has been reported in cervical cancer, suggesting that HIF-1 α might be used as a surrogate for tumour hypoxia [18]. By using HIF-1 α as a marker for hypoxia, approximately 25–40% of all invasive breast cancer samples are hypoxic; the frequency of HIF-1 α -positive cells increases in parallel with increasing pathologic stage and is associated with a poor prognosis [19-21].

Clear-cell renal cell carcinoma (ccRCC), the most frequent subtype of renal cancer, is characterized by inactivation of the von Hippel–Lindau (VHL) tumour suppressor gene in about 70% of the tumours. The VHL protein binds to HIF and targets it for ubiquitination and degradation. Therefore, loss of VHL in these tumours leads to persistently elevated levels of HIF expression [22]. Loss of VHL function in ccRCC also results in strongly enhanced transcription of the HIF-inducible, G-protein-coupled, CXC motif, chemokine receptor 4 (CXCR4), and its cognate, stromal derived factor1 α (SDF1 α) [22, 23].

CXCR4 is a G-protein coupled receptor that is expressed constitutively in a wide variety of normal tissues, including lymphatic tissues, thymus, brain, spleen, stomach, and small intestine [24]. This receptor is also expressed in normal stem cells from a variety of tissues, including mammary stem cells [25]. The fact that CXCR4 is present in normal mammary stem cells suggests that this molecule may be essential for stem cells that appear to be progenitors of breast carcinoma [26]. Signalling through CXCR4 activates a number of downstream effector molecules, including molecules that regulate key processes such as cell cycle control and apoptosis. The chemokine SDF1 α is also expressed constitutively in a variety of tissues, including lung, liver, lymph nodes, bone marrow, and adrenal glands [24, 27, 28]. Of particular relevance to breast cancer, many of the organs with highest expression levels of SDF1 α correlate with common sites of metastatic breast cancer, such as bone, liver lung and lymph nodes. Muller et al [9] investigated functions of chemokines and chemokine receptors in breast cancer. These investigators found that high levels of SDF1 α are produced in many organs and tissues commonly affected by metastatic breast cancer, while CXCR4 appears to be expressed in human breast cancer cells and metastatic lesions. CXCR4 signalling in response to SDF1 α induces chemotaxis and migration of breast cancer cells. In a mouse model of breast cancer, neutralizing antibodies to CXCR4 significantly limited metastases to lymph nodes and lung [9]. Their data were the first to identify a key function for SDF1 α –CXCR4 in metastatic breast cancer. Our hypothesis was that the hypoxia commonly found in primary breast cancers would induce an increased expression of CXCR4, and that this increase in CXCR4 expression would increase breast cancer cells metastatic ability.

Methods

Cell lines and cultures

MCF7 and MDA-MB-231, two human breast cancer cell lines, and 4T1, a murine breast cancer cell line, were maintained in Dulbecco-modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and glutamine (2 mM). Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. The cells in hypoxic experiments were exposed to 2% O₂ with 5% CO₂ at 37°C for 48 hrs. All culture medium and reagents for cell cultures were purchased from Invitrogen Life Technologies (Paisley, Scotland, UK).

Cell surface expression of CXCR4

After being cultured in either normoxic condition or hypoxic condition for 48 hrs, cells were stained with PE-conjugated anti-CXCR4 mAb (FAB170P, Clone 12G5) (R&D Systems, Minneapolis, MN). PE-conjugated mouse IgG_{2a} isotype-matched mAb (IC003P, R&D Systems) was used as the negative control. FACSscan analysis was performed from at least 5,000 events for detecting cell surface expression of CXCR4 using CellQuest software (BD Biosciences, San Jose, CA).

Tumour cell migration and invasion assay

Tumour cell migration through a microporous membrane and invasion through an extracellular matrix were assessed based on the Boyden chamber principle using the fluorescent CyQuant GR dye (Chemicon, Temecula, CA). Cells were incubated with

culture medium as the control, 10 µg/ml control IgG_{2b} mAb (MAB004, R&D Systems), 10 µg/ml CXCR4 blocking mAb (MAB172, R&D Systems), or 100 nM CXCR4 antagonist AMD3100 (Sigma-Aldrich, St. Louise, MO) for 1 hr, and then plated onto the top chamber. Culture medium containing 100 ng/ml of either recombinant mouse SDF1α (460-SD, R&D Systems) or recombinant human SDF1α (350-NS, R&D Systems) was added into the lower chamber. The plate was incubated at 37°C, 5% CO₂ for 18 hrs. The migrated or invaded cells were dislodged from the underside of the chamber using a detachment solution. A lysis buffer and dye solution was added and the plate was read with a fluorescence plate reader using a 480/520 nm filter set (Thermo Labsystems, Cheshire, England, UK).

Cell viability

Cell viability was assessed by measuring the ability of cells to metabolize MTT (Sigma-Aldrich), a water-soluble tetrazolium salt, into a water insoluble formazan product. In brief, tumour cells (5x10³/well) were plated into 96-well plates at 37°C, 5% CO₂ for 12 hrs. Cells were then treated with culture medium, 10 µg/ml control IgG_{2b} mAb (MAB004, R&D Systems), 10 µg/ml CXCR4 blocking mAb (MAB172, R&D Systems), or 100 nM AMD3100 (Sigma-Aldrich) at varying doses. After 24 hrs, 50 µl of 1 mg/ml of MTT solution was added to each well and incubated for 3 hrs. The supernatant was then gently removed and 100 µl DMSO (Sigma-Aldrich) added to each well. The OD value in each well was measured with a micro-plate reader (Dynatech Laboratories, Chantilly, VA) at absorption wavelength of 570 nm with a reference wavelength of 650 nm.

Statistical analysis

All data are presented as the mean \pm SD. The significance of the difference was analysed with a two-sided Student's *t* test, and a *p* value < 0.05 was considered statistically significant.

Results

Hypoxia facilitates breast cancer cell migration and invasion

To examine the effect of hypoxia on breast cancer cell metastatic ability, two human breast cancer cell lines MCF7 and MDA-MB-231, and one murine breast cancer cell line 4T1 were exposed to either normoxia or hypoxia for 48 hrs. Breast cancer cell migration through a microporous membrane and invasion through an extracellular matrix were assessed under both normoxic and hypoxic conditions. It was observed that exposure of these three breast cancer cell lines to hypoxic conditions resulted in significantly increased migration (Figure 1A) and invasion (Figure 1B) in vitro.

Following confirmation of increased metastatic ability in hypoxia-treated breast cancer cells, we wished to investigate whether CXCR4 played a role in this increased capacity, as CXCR4 has been implicated in breast cancer metastases and linked to tumours with increased levels of HIF1 α (a surrogate of hypoxia). We again treated cells to the same hypoxic and normoxic conditions and assessed cell surface expression of CXCR4 by FACSscan analysis. Exposure of two human breast cancer cell lines MDA-MB 231 and MCF7 to hypoxia led to a statistically significant increase in cell surface expression of CXCR4 by 27.5% ($p < 0.05$) and 67.5% ($p < 0.05$), respectively, when compared to cells exposed to normoxia (Figure 2 A-C).

Blocking CXCR4 attenuates breast cancer cell migration and invasion

To determine if the increased metastatic potential seen following hypoxia treatment was associated with the increased cell surface expression of CXCR4, we repeated the

migration and invasion experiments in two human breast cancer cells lines, MDA-MB 231 and MCF7. Cells were exposed to either 48 hrs of the same normoxic or hypoxic conditions. Before assessment of cell migration and invasion, both MDA-MB-231 and MCF7 cells were incubated with either a CXCR4 blocking mAb, MAB172 (10 µg/ml), or a CXCR4 antagonist, AMD3100 (100 nM) for 1 hr. Culture medium and control IgG_{2b} mAb, MAB004 (10 µg/ml) were used as the control. It was observed that attenuation of CXCR4 with either the blocking mAb, MAB172 or the antagonist, AMD3100 led to statistically significant attenuation of breast cancer cell migration in response to SDF1α in MCF7 (Figure 3A) and MDA-MB-231 (data not shown) cells. This effect was not seen with the isotype control mAb.

Similarly, CXCR4 inhibition with the CXCR4 blocking mAb led to statistically significant attenuation of cell invasion through an extracellular matrix in response to SDF1α observed in hypoxia-treated MCF7 (Figure 3B) and MDA-MB-231 (data not shown) cells. The CXCR4 antagonist, AMD3100 was also observed to have a similar effect as the CXCR4 blocking mAb with attenuation of extracellular matrix invasion of hypoxia-treated MCF7 (Figure 3B) and MDA-MB-231 (data not shown) cells.

To assure that the effects of CXCR4 attenuation on breast cancer cell migration and invasion was not due to decreased cell viability resulted from the treatment with the CXCR4 blocking mAb or the CXCR4 antagonist; we assessed the effect of CXCR4 blockade on cell viability with an MTT assay. AMD3100 was tested at varying concentrations from 0.5 to 100 nM and the blocking mAb was tested at concentrations

from 2.5 to 20 $\mu\text{g/ml}$. As shown in Figure 4A and 4B, treatment of breast cancer cells with AMD3100, the blocking mAb or the isotype control mAb did not significantly affect cell viability *in vitro*. Lower doses of CXCR4 blockade seemed to have a mild proliferative effect and this was significant at a dose of 5 nM of AMD and at a dose of 2.5 $\mu\text{g/ml}$ of the blocking mAb. The higher dose of 20 $\mu\text{g/ml}$ of the mAb did show a significant negative effect on cell viability, but the treatment dose of 10 $\mu\text{g/ml}$ did not.

Discussion

It has been proposed that molecules regulating the metastatic dissemination of tumour cells in an organ specific manner would need to fulfil certain criteria [2, 29]. They would have to be constitutively expressed in the target organs of metastasis. Adhesion of the circulating tumour cells to the endothelium and their transendothelial migration would need to be promoted. These molecules would need to be capable of mediating the invasion of tumour cells into tissues that would provide supportive microenvironments. Lastly, this process would require the expression of a distinct receptor repertoire by the target cells, depending on their metastatic profile. Given their well-established roles in leukocyte trafficking and homeostasis, chemokines are perfectly positioned to fulfil these criteria [30-37].

CXCR4 with its unique ligand SDF1 α has been and continues to be a source of investigation into the organ specific metastases of several types of cancer including breast cancer. Muller et al [9] were the first to investigate the functions of chemokines and chemokine receptors in breast cancer. They found that high levels of SDF1 α are produced in many organs and tissues commonly affected by metastatic breast cancer, while CXCR4 appears to be expressed in human breast cancer cells and metastatic lesions. CXCR4 signalling in response to SDF1 α was found to induce chemotaxis and migration of breast cancer cells. These data were the first to elucidate key roles for the CXCR4-SDF1 α receptor-ligand axis in metastatic breast cancer. The hypothesis now proposed holds that organ-specific attractant molecules such as SDF1 α stimulate the migrating tumour cells to invade the walls in blood vessels and enter the target organs.

This theory is widely known as “chemoattraction” theory which is similar with Paget’s original paper looking at the site specific pattern of breast cancer metastases proposing “seed and soil theory” [38].

Hypoxia is a primary factor in the pathology of many disease states, including solid tumours [12-14]. In this paper we propose that in solid tumours such as breast cancer, in addition to genetic alterations such as mutations of VHL [22], PTEN [39], or p53 [40] that are associated with increased levels of HIF1 transcriptional activity, tumour microenvironmental hypoxia may increase CXCR4 expression and thus the metastatic potential of cancer cells. The results in this paper support other findings in the literature that suggest that hypoxia plays a key role in CXCR4 expression. Schioppa T et al [41] also looked at the role of oxygen levels on CXCR4 expression. They found that low oxygen concentration induced high expression of SDF1 α and CXCR4, in different cell types (monocytes, monocyte-derived macrophages, tumour-associated macrophages, endothelial cells, and cancer cells). Our data support these findings showing that breast cancer cells are sensitive to the microenvironment, in particular oxygen levels which can lead to upregulation of CXCR4 cell surface expression.

We further demonstrated that upregulation of CXCR4 in breast cancer cells secondary to hypoxia is associated with increased chemotactic ability and metastatic potential, and that this effect can be abrogated by CXCR4 attenuation. This finding may indicate a potential therapeutic role for CXCR4 inhibition. Certainly, in vivo studies [9, 42] have confirmed this theory with CXCR4 inhibition in mouse models of cancer showing decreased

metastases. Immunohistochemistry studies of primary breast tumours have also demonstrated a relationship between CXCR4 expression and metastases [43-47]. Tumours with high expression of CXCR4 are associated with more aggressive phenotypes showing increased rates of distant metastases and poorer clinical outcome. Organ-specific metastases to liver [46] and bone marrow [47] have been shown again consistent with the “chemoattraction” theory. Further reason to believe that interference with the CXCR4-SDF1 α receptor-ligand axis could be of potential therapeutic benefit in the treatment or prevention of breast cancer metastases.

The perioperative period may afford a window of opportunity for treatment using interference of the CXCR4-SDF1 α axis. There are many factors during the perioperative period that are thought to affect tumour development [48]. One of these is the possibility of increased circulating tumour cells postoperatively [49, 50]. Therefore, we would propose that treatment with CXCR4 antagonists such as AMD3100 in the perioperative period would prevent the migration and invasion of CXCR4 expressing breast cancer cells into organs of metastases that are rich in SDF1 α . AMD3100 is safe, in December 2008, Plerixafor, the new trade name for AMD3100, was approved by the FDA of United States for use in patients with non-Hodgkin’s lymphoma and multiple myeloma [51]. It is used in combination with granulocyte colony-stimulating factor in these patients to mobilize haematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation.

Conclusion

In summary, CXCR4-SDF1 α receptor-ligand axis plays an important role in the metastatic ability of breast cancer cells. CXCR4 is expressed on breast cancer cells and exposure to hypoxia upregulated this expression. From the literature we know that hypoxic breast cancer primary tumours correlate with later stage tumours and metastases and are associated with patients of poor prognosis. We propose that one of the mechanisms underlying this increased metastatic ability and poorer prognosis is that tumour hypoxia through upregulation of CXCR4 cell surface expression leads to increased metastatic potential of breast cancer cells. We would also suggest that further work in this area should look at utilising the perioperative window as the possible timing of interference in this axis as a potential metastases preventative strategy.

Competing Interests

The authors declare that they have no competing interests.

Contributing Authors

PAC and JHW carried out the experiments described in the study while the study was designed and analysed by PAC, JHW and HPR. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. Migration of breast cancer cells through a microporus membrane (A) and invasion through an extracellular matrix (B) in response to SDF1 α in normoxia and hypoxia conditions.

Three cell lines MDA-MB-231, MCF7, and 4T1 cells were exposed to either normoxia or hypoxia (2% O₂ with 5% CO₂) for 48 hrs. Cell migration and invasion were assessed as described in the Methods and expressed as relative fluorescence units (RFU). Data are presented as the mean \pm SD of duplicate samples and are representative of three independent experiments. *p<0.05 versus normoxia-treated cells.

Figure 2. Cell surface expression of CXCR4 in normoxia and hypoxia treated MDA-MB-231 (A) and MCF7 (B) cells.

Human breast cancer cell lines, MDA-MB-231 and MCF7 cells were exposed to either normoxia or hypoxia (2% O₂ with 5% CO₂) for 48 hrs. Cell surface expression of CXCR4 on breast cancer cells was assessed by FACScan analysis, and expressed as mean channel fluorescence (MCF). (A-B) Results shown represent one experiment from a total of three independent experiments. The grey filled indicates CXCR4 expression on normoxia-treated cells, whereas the grey line indicates CXCR4 expression on hypoxia-treated cells. (C) Data are expressed as the mean \pm SD of three separate experiments. *p<0.05 versus normoxia-treated cells.

Figure 3. The effect of CXCR4 inhibition on cell migration (A) and invasion (B) in hypoxia-treated breast cancer cells.

Human breast cancer cell line, MCF7 cells were exposed to hypoxia (2% O₂ with 5% CO₂) for 48 hrs, and further incubated with either a CXCR4 blocking mAb, MAB172 (mAb, 10 µg/ml), or a CXCR4 antagonist, AMD3100 (AMD, 100 nM) for 1 hr. Culture medium (CM) and control IgG_{2b} mAb, MAB004 (IgG, 10 µg/ml) were used as the control. Cell migration and invasion were assessed as described in the Methods and expressed as (relative fluorescence units RFU). Data are presented as the mean ± SD of duplicate samples and are representative of three independent experiments. *p<0.05 versus CM or IgG treated cells.

Figure 4. Cell viability of MCF7 breast cancer cells following treatment with either AMD3100 or the CXCR4 blocking mAb.

Cells were treated with (A) CXCR4 antagonist AMD3100 (0.5 - 100 nM) and (B) the blocking mAb, MAB172 (2.5 - 20 µg/ml) for 24 hrs at 5% CO₂ at 37°C. Control IgG_{2b} mAb, MAB004 (10 µg/ml) was used as the control. Data are expressed as the mean ± SD of duplicate samples and are representative of three independent experiments. *p<0.05 versus (A) culture medium (CM) or (B) control mAb (IgG) treated cells.

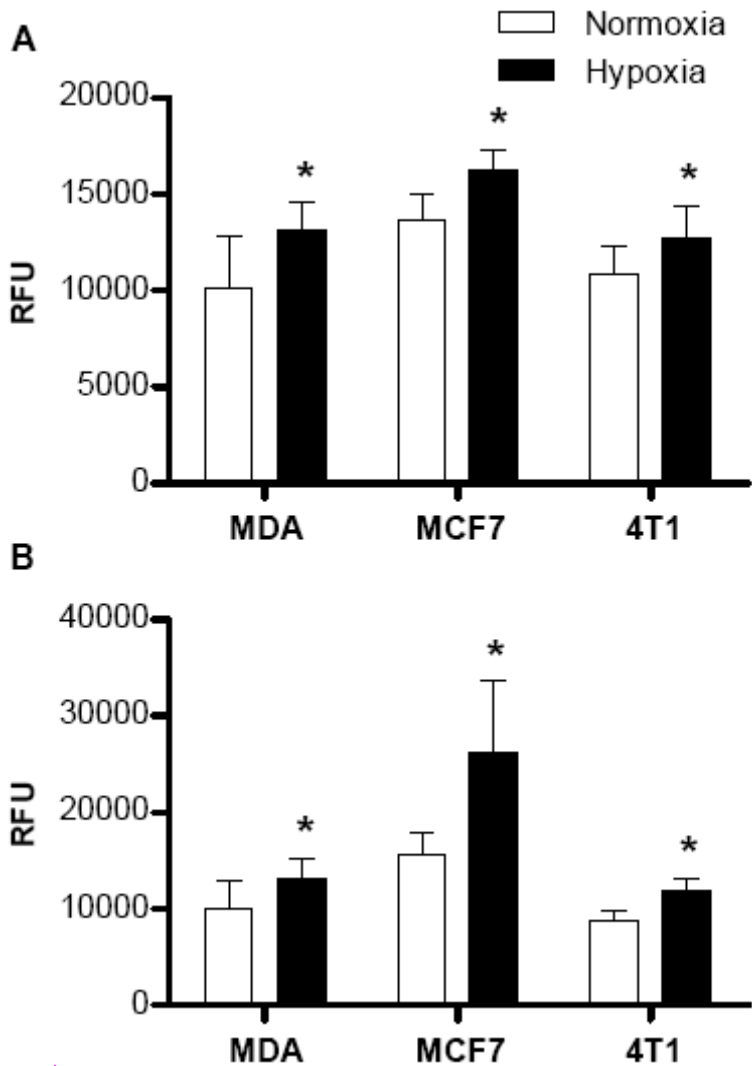


Figure 1

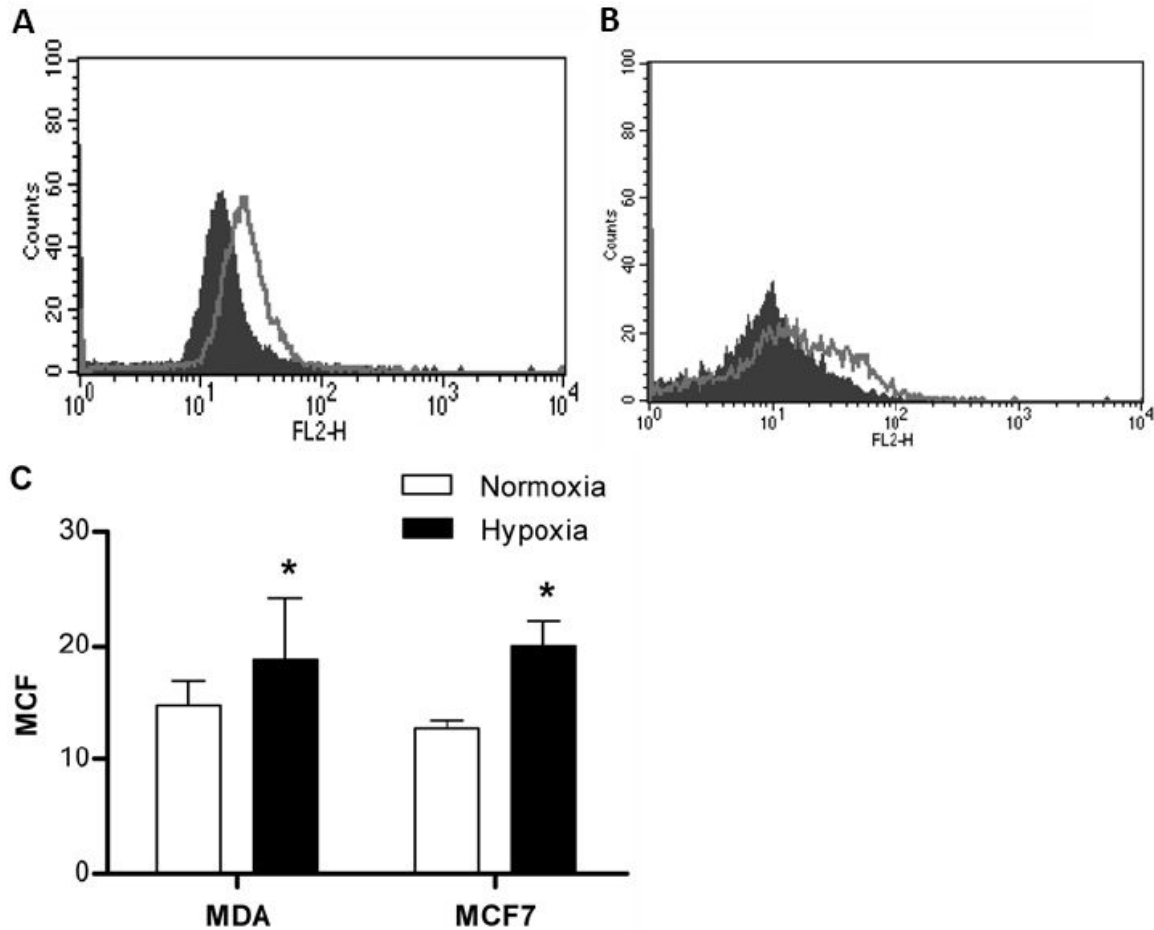


Figure 2

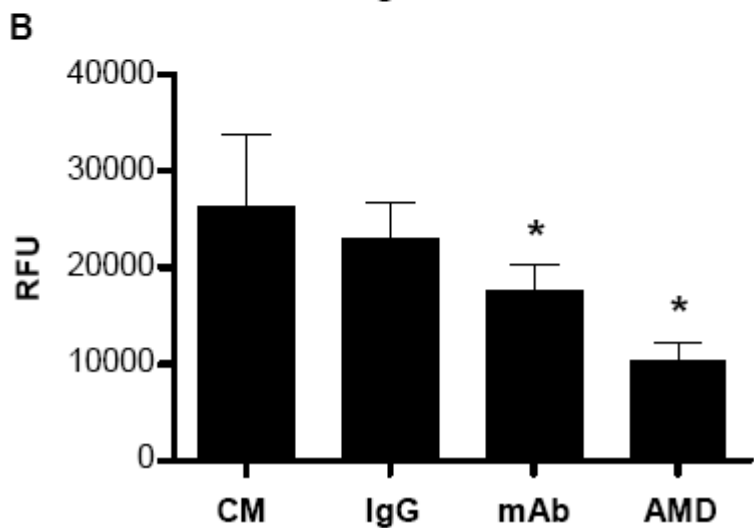
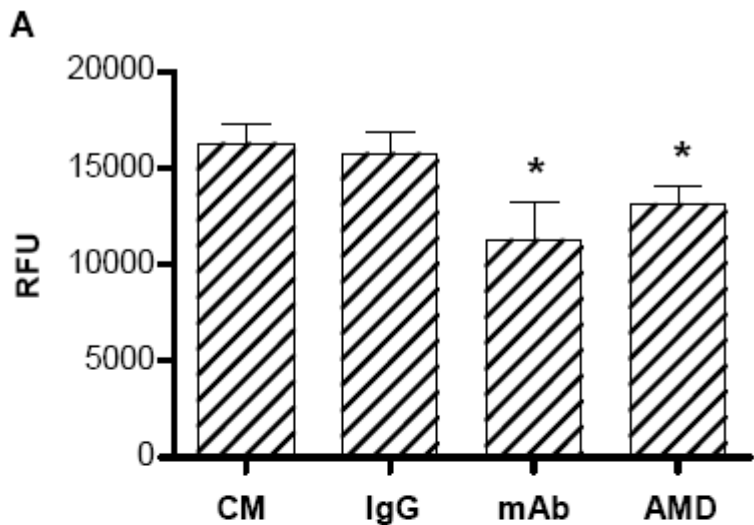


Figure 3

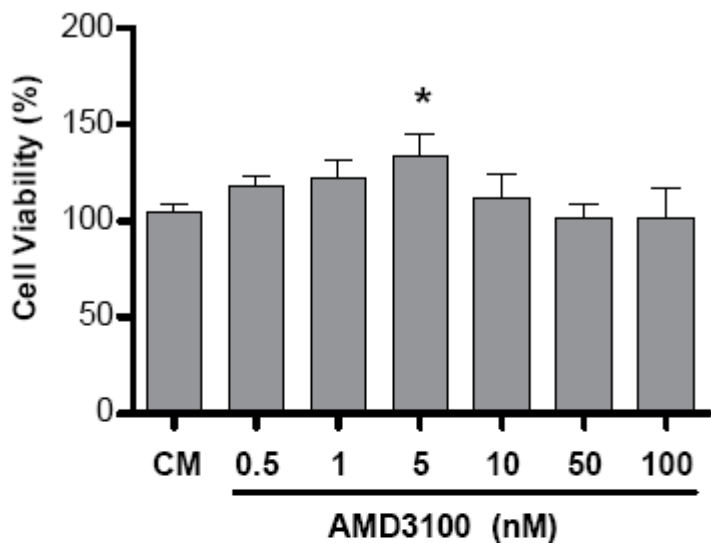
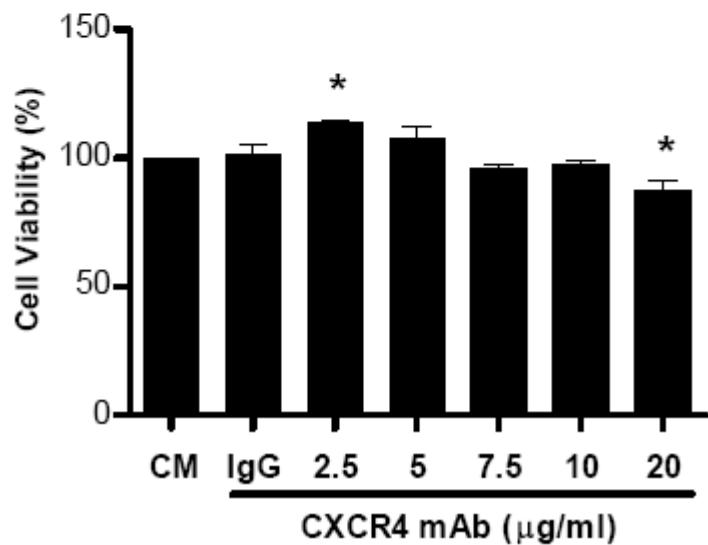
A**B**

Figure 4