Exposure to 60% oxygen promotes migration and upregulates angiogenesis factor secretion in breast cancer cells

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Abstract

Peri-operative factors, including anaesthetic drugs and techniques, may affect cancer cell biology and clinical recurrence. In breast cancer cells, we demonstrated that sevoflurane promotes migration and angiogenesis in high fractional oxygen but not in air. Follow-up analysis of the peri-operative oxygen fraction trial found an association between high inspired oxygen during cancer surgery and reduced tumor-free survival. Here we evaluated effects of acute, high oxygen exposure on breast cancer cell viability, migration and secretion of angiogenesis factors *in vitro*. MDA-MB-231and MCF-7 breast cancer cells were exposed to 21%, 30%, 60%, or 80% v/v O_2 for 3 hours. Cell viability at 24 hours was determined by MTT and migration at 24 hours with the Oris™ Cell Migration Assay. Secretion of angiogenesis factors at 24 hours was measured *via* membrane-based immunoarray. Exposure to 30%, 60% or 80% oxygen did not affect cell viability. Migration of MDA-MB-231 and MCF-7 cells was increased by 60% oxygen ($P = 0.012$ and $P = 0.007$, respectively) while 30% oxygen increased migration in MCF-7 cells ($P = 0.011$). These effects were reversed by dimethyloxaloylglycine. In MDA-MB-231 cells high fractional oxygen increased secretion of angiogenesis factors monocyte chemotactic protein 1, regulated on activation normal T-cell expressed and vascular endothelial growth factor. In MCF-7 cells, interleukin-8, angiogenin and vascular endothelial growth factor secretion was significantly increased by high fractional oxygen. High oxygen exposure stimulates migration and secretion of angiogenesis factors in breast cancer cells *in vitro*.

Key words: breast cancer; oxygen; angiogenesis; metastasis; anaesthesia; hyperoxia; migration; viability

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Introduction

Breast cancer is the leading cause of cancer and cancer-related mortality among women in Europe.[1] Surgical resection of the tumor is the primary mode of treatment and post-surgical metastases account for breast cancer associated morbidity and mortality.[2] It has been hypothesised that peri-operative factors, such as anaesthesia, may influence the occurrence of these metastases, affecting the immune response and tumor cell dissemination and growth.[3,4]

Supplemental oxygen is commonly used during general anaesthesia to offset any impairment of cardiac or pulmonary function.[5] It has been suggested that high levels of inspired oxygen may decrease the incidence of post-operative wound infection.[6-8] However, this effect has not been replicated in other clinical trials and a recent Cochrane review concluded that there is no evidence that high inspired oxygen during surgery prevents surgical site infection.[9,10] Conversely, there is a growing concern about the overuse of

high inspired oxygen in emergency settings, with hyperoxia having been linked to increased mortality and morbidity in cardiac arrest,[11] pulmonary disease,[12] and stroke.[13]

Routine anaesthetic practice often combines volatile general anaesthetic agents with high inspired oxygen (FiO_2) during cancer surgery.[14] Hyperoxia and its consequent cellular damage have been suggested to increase levels of angiogenesis factors.[15] Hyperbaric oxygen (HBO) has been shown to have no cancer promoting effect in many cancers; however in breast cancer HBO has been shown to inhibit cell proliferation.[16,17] Conversely, without the use of HBO, a follow-up analysis of the peri-operative oxygen fraction (PROXI) trial, found an association between high FiO_2 in the peri-operative period and reduced tumor free survival in abdominal laparotomy patients.[18] We recently observed that sevoflurane, when delivered in a high oxygen content, significantly increased cell migration in human breast adenocarcinoma cells, compared with normal oxygen concentrations.[19] If this effect was replicated clinically, high FiO_2 in the peri-operative period might facilitate tumor cell migration and metastases independent of anaesthesia *per se*.

The objective of this study was to investigate directly the effect of different concentrations of oxygen on migration and secretion of angiogenesis factors in oestrogen receptor positive (ER^+) MCF-7 and oestrogen receptor negative (ER^-) MDA-MB-231 breast cancer cells *in vitro*.

Materials and Methods

Cell lines

MDA-MB-231 is an ER^- human breast adenocarcinoma cell line. MCF-7 is an ER^+ and progesterone-receptor positive human breast adenocarcinoma cell line. MDA-MB-231 cells were routinely cultured in L-Liebowitz 15 medium supplemented with 15% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin solution, while MCF-7 cells were routinely cultured in minimum essential medium eagle with Earle's salts and sodium bicarbonate, supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin-streptomycin solution. Both cell lines were grown in T175 flasks (CellStar filter cap flask) at 37°C in a humidified atmosphere containing 5% CO_2 and were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

Oxygen

Four concentrations of oxygen were used in this study 21%, 30%, 60%, and 80% (v/v) O_2. Medical air (21% v/v O_2, 79% v/v N_2) was used as control and 100% O_2 was added to medical air until the oxygen content equalled the desired concentration. Oxygen concentration was monitored using a Hewlett Packard HP M1026A Anaesthesia Airway Gas Monitor (Hewlett Packard, Palo Alto, CA, USA). All gases were supplied by BOC Industrial Gases (Dublin, Ireland).

Gas exposure

Cells were exposed to gas in a hermetic chamber (Enzyscreen BV, Heemstede, Netherlands), of which there were two to allow for concurrent gas exposure with experimental or control gas. Before introducing the cells to the chamber, cell line-appropriate FBS-free medium was enriched with the experimental gases (30%, 60%, or 80% O_2 or medical air) using a glass gas wash bottle (Dreschel pattern) at a flow rate of 1–2 L/min for 20 minutes, adjusted to lowest flow that maintained bubbling through the filter disc. The removal of the FBS during this procedure was to prevent frothing. Separate gas wash bottles were used for each cell line, and gas wash bottles were cleaned prior to the next gas enrichment. FBS in cell line-appropriate concentrations was subsequently added to the enriched media for the migration studies outlined below.

Non-treated medium was decanted from the cell cultures, the cells were washed with phosphate buffered saline (PBS) and enriched media was introduced (appropriate to the cell lines). The plates were introduced to the chamber and the atmosphere of the chamber was replaced by the desired experimental gas mixture using a flow rate of 15–20 L/min for 3 minutes. Where indicated, a paired medical air plate and chamber were similarly prepared. The chambers were sealed prior to closure of gas supplies. The hermetic chambers were then introduced into an incubator for a 3-hour period at 37°C. The gas-exposed cultures (experimental or control) were removed from the chambers and returned to an incubator at 37°C in 5% CO_2 and allowed to grow for 24 to 48 hours, before analysis of their viability, migration or angiogenesis properties, as outlined below.

Dimethyloxaloylglycine (DMOG) exposure

DMOG, a powerful inhibitor of 2-oxoglutarate-dependent dioxygenases,[20] was used to artificially impose hypoxic conditions on the cells to reverse the effects of oxygen exposure. DMOG was dissolved in water and 1% of 100 mM stock solution was added to the enriched media during cell seeding, to achieve a final concentration of 1 mM DMOG. Water (1% v/v) was added to vehicle-treated wells as a control. Migration and viability experiments conducted with DMOG were carried out in 60% O_2 and medical air as this is where the greatest migratory effect was seen in our

migration experiments in both cell lines.

Evaluation of cell viability

The MTT assay was used to determine effects on cell viability. This assay detects living, but not dead, cells and measures the reduction of the cell permeable yellow salt, MTT, to non-permeable purple formazan crystals by active mitochondrial dehydrogenases in viable cells.[21] Cells were seeded into 96-well plates at a density of 2×10^4 cells/cm^2 and allowed to adhere overnight. Plates were placed in the hermetically sealed chambers and gas exposure was conducted as described above and viability was determined at 24 hours after gas exposure was complete.

MTT solution was prepared by adding 0.5 mg/L MTT to cell line-appropriate FBS-containing media. Experimentally-enriched media was removed from the 96-well plate and a 100-µL aliquot of MTT solution was added to each of the wells and allowed to incubate for 3 hours at 37°C in 5% CO_2. The solution was removed from the wells and the formazan crystals were solubilised by the addition of 100 µL of dimethyl sulphoxide (DMSO), after which the plate was left for 20 minutes on a rocker. The absorbance of each well was read at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Viability was expressed as a percentage of control.

Migration assay

The effect of experimental gases on breast cancer cell migration on a collagen substrate was determined using the Oris™ Cell Migration Assay (Platypus Technologies, Madison, WI, USA), according to the manufacturer's protocol. This assay involved measuring the migration of cells into an exclusion zone andis superior to the scratch assay, as it does not involve injury to the cells or the extracellular matrix.

Oris™ Cell Migration Assembly Kit-FLEX 96-well plates were prepared by coating each well with 7–8 µg/cm^2 rat tail collagen I (Sigma, Dublin, Ireland). Cells grown to 90–99% confluence in T75 flasks were removed by trypsinisation, resuspended in appropriate medium, and seeded into collagen-coated plates. Each separate and independent experiment employed two 96-well plates, one each for medical air and oxygen (30%, 60%, or 80% O_2). These two plates were seeded at the same time with the same passages of cells. Each well had an Oris™ cell seeding stopper in situ to restrict cell seeding to the outer regions of the well. The plate was seeded with a concentration of 100,000 cells in 100 µL appropriate medium per well. The seeded plates were incubated overnight at 37°C in 5% CO_2 to allow cell attachment.

For each cell line, stoppers for the experimental wells were then removed to create a detection zone of 2-mm diameter into which cells could migrate. This was done immediately prior to placement into the hermetically sealed chambers as described in the gas exposure protocol. Oxygen and paired medical air control plates were exposed to gas as described above. After each gas exposure, the 96-well plates were incubated for 24 hours at 37ºC in 5% CO_2 to allow time for migration. Following this incubation period, the stoppers were removed from the negative control wells immediately prior to cell staining, such that no migration could have occurred in these wells.

Staining and fixation was conducted with Coomassie blue (1% w/v Coomassie blue in 40% v/v methanol, 10% v/v acetic acid). The medium was removed and the wells washed with PBS, following which 100 µL of filtered Coomassie blue was applied for 10 minutes. The stain was carefully decanted and the cells were washed twice with PBS and allowed to dry. Cells migrating into the detection zone were quantified by measuring absorbance using a bottom-reading colorimetric plate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA); using a 570 nm measurement filter. A template mask (Oris™) was used to shield all regions of the wells, other than the 2 mm detection zone. Absorbance values were averaged across the eight experimental wells for each passage to obtain a single experimental absorbance. Background absorbance values were averaged across all negative control wells for each cell line and this value was subtracted from the mean experimental absorbance to obtain a single absorbance value, indicative of cell migration, for each passage of cells. This procedure was repeated for both experimental gas and medical air plates. The results of each migration study are presented as a percentage of medical air migration on parallel, matched plates. Three or four separate and independent experiments were conducted for each experimental gas.

At the end of the migration assay, Coomassie blue-stained cells were solubilised with 100 µL of DMSO and absorption at 570 nm was determined to provide an indication of cell density.

Angiogenesis assay

The presence and potential alteration of, angiogenesis markers in conditioned medium was assessed using the Human Angiogenesis Array C1 (RayBiotech, GA, USA). MDA-MB-231 cells or MCF-7 cells were seeded at a density of 2×10^5 cells/cm^2 in T25 flasks (Greiner, Austria) and grown to ~90% confluency. Medium was removed and the cells were washed with PBS. Two millilitres of gas-enriched medium (30%, 60%, and 80% O_2 or medical air), prepared as per the gas exposure protocol, were added to each flask. The flasks (with loosened caps) were then exposed to the

corresponding gases in the hermetic chambers, as per the gas exposure protocol, for 3 hours. Flasks were then removed, caps tightened and they were incubated for a further 24 hours at 37°C in 5% CO_2. After each exposure period the conditioned medium was removed and frozen at –80°C in a 2-mL cryotube vial (Sigma).

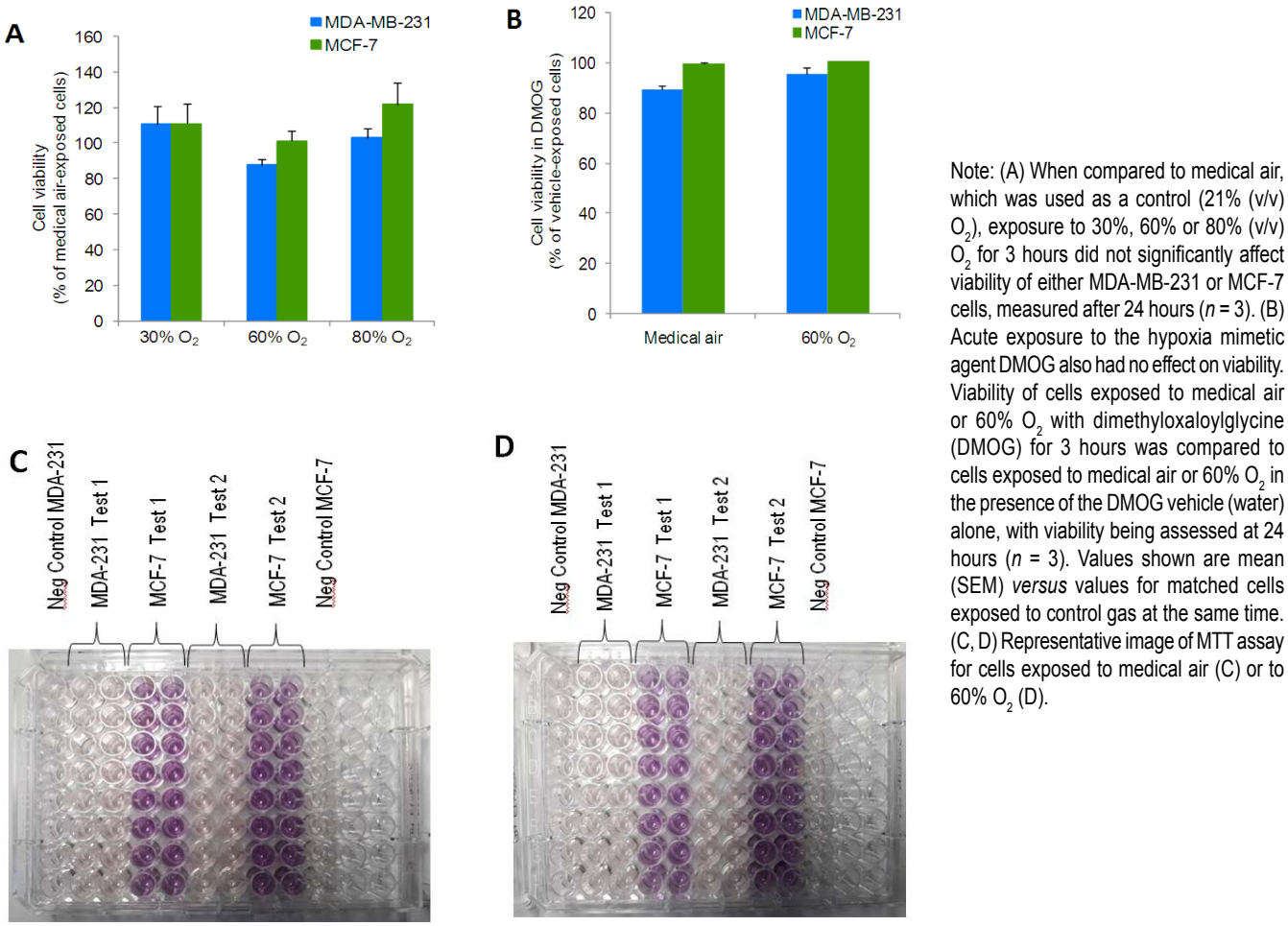
Once samples of all four conditioned media had been collected, they were defrosted and the release of key angiogenesis factors into the medium was determined using the RayBio human angiogenesis array C1 using the manufacturer's protocol. Scanning densitometry was employed to provide a semi-quantitative indication of dot intensity using the GelEval software programme v1.33 (Frogdance Software, Dundee, Scotland). Replicate spot density values for individual angiogenesis factors were then averaged and normalized to the average density of the control dots on the same array to control for array-to-array variations.

Statistical analysis

Cell viability and migration in oxygen-exposed cells are summarized as percentage values relative to medical air. For the DMOG study, values for DMOG-exposed cells were compared to vehicle-treated cells with identical gas exposures (either medical air or 60% O_2). Data was analysed using Prism v6 (Graphpad Software, La Jollla, CA, USA). Data are expressed as the mean ± SD (or SEM), and was evaluated for distribution using the Kolmogorov-Smirnov test. Being non-normally distributed, comparisons between groups were analysed using the Kruskal-Wallis test. A value of $P \leq 0.05$ was considered significant.

Results

Viability of MCF-7 and MDA-MB-231 cells was not significantly affected by a 3-hour exposure to 30%, 60%, or 80% O_2, as determined by MTT assay, conducted 24 hours after completion of the gas exposure period (**Figure 1A**).



Note: (A) When compared to medical air, which was used as a control (21% (v/v) O_2), exposure to 30%, 60% or 80% (v/v) O_2 for 3 hours did not significantly affect viability of either MDA-MB-231 or MCF-7 cells, measured after 24 hours (n = 3). (B) Acute exposure to the hypoxia mimetic agent DMOG also had no effect on viability. Viability of cells exposed to medical air or 60% O_2 with dimethyloxaloylglycine (DMOG) for 3 hours was compared to cells exposed to medical air or 60% O_2 in the presence of the DMOG vehicle (water) alone, with viability being assessed at 24 hours (n = 3). Values shown are mean (SEM) *versus* values for matched cells exposed to control gas at the same time. (C, D) Representative image of MTT assay for cells exposed to medical air (C) or to 60% O_2 (D).

Figure 1: Influence of oxygen and dimethyloxaloylglycine (DMOG) on cell viability in MCF-7 and MDA-MB-231 breast adenocarcinoma cell lines, as determined by MTT assay.

Similarly, co-exposure to 1 mM DMOG did not significantly affect viability in either 60% oxygen or in medical air (**Figure 1B**).

The influence of an acute oxygen exposure (3 hours) on subsequent migration of breast cancer cells was determined by measuring migration into the detection zone 24 hours following completion of the gas exposure protocol. A 3-hour exposure to 30% O_2 increased migration of MCF-7 cells by 24% (n = 6, P = 0.01) relative to migration in medical air. Similarly, when exposed to 60% O_2, migration of both MDA-MB-231 and MCF-7 cells was significantly increased relative to medical air by 21% and 54%, respectively (P = 0.01 and P = 0.007). When exposed to 80% oxygen, no increase in cell migration was observed relative to medical air in either cell line (**Figure 2**). Addition of 1 mM DMOG affected the migration of MDA-MB-231 and MCF-7 cells in both medical air and 60% O_2. Migration in wells containing DMOG was decreased by 30–40% when compared to wells without DMOG for both cell lines (**Figure 3A**). To confirm that this decreased cell migration did not reflect changes in overall cell density in the wells, the Coomassie blue-stained cells, used for the migration study, were solubilised with DMSO and absorption was measured. This confirmed that cell density was identical in DMOG-exposed and vehicle-exposed wells (**Figure 3B**). Nine angiogenesis factors were detected in conditioned medium harvested from MDA-MB-231 cells at 24 hours, following a 3-hour exposure to varying concentrations of oxygen (**Table 1**, **Figure 4A**). Relative to medical air, secretion of the pro-angiogenic cytokines– regulated on activation normal T cell expressed (RANTES) and vascular endothelial growth factor (VEGF)–was increased by a 3-hour exposure to 60% O_2. Expression of monocyte chemotactic protein 1 (MCP-1) was increased by 30%, 60% and 80% oxygen relative to medical air. In MCF-7 conditioned medium, secretion of pro-angiogenic interleukins 6 and 8 (IL-6, IL-8) were significantly increased by a 3-hour exposure to 30%

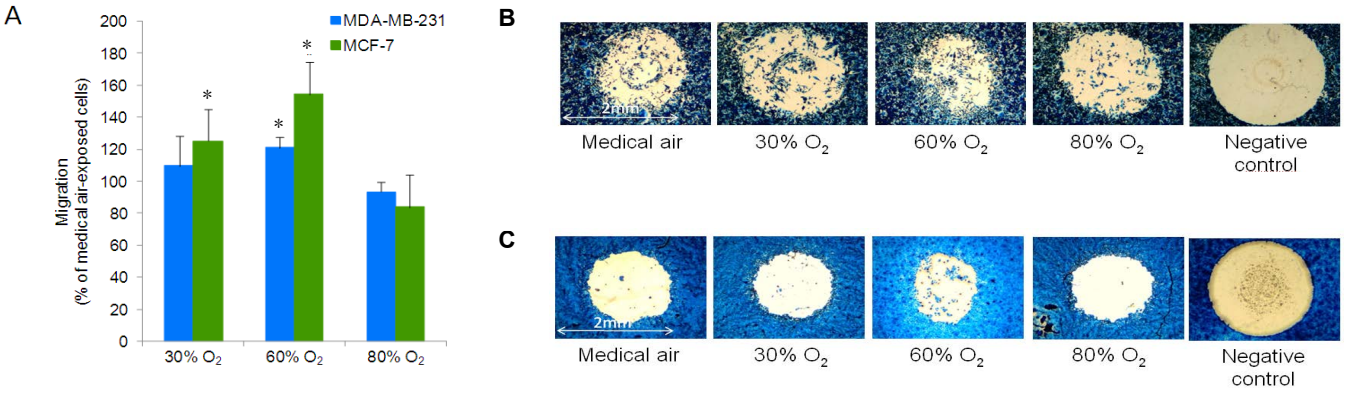
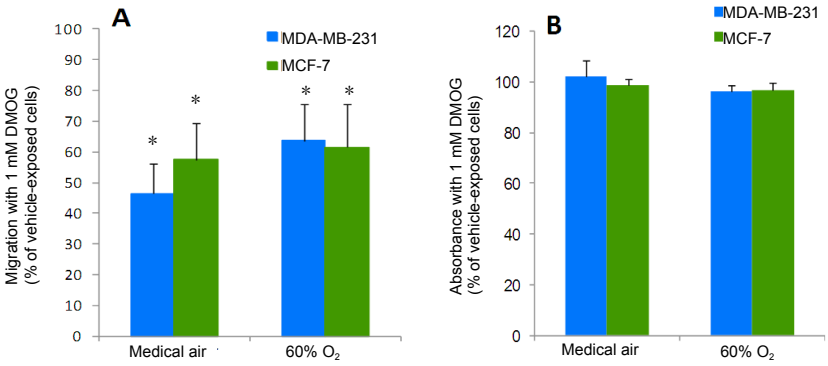


Figure 2: Influence of oxygen on cell migration on a collagen substrate in the MDA-MB-231 and MCF-7 breast adenocarcinoma cell lines, as determined using the Oris™ Cell Migration assay.

Note: (A) Acute (3-hour) exposure of MCF-7 cells to 30% or 60% O_2 significantly enhanced subsequent migration into the exclusion zone; MDA-MB-231 migration was similarly increased by 60% O_2. Values shown are mean (SEM) *versus* values for matched cells exposed to control gas at the same time ($5 \le n \ge 6$). *$P \le 0.05$, *vs.* control gas. (B) Representative images of MDA-MB-231 migration in the Oris™ Cell Migration Assay; from left to right: medical air, 30% O_2, 60% O_2, 80% O_2, and negative control. (C) Representative images of MCF-7 migration in the Oris™ Cell Migration Assay; from left to right medical air, 30% O_2, 60% O_2, 80% O_2, and negative control.



Note: (A) Cells were incubated with either DMOG (1 mM) or a vehicle control (water) and the whole plate was exposed to either 60% O_2 or control gas for 3 hours, following which migration was measured at 24 hours as described above. DMOG reduced migration of both cell lines in control gas and also reduced migration of MCF-7 cells in 60% O_2. (B) Reduced migration did not reflect decreased cell density in the DMOG-exposed wells, as solubilisation of Coomassie blue-stained cells in plates used for migration studies did not reveal any difference in absorbance at 570 nm (n = 3). Values shown are mean (SEM) *versus* values for matched cells exposed to vehicle control at the same time *$P \le 0.05$, *vs.* vehicle control.

Figure 3: Reversal of oxygen-mediated effects on MDA-MB-231 and MCF-7 cell migration by dimethyloxaloylglycine (DMOG).

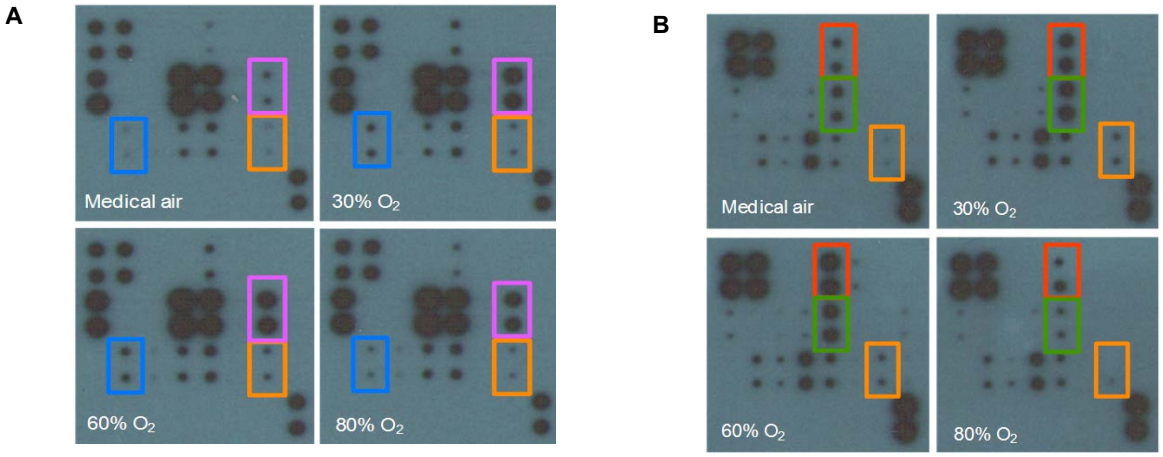


Figure 4: Representative images showing the influence of high fractional oxygen on the secretion of angiogenesis factors from MDA-MB-231 (A) and MCF-7 (B) breast adenocarcinoma cells.

Note: Cells were exposed to medical air, 30%, 60% or 80% O_2 for 3 hours as described in Materials and Methods, following which they were returned to the incubator for 24 hours. Conditioned medium was collected and applied to a commercially available membrane-based angiogenesis array and expression of secreted angiogenesis factors was detected as per the manufacturer's protocol. (A) In comparison to medical air, increased secretion of regulated on activation normal T cell expressed (RANTES) (blue), monocyte chemotactic protein 1 (MCP-1) (pink), and vascular endothelial growth factor (VEGF) (orange) angiogenesis factors were detected in medium from MDA-MB-231 cells exposed to high fractional oxygen. (B) In comparison to medical air, increased secretion of angiogenin (red), interleukin-8 (green), and VEGF (orange) was detected in medium from MCF-7 cells exposed to high fractional oxygen. Semi-quantitative scanning densitometry confirmed these observations (**Table 1**).

Table 1: Influence of different fractional oxygen on the secretion of angiogenesis factors from MDA-MB-231 and MCF-7 breast cancer cells

Angiogenesis factor	Medical air (21% O_2)	30% O_2	60% O_2	80% O_2
MDA-MB-231				
Growth regulated oncogene	156.5±31.2	177.6±3.9	168.7±9.9	168.7±8.6
Regulated on activation normal T cell expressed (RANTES)	9.8±5.1	26.2±3.4	38.0±5.3*	13.2±2.8
Interleukin 6	240.1±12.1	211.6±17.0	211.5±14.8	204.2±10.6
Interleukin 8	210.2±7.7	185.0±14.4	202.9±9.6	190.3±2.9
Angiogenin	30.8±1.9	49.0±25.9	61.1±32.5	35.8±24.0
Tissue inhibitor of metalloproteinase1	49.6±19.9	32.1±10.0	50.4±13.8	50.8±16.6
Tissue inhibitor of metalloproteinase2	52.1±17.2	52.3±10.5	67.1±16.3	62.9±16.2
Monocyte chemotactic protein 1	33.5±4.8	117.6±8.2*	127.8±3.9*	89.7±5.0*
Vascular endothelial growth factor	10.7±1.1	18.0±4.2	25.9±3.3*	14.3±1.9
MCF-7				
Growth regulated oncogene	18.7±8.3	22.0±6.9	25.0±5.3	16.0±6.3
Regulated on activation normal T cell expressed (RANTES)	15.2±0.9	18.4±0.4	21.6±2.8	11.8±1.0
Interleukin 6	13.9±0.3	28.2±1.9*	32.7±3.0*	9.7±1.8
Interleukin 8	79.5±4.7	110.3±1.2*	116.5±0.5*	82.9±3.8
Angiogenin	46.0±2.0	76.5±3.1*	77.3±14.1	31.4±1.1
Tissue inhibitor of metalloproteinase1	60.7±2.5	56.7±4.7	70.5±7.4	47.3±4.9
Tissue inhibitor of metalloproteinase2	51.4±9.6	56.2±18.9	64.2±16.9	34.4±6.3
Monocyte chemotactic protein 1	7.8±0.6	15.5±3.8	13.3±0.2*	0.1±2.6
Vascular endothelial growth factor	13.8±0.2	20.1±6.4	23.3±0.4*	0.8±2.6

Note: Cells were exposed to medical air, 30%, 60% or 80% O_2 for 3 hours, following which they were returned to the incubator for 24 hours. Conditioned medium was collected and applied to a commercially available membrane-based angiogenesis array and expression of secreted angiogenesis factors was detected as per the manufacturer's protocol. Semi-quantitative scanning densitometry was performed using the GelEval software package and values were normalised to positive control dots on each array. Values shown are the mean ± SD, $^*P \leq 0.05$, *vs.* medical air.

and 60% O_2. Angiogenin was increased by 30% O_2, relative to medical air (**Table 1**, **Figure 4B**). Secretion of VEGF and MCP-1 was also significantly upregulated by 60% O_2. There were no other significant differences in cytokine secretion.

Discussion

We have shown that an acute (3-hour) exposure of human breast cancer cells to 30% and 60% O_2, increases cell migration in both ER^+ and ER^- breast cancer cells, compared to cells exposed to medical air. Importantly, this effect was reversed by co-exposure to DMOG, a hypoxia mimetic agent. In addition, secretion of several angiogenesis factors was up-regulated in MCF-7 and MDA-MB-231 cells following acute exposure to high oxygen, when compared to standard medical air. At 80% O_2 migration was not increased unlike at 60% O_2 although viability was not significantly affected. The cause of this was not found in this study.

Mortality associated with breast cancer is due invariably to the burden of metastatic disease, rather than the primary breast tumor. Virtually all patients with breast cancer undergo surgery at some point, be that before, during or after chemotherapy and/or radiation therapy. A number of peri-operative factors are important in minimising metastatic risk during tumor resection, because manipulation of the primary tumor during surgery may result in release of tumor cells into the blood or lymphatic system.[22] Furthermore, undetected micro-metastases may be present in patients who appear to be lymph node-negative at the time of surgery.[23] Although the exposure of cells to oxygen in this experimental model is not the same as in patients in a surgical setting it is difficult to adequately replicate the process of gaseous exchange into the blood stream *in vitro*. This study was carried out to see the effects of oxygen on cancer cells *ex vivo* in order to investigate if there was any direct change on cancer cell behaviour.

Progression of micro-metastases into future disease can be reduced by effective immune surveillance; however peri-operative factors including anaesthetic technique and high FiO_2 have been shown to compromise immune function *in vivo*.[24,25] In a recent study we demonstrated that serum derived from patients who had undergone general anaesthesia with sevoflurane and opioid analgesia for breast tumor resection inhibited the apoptosis of human breast cancer cells *in vitro* when compared with either pre-operative serum or serum from patients anaesthetized with paravertebral anaesthesia and propofol.[26] This suggests that factors secreted into the bloodstream in the peri-operative period may be important in directly affecting micrometastases and thereby dictating the outcome of breast cancer surgery. Furthermore, it suggests that reduced use of volatile agents and/or opioids may have beneficial, anti-metastatic effects. Although volatile anaesthetic agents are routinely delivered in a high oxygen content, this is not necessarily an evidence-based practice. A 3.5-year follow-up of the evaluation of nitrous oxide in the gas mixture for anaesthesia (ENIGMA) trial comparing fractional inspired nitrous oxide FiN_2O = 0.7 with FiO_2 = 0.8 oxygen during non-cardiac surgery lasting more than 2 hours, found no significant difference in long-term mortality.[27] Moreover, concerns have been raised that hyperoxia might contribute to adverse outcome both post-operatively and in emergency settings.[28,29]

We observed that hyperoxia directly increases the migration of two breast cancer cell lines. The hypoxic mimetic agent, DMOG, which is a 2-oxoglutarate-dependent dioxygenase inhibitor[30] that also stabilizes the hypoxia inducible factor (HIF)1α pathway,[31] reversed this effect. At 30% O_2 MCF-7 cell migration was significantly increased while MDA-MB-231 migration was not. At 60% O_2 the migration of both cell lines was significantly increased compared to medical air-exposed controls, although the increased migration of MCF-7 cells appeared more pronounced than MDA-MB-231 cells it is not significantly so. MCF-7 cells are ER^+ and the estrogen receptor has been linked to increased expression of HIF1α.[32] This may account for the observed differences between MCF-7 and MDA-MB-231 cells as MCF-7 cells may express more HIF1α due to ER^+ expression. Yahara et al.[33] found that MDA-MB-231 preferentially migrated along a gradient towards oxygen from a hypoxic environment. This study in theory supports our migratory results however they stated that gradients of other factors such as glucose and metabolites could also have had an effect in their model; which should not be present under the conditions in our study. In the follow-up analysis of the PROXI trial, tumor-free survival time was reduced in patients undergoing abdominal laparotomy who received FiO_2 = 0.8 compared with FiO_2 = 0.3 during surgery and in the immediate post-operative period.[18] There are some limitations to this study; a small number of the control patients received more than FiO_2 = 0.3 in order to maintain arterial oxygen saturation, administration of antibiotics and analgesia varied slightly from patient to patient, and it is possible that a mix of different surgical procedures may have affected results. This is still the only current evidence of an effect of increased oxygen on patient outcome in cancer surgery and due to the large sample size is a useful indicator of a potential ramification of high inspired oxygen. However, there are no prospective, randomized, clinical trials of fractional inspired oxygen in breast cancer. There are obvious difficulties in directly comparing oxygen concentrations *in vitro* and in the clinical setting. In our system, oxygen is not inspired into a human lung and it is therefore possible that

much higher levels of oxygen actually reach the cancer cell than might be expected during tumor resection surgery. At very high levels of oxygen, the production of reactive oxygen species (ROS) might require consideration; however interestingly we did not observe any effects on cell viability in our model of acute oxygen exposure.

Our finding of enhanced migration of breast cancer cells by hyperoxia contrasts somewhat with the accepted view that tumors have evolved to utilize hypoxic stress to their own advantage by activating key pro-metastasic pathways. Intratumoral hypoxia is proposed to drive motility and invasion in many tumor types and increased migration of renal tumor cells by the anesthetic vapor isoflurane *in vitro* has previously been attributed to upregulation of hypoxia inducible factors.[34] The apparent discrepancy may be attributable to differences in migratory behavior between types of tumor cell. It is also likely that our findings are more relevant to circulating tumor cells, specifically those cells released peri-operatively, and these are not necessarily attempting to thrive in the hypoxic conditions of the tumor core. To our knowledge, this is the first study to evaluate directly the effect of different oxygen concentrations alone on isolated cancer cells, whereas previous studies have evaluated normoxia *versus* hypoxia. We adopted this approach in an effort to understand how hyperoxia might directly alter cancer cell behavior.

Metastases require neovascularization to grow at their new sites, and consequently tumor cells secrete angiogenesis factors to recruit hematopoietic cells and drive the production of new vessels.[35] Increased secretion of these factors by cancer cells could promote the survival of micro-metastases and may be a modifiable factor in the peri-operative period. Notably, peri-operative hyperoxia has been shown to promote vascularization *via* an increase in angiogenesis factor secretion in wound healing.[36] In this study, we have observed that hyperoxia can stimulate the release of angiogenesis factors from human breast cancer cells. Interestingly, MCF-7 and MDA-MB-231 cells showed overlapping, but distinct, changes in angiogenic factor secretion when a highly sensitive array-based system was used to detect these factors in conditioned medium. In both cell lines, secretion of VEGF was significantly increased by acute exposure to 60% O_2. Since VEGF is a well established poor prognostic indicator metastatic disease,[37] and may also affect the immune response,[38] our observation suggests that high inspired oxygen may promote angiogenesis *via* a direct effect on VEGF. Vascular endothelial growth factor has also been implicated in the triggering of an autocrine loop in breast cancer cells, thereby promoting migration.[39] The increases in migration seen in our cells may be, at least in part, caused by the up-regulation of VEGF by oxygen exposure. In the ER- MDA-MB-231 cells, MCP-1 and RANTES secretion was also increased as a consequence of hyperoxia. RANTES is increased in several breast cancer subtypes[40] and has been associated with breast cancer progression.[41] We recently showed that RANTES expression is decreased in breast cancer cells following exposure to the noble anaesthetic gas, xenon.[19] MCP-1 is a critical chemo attractant responsible for the recruitment of macrophages and angiogenesis in breast cancer and it may also contribute to indirect crosstalk between the immune system and cancer cells *via* recruitment of tumor-resident macrophages.[42]

Angiogenin and IL-8 secretion was upregulated by hyperoxia in MCF-7 cells. Angiogenin regulates rRNA production during angiogenesis and although early studies found that increases in its production had no bearing on the severity of breast cancer,[43] later findings have indicated that it interacts with members of the plasminogen activation system and that it is positively associated with migration.[44] IL-8 is a pro-inflammatory chemokine, which is associated with pleotropic effects on cancer cell biology. It may promote angiogenic response, increase proliferation and survival of endothelial and cancers cells, and potentiate cancer cell migration.[45] That secretion of IL-8 and angiogenin was increased by high oxygen in MCF-7, but not MDA-MB-231 cells, may further explain differences in migration seen between the two cell lines, as both cytokines are implicated in affecting migration. If these changes in angiogenesis factor secretion were replicated *in vivo* by high fractional oxygen during surgery, they could obviously increase the likelihood that micrometastases survive and ultimately result in disease recurrence.

In conclusion, exposure of both ER^+ and ER^- human breast cancer cells to controlled hyperoxic environments enhanced migration without affecting cell viability. Furthermore, hyperoxia promoted the secretion of several pro-metastatic angiogenesis factors *in vitro*. These results may provide a mechanistic basis for the observation of shorter disease-free survival times following surgery in patients exposed to higher FiO_2 during anesthesia and may have implications for the clinical use of oxygen in surgery to resect breast and other tumors. Prospective *in vivo* and clinical studies are warranted to indicate best practice for oxygen delivery in a surgical setting.

Author contributions

PDC: Primary experimental researcher & data analyser, article author. VS and E O'C: Assisted in experiments and data analysis. SAA: Assisted in experiments and initial design. DJB: Co-supervisor, study design, article editing. HCG: Co-supervisor, data analysis, study design, article editing. All the authors approved the final version of the manuscript for publication.

Conflicts of interest
PDC, VS, EO'C, and SAA: No interest declared. DJB: Received unrestricted research grant funding from Air Liquide, manufacturer of xenon, on the editorial board of BJA. HCG: Received unrestricted research grant funding from Air Liquide, manufacturers of xenon.
Data sharing statement
Datasets analyzed during the current study are available from the corresponding author on reasonable request.
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References

1. European Cancer Observatory. Most Frequent Cancers in Women, 2012. 2012; http://eco.iarc.fr/EUCAN/Country.aspx?ISOCountryCd=930. Accessed 15 January 2015.
2. Rashid OM, Takabe K. Does removal of the primary tumor in metastatic breast cancer improve survival? *J Womens Health (Larchmt)*. 2014;23:184-188.
3. Heaney A, Buggy DJ. Can anaesthetic and analgesic techniques affect cancer recurrence or metastasis? *Br J Anaesth*. 2012;109 Suppl 1:i17-i28.
4. Ash SA, Buggy DJ. Does regional anaesthesia and analgesia or opioid analgesia influence recurrence after primary cancer surgery? An update of available evidence. *Best Pract Res Clin Anaesthesiol*. 2013;27:441-456.
5. Grecu L. Oxygen use in the perioperative period. Should we change our practice? *Analg Resusc*. 2012;1:1-2.
6. Hopf HW, Holm J. Hyperoxia and infection. *Best Pract Res Clin Anaesthesiol*. 2008;22:553-569.
7. Stall A, Paryavi E, Gupta R, Zadnik M, Hui E, O'Toole RV. Perioperative supplemental oxygen to reduce surgical site infection after open fixation of high-risk fractures: a randomized controlled pilot trial. *J Trauma Acute Care Surg*. 2013;75:657-663.
8. Belda FJ, Aguilera L, Garcia de la Asuncion J, et al. Supplemental perioperative oxygen and the risk of surgical wound infection: a randomized controlled trial. *JAMA*. 2005;294:2035-2042.
9. Meyhoff CS, Wetterslev J, Jørgensen LN, et al. Effect of high perioperative oxygen fraction on surgical site infection and pulmonary complications after abdominal surgery: the PROXI randomized clinical trial. *JAMA*. 2009;302:1543-1550.
10. Wetterslev J, Meyhoff CS, Jørgensen LN, Gluud C, Lindschou J, Rasmussen LS. The effects of high perioperative inspiratory oxygen fraction for adult surgical patients. *Cochrane Database Syst Rev*. 2015:CD008884.
11. Kilgannon JH, Jones AE, Shapiro NI, et al. Association between arterial hyperoxia following resuscitation from cardiac arrest and in-hospital mortality. *JAMA*. 2010;303:2165-2171.
12. Austin MA, Wills KE, Blizzard L, Walters EH, Wood-Baker R. Effect of high flow oxygen on mortality in chronic obstructive pulmonary disease patients in prehospital setting: randomised controlled trial. *BMJ*. 2010;341:c5462.
13. Rincon F, Kang J, Maltenfort M, et al. Association between hyperoxia and mortality after stroke: a multicenter cohort study. *Crit Care Med*. 2014;42:387-396.
14. Strachan L, Noble DW. Hypoxia and surgical patients--prevention and treatment of an unnecessary cause of morbidity and mortality. *J R Coll Surg Edinb*. 2001;46:297-302.
15. Hopf HW, Gibson JJ, Angeles AP, et al. Hyperoxia and angiogenesis. *Wound Repair Regen*. 2005;13:558-564.
16. Moen I, Stuhr LE. Hyperbaric oxygen therapy and cancer--a review. *Target Oncol*. 2012;7:233-242.
17. Granowitz EV, Tonomura N, Benson RM, et al. Hyperbaric oxygen inhibits benign and malignant human mammary epithelial cell proliferation. *Anticancer Res*. 2005;25:3833-3842.
18. Meyhoff CS, Jorgensen LN, Wetterslev J, Siersma VD, Rasmussen LS. Risk of new or recurrent cancer after a high perioperative inspiratory oxygen fraction during abdominal surgery. *Br J Anaesth*. 2014;113 Suppl 1:i74-i81.
19. Ash SA, Valchev GI, Looney M, et al. Xenon decreases cell migration and secretion of a pro-angiogenesis factor in breast adenocarcinoma cells: comparison with sevoflurane. *Br J Anaesth*. 2014;113 Suppl 1:i14-21.
20. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science*. 2002;295:858-861.
21. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55-63.
22. Sessler DI. Long-term consequences of anesthetic management. *Anesthesiology*. 2009;111:1-4.
23. Dowlatshahi K, Fan M, Snider HC, Habib FA. Lymph node micrometastases from breast carcinoma: reviewing the dilemma. *Cancer*. 1997;80:1188-1197.
24. Buckley A, McQuaid S, Johnson P, Buggy DJ. Effect of anaesthetic technique on the natural killer cell anti-tumour activity of serum from women undergoing breast cancer surgery: a pilot study. *Br J Anaesth*. 2014;113 Suppl 1:i56-62.
25. Qadan M, Battista C, Gardner SA, Anderson G, Akca O, Polk HC, Jr. Oxygen and surgical site infection: a study of underlying immunologic mechanisms. *Anesthesiology*. 2010;113:369-377.
26. Jaura AI, Flood G, Gallagher HC, Buggy DJ. Differential effects of serum from patients administered distinct anaesthetic techniques on apoptosis in breast cancer cells in vitro: a pilot study. *Br J Anaesth*. 2014;113 Suppl 1:i63-67.
27. Leslie K, Myles PS, Chan MT, et al. Nitrous oxide and long-term morbidity and mortality in the ENIGMA trial. *Anesth Analg*. 2011;112:387-393.
28. Martin DS, Grocott MP. III. Oxygen therapy in anaesthesia: the yin and yang of O2. *Br J Anaesth*. 2013;111:867-871.
29. Rothen HU. Oxygen: avoid too much of a good thing! *Eur J Anaesthesiol*. 2010;27:493-494.

30. Elvidge GP, Glenny L, Appelhoff RJ, Ratcliffe PJ, Ragoussis J, Gleadle JM. Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. *J Biol Chem*. 2006;281:15215-15226.
31. Ayrapetov MK, Xu C, Sun Y, et al. Activation of Hif1alpha by the prolylhydroxylase inhibitor dimethyoxalyglycine decreases radiosensitivity. *PLoS One*. 2011;6:e26064.
32. Bos R, van Diest PJ, van der Groep P, Shvarts A, Greijer AE, van der Wall E. Expression of hypoxia-inducible factor-1alpha and cell cycle proteins in invasive breast cancer are estrogen receptor related. *Breast Cancer Res*. 2004;6:R450-459.
33. Yahara D, Yoshida T, Enokida Y, Takahashi E. Directional Migration of MDA-MB-231 Cells Under Oxygen Concentration Gradients. *Adv Exp Med Biol*. 2016;923:129-134.
34. Benzonana LL, Perry NJ, Watts HR, et al. Isoflurane, a commonly used volatile anesthetic, enhances renal cancer growth and malignant potential via the hypoxia-inducible factor cellular signaling pathway in vitro. *Anesthesiology*. 2013;119:593-605.
35. Furuya M, Yonemitsu Y. Cancer neovascularization and proinflammatory microenvironments. *Curr Cancer Drug Targets*. 2008;8:253-265.
36. Sheikh AY, Rollins MD, Hopf HW, Hunt TK. Hyperoxia improves microvascular perfusion in a murine wound model. *Wound Repair Regen*. 2005;13:303-308.
37. Duda DG, Batchelor TT, Willett CG, Jain RK. VEGF-targeted cancer therapy strategies: current progress, hurdles and future prospects. *Trends Mol Med*. 2007;13:223-230.
38. Lissoni P, Malugani F, Bonfanti A, et al. Abnormally enhanced blood concentrations of vascular endothelial growth factor (VEGF) in metastatic cancer patients and their relation to circulating dendritic cells, IL-12 and endothelin-1. *J Biol Regul Homeost Agents*. 2001;15:140-144.
39. Perrot-Applanat M, Di Benedetto M. Autocrine functions of VEGF in breast tumor cells: adhesion, survival, migration and invasion. *Cell Adh Migr*. 2012;6:547-553.
40. Gonzalez RM, Daly DS, Tan R, Marks JR, Zangar RC. Plasma biomarker profiles differ depending on breast cancer subtype but RANTES is consistently increased. *Cancer Epidemiol Biomarkers Prev*. 2011;20:1543-1551.
41. Velasco-Velázquez M, Pestell RG. The CCL5/CCR5 axis promotes metastasis in basal breast cancer. *Oncoimmunology*. 2013;2:e23660.
42. Ueno T, Toi M, Saji H, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res*. 2000;6:3282-3289.
43. Sheen-Chen SM, Eng HL, Chen WJ, Chou FF, Chen HS. Serum level of angiogenin in breast cancer. *Anticancer Res*. 2000;20:4769-4771.
44. Dutta S, Bandyopadhyay C, Bottero V, et al. Angiogenin interacts with the plasminogen activation system at the cell surface of breast cancer cells to regulate plasmin formation and cell migration. *Mol Oncol*. 2014;8:483-507.
45. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res*. 2008;14:6735-6741.