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Hypoxia and Hypoxia-Inducible factors in Breast Cancer: Targeting Breast Cancer Stem Cells

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Abstract

Background: Breast cancer is one of the most common cancers in western women. The cancer stem cell hypothesis say that in tumors, including breast cancer, only a small subpopulation of cells called cancer stem cells (CSCs), can induce invasion and metastasis. These CSCs shows resistance to radiation therapy and some chemotherapy and later on recurrence may take place. Hypoxia has been proposed to be one of the driving forces of CSCs. Hypoxia is associated with poor prognosis in cancer. Cell adaptation to hypoxic conditions is mainly directed by the activity of two hypoxia induced transcription factors, HIF-1a and HIF-2a that are accumulated under low oxygen conditions. Epithelial-to-mesenchymal-transition is a prerequisite for tumor spread and has been proposed to give rise to cancer stem cells.

Aim:

- To study if the frequency of the proposed CSC population is altered by oxygen conditions.
  - Optimize method and study in cell lines
  - Transfer to primary cells from tumors and secondary sites i.e. pleural effusions
- To set up and optimize method for cell sorting of CSCs based on at first CD44/CD24 phenotype.
- To compare the phenotype of stem and non-stem cancer cells (first cell lines later primary cells).

Methods: Culture cells in hypoxia to mimic *in vivo* conditions and then FACS analysis was performed for the expression of CD markers (CD44+/CD24- and E-cadherin) and Aldefluor-activity assay. Expressions of HIFs are monitored by western blotting and their transcriptional activity by QPCR.

Results and Conclusions: We found with FACS that, a population at hypoxia a population of MCF-7 cells showed loss of E-cadherin i.e. traits of epithelial-to-mesenchymal-transition. To find out whether these cells are cancer stem cells, we will sort them out and inject into the NOD-SCID mice.

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Introduction

Cancer

Cancer is the combination of about 200 diverse diseases; it happens when the ideal balance of cell division and cell death are disturbed and an uncontrolled cell growth takes place. Tumors can be sub divided into two groups: 1) Benign and 2) Malignant. A malignant tumor has the ability to metastasize, grow to the distinct anatomical sites after intravasation through lymphatic or blood microvessels, invade and destroy adjacent tissues [1]. This is what makes malignant tumors so dangerous and lethal. The most common cancers are: lung, prostate (men), breast (women), colorectal, liver, and stomach cancer [2]. In 2007-2008, 7.9 million humans died due to cancer, which is about 13% of all human deaths worldwide. Reports indicate that the cancer frequency is increasing day by day as the lifestyle changes in the developing world [3]. Cancer is the second most important cause of death in Sweden after cardiovascular disease [2].

Cancer is a multistep process, Douglas Hanahan and Robert A. Weinberg [4] proposed the 6 hallmarks of cancer during the developmental growth of human tumors. The 6 hallmarks are sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [4].

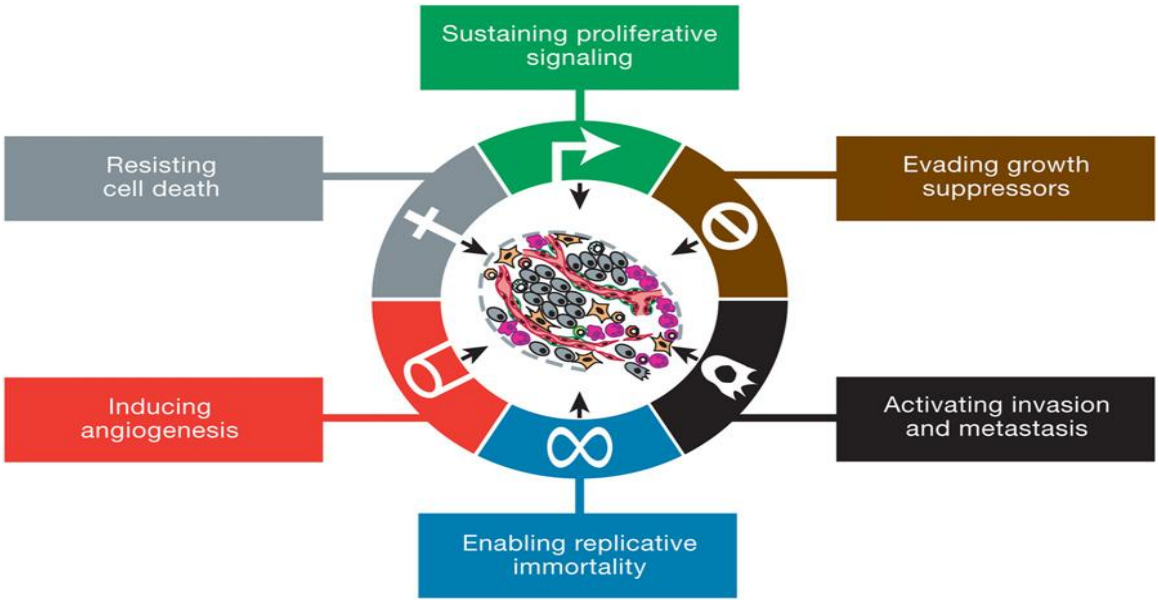

Figure 1. The Hallmarks of cancer during the developmental growth of human tumors [4].

Cancer Stem/Initiating Cell

Since the last several years, a breakthrough for cancer researcher is the indication that tumors are regenerated by their own stem cells called 'cancer stem cells' [5]. In a manner similar to all other differentiated cells in a body, which arise from a progenitor cell generally known as ''stem cell'', also tumor cells are hypothesized to be generated from a smaller pool of self-regenerating and less differentiated cells. What is a stem cell? The answer is ''the special type of cells, which has an ability to differentiate into different specialized cells (e.g. nerve, skeletal, cardiac, blood cells etc.) and also regenerate itself. Stem cells are classified into different subtypes e.g. totipotent (differentiate into all specialized cells), pluripotent (all except embryonic membrane cells), bipotent (has the ability to form two different cell types) [6]. Stem cells has the ability of self-renewal, cancer also grow by a limited number of stem cell/initiating cells called Cancer Stem Cells or Cancer Initiating Cells. According to the cancer stem cell hypothesis only this specific subpopulation of cancer cells are able to propagate the tumor and form metastases [7].

In 2003, Al Hajj et al. [7] proposed the separation of breast cancer cells according to their expression of cell-surface markers and found a population of cells with higher tumor forming capacity in immune-deficient mice, i.e. containing breast cancer stem cells (CSC) or so called tumor initiating cells (TIC) in the subpopulation of cells with the $EPCAM^{+}/CD44^{+}/CD24^{-/low}/lineage^{-}$ phenotype (surface markers to separate and sort out the specific cell population) in eight out of nine tumors. They showed that tumors were formed by as few as 100 $EPCAM^{+}/CD44^{+}/CD24^{-/low}$/lineage- cells in immune-deficient mice while 20,000 cells of $EPCAM^{+}/CD44^{+}/CD24^{+/high}/lineage^{-}$ phenotype did not [7]. 58 more mice were trailed with $EPCAM^{+}/CD44^{+}/CD24^{+/high}/lineage^{-}$ cells of about 1,000-50,000 cell/administration, and no tumor was observed after 16-29 weeks. From $EPCAM^{+}/CD44^{+}/CD24^{-/low}/lineage^{-}$ tumor cells they could propagate all of the populations $EPCAM^{+}/CD44^{+}/CD24^{-/low}/lineage^{-}$ cells and non-tumorigenic cells of the other phenotypes (same as above) as well. On several propagations, the cells sustain the population of $EPCAM^{+}/CD44^{+}/CD24^{-/low}$ phenotype and the capability of tumor formation. Extractions of $EPCAM^{+}/CD44^{+}/CD24^{-/low}/lineage^{-}$ cells were from metastases in 8 out of 9 cases and one was from the primary tumor [7]. To characterize the presence of CSCs in primary tumors more investigations are required.

An alternative way of selecting for stem cells is detection of Aldehyde dehydrogenase (ALDH) activity by using an aldeflour flow cytrometry-based assay [8]. Cancer stem cells can be detected by the expression of ALDH1, however this marker is not exclusively detecting tumor stem cells. $EPCAM^{+}/CD44^{+}/CD24^{-/low}/lineage^{-}$ cells with ALDH-activity showed that these could induce tumors even when there were only 20 cells injected in the immune-deficient mice, however the overlapping population, i.e. $EPCAM^{+}/CD44^{+}/CD24^{-/low}/lineage^{-}$ cells with ALDH positive activity, was very small [9]. In addition, ALDH-activity plays a key role in the detection of both normal and cancer stem cells from different human tissues, and in human breast carcinoma the presence of ALDH1 activity associated to poor clinical outcome [9].

Breast Cancer

Breast cancer is the most common cancer in western women. It is also present in men, but very rare as compared to the incidence in women. In Sweden, breast cancer accounts for about 30% of all cancer diagnoses in women [10]. In a 2010 report, 7400 Swedish women were diagnosed with breast cancer [10]. In US, invasive breast cancer rate is about 12%, which means that 1 out of 8 women get breast cancer in their lifespan [11]. According to US report of breast cancer, in 2011, an estimated calculation of new cases of invasive breast cancer in women is 230,480 and 2,140 in men, while 57,650 new cases of non-invasive (in situ) were diagnosed [11]. However, new therapeutic treatments and improvements in clinical diagnostics are leading towards better survival rates as today the 5 year survival rate is about 87% and 78% survival for 10 years [10, 12]. Due to this improvement, reports indicate that in the last 2 decades the incidence rate is increasing but mortality rate is decreasing [13]. The incidence risk of breast cancer in women is almost twice for a woman that has a first-relative (mother, sister or daughter) with breast cancer [11]. Breast cancer risk factors comprise of many features and the most known are age, family background (inheritance), previous breast cancer, global location, ionizing radiation and hormonal balance [14, 15]. Mutation in BRCA1 (chromosome 17) and BRCA2 (chromosome 13) genes is the most well-known mutations in hereditary breast cancer [16]. One third of all affected by breast cancer have a close relative with breast cancer i.e. familial breast cancer. Out of these 30%, 10% shows the clear indication of inheritance called hereditary breast cancer while the rest about 70% is sporadic; those who do not have family background of breast cancer [11]. Today, the research is focusing much on the effect of diet, weight, alcohol consumption and physical training and it is clearly visible that physical activity has a sound effects on decreasing the risk of breast cancer while rest of the risk factors are increasing the risk [15].

Treatment

Breast cancer can be treated in different ways e.g. surgery, chemotherapy, radiation therapy, anti-estrogen therapy and Human epidermal growth factor receptor 2 (HER-2) antagonist treatment, depending on the characteristics of the cancer [17]. However, the most common is surgical removal of the tumor, either full mastectomy or breast conservatory surgery, leaving parts of the breast. The most important characteristics for determining therapeutical strategy in breast cancer is the expression of estrogen receptor (ER), HER2, and if the cancer has spread to nearby lymph nodes or not [18, 19]. Therefore, this is always analyzed when surgery is performed. Often, the combination of surgery with radiotherapy takes place. Radiation therapy results both in direct DNA damages and generates reactive oxygen species eventually leading towards cell death [17]. Radiation therapy is a vital tool in cancer therapy and its efficiency is dependent on tumor oxygenation status. Adjuvant chemotherapy is in use from the late 1950's, it is used to make the cells less resistant against the drug effect, and later polychemotherapy was introduced and new drugs were came into existence [20]. Anthracyclin-based polychemotherapy in combination with taxanes is the most effective chemotherapy until

date[21]. Trastuzumab, a monoclonal antibody is used as an adjuvant for treatment of HER-2 positive tumors [22].

Some designed targeting drugs, disturb several hormonal pathways and endocrine therapy triggers the induced signaling between and within the cells and is used to treat hormone responsive breast cancer, Tamoxifen is an example of dysregulating drug in ER-positive breast cancer [17]. Estrogen induction can be effected either by blocking or modulating the receptors (e.g. by Tamoxifen) or by lowering the quantity of estrogens in body (e.g. Fulvestrant). Tamoxifen is a selective estrogen receptor modulator (SERM) and it is an ER-antagonist in some hormone responsive tissues including the breast, hence it binds and inhibits the ER activation. About 75% of all invasive breast cancer expresses ER [23].

Breast Cancer Cell Lines

Cancer cell lines are immortalized; i.e. they can grow up to unlimited numbers and infinitely. For the last 40 years such cancer cell lines are used for cancer research. Until date, more than 100 cancer cell lines have been established, which help to study the cell and protein functions i.e. how they regulate their functions and signaling, giving important biological insight of the cell.

Hypoxia

Hypoxia is the condition in, which oxygen is deprived and adequate energy supply is destroyed. Hypoxia is an unbalanced demand of oxygen supply compared to the demand also known in limited O_2 supply to the organs, tissues or cells, which causes the damage of their normal functions [24].

Tumor Hypoxia

In solid tumors hypoxia is often induced due to the tumor size increment and malfunctioned angiogenesis leading to disturbed delivery of the required oxygen and nutrients [25] [26]. According to the requirement, every organ has their own partial oxygen pressure (pO_2), which is quite different from each other, it varies from high arterial (120 mmHg, 16% O_2) to the lowest at the end-capillaries (45-50 mmHg, 5-6% O_2) depends on their need and it is considered as a normal oxygen pressure as the requirements is full filled for organ/cell functions. This normal demand and supply of oxygen is termed as “normoxia” [27]. To define hypoxia threshold it is difficult to set a fixed oxygen partial pressure as hypoxic, since it depends on the tissue context and the normal variation in the body, whereas metabolic hypoxic threshold is often set at 8-10 mmHg, 1.3% O_2 [24, 27], and 65% pO2 in normal breast tissue [28]. Hypoxia is an obstacle both for malignant and normal cells, but tumor cells may undergo genetic changes and mutations/selection allowing them to survive and propagate even in an oxygen deprived environment [25]. For example, hypoxic conditions select for cells with p53 mutations and hence less prone to apoptosis and senescence. Due to some abnormalities in function and shape of microvessels the oxygen flow is dropped, which leads towards proper shut down of microvessel, known as acute hypoxia [29]. When

tumor cells become hypoxic, the gene expression alters, which plays a vital role in regulation of angiogenesis, immune invasion, genetic instability, metastasis and invasion, metabolism, treatment resistance and stem cell maintenance [30]. In hypoxia, it has been reported that hypoxia encourage the de-differentiation of tumor cells [31]. To analyze the hypoxic cellular expression in cell culture, hypoxic condition is achieved by providing 1% O2 to the hypoxic work-station.

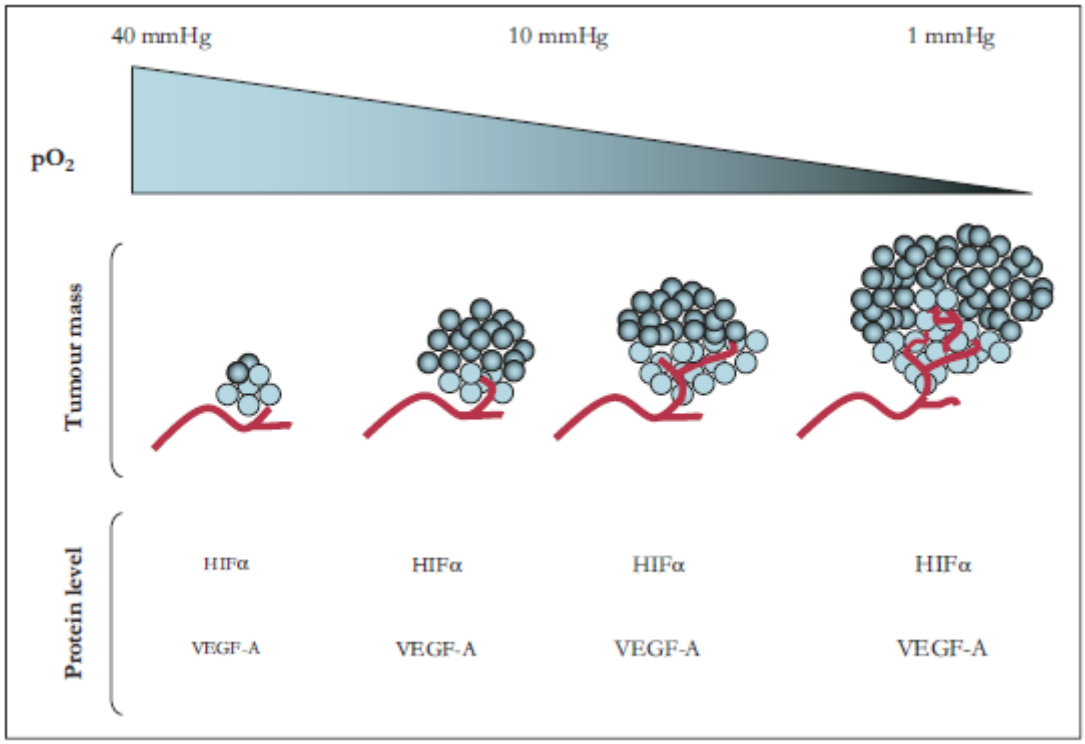

Figure 2. Tumor cells under hypoxic stress: As the tumor size increasing the concentration of O_2 is decreasing, at the same time the hypoxia inducible factor HIFα and VEGF (a gene responsible for angiogenesis) protein is increasing [32].

Hypoxia-Inducible Factors

There are two Hypoxia-Inducible factors (HIFs), HIF-1 α and HIF-2 α, bHLH-PAS transcription factors activated at hypoxia. HIFs are continuously synthesized in the cells, but are stabilized and activated during hypoxic condition, whereas in normal conditions they are degraded by the proteasome [28, 33]. In hypoxia, HIF α-subunits bind with β-subunit (also called ARNT, aryl hydrocarbon nuclear translocator) and transfer to the nucleus, where these subunits form dimers combine with hypoxia responsive element (HRE) in the promoter region to start gene transcription [33, 34]. Hypoxia induced transcription was initially reported as a cell regulator in erythropoietin production [35].

Depending on the surrounding environment of the HIF-α, hypoxia is regulated with the supply of O_2, illustrated in figure 2. In normoxic condition, the prolyl hydroxylase domain (PHDs) hydroxylates the HIF-α. The hydroxyl groups create a binding site for von Hippel-Lindau protein (pVHL), and combines with elongin C, elongin B, cullin-2, and Rbx1 developing an E3 ubiquitin ligase complex and targets HIFα for proteasomal

degradation [36]. On the other hand, another factor, factor inhibiting HIF-1α protein (FIH-1) can hydroxylate HIF-α, which restrict the binding of the 300-kilodalton co-activator protein (p300) and CREB binding protein (CBP) and so initiates the gene expression [30, 33, 37].

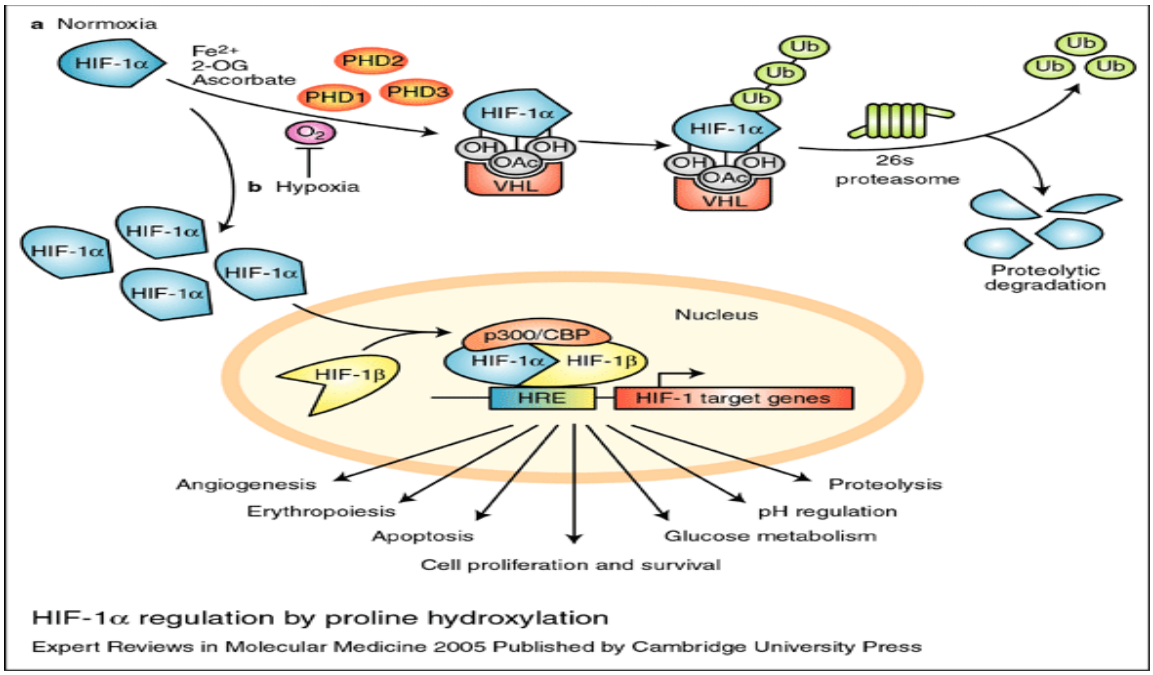

Figure 3. HIF-1α regulation by proline hydroxylation: **a**. Under normoxic condition the the oxygen-dependent prolyl hydroxylase domain (PHD) and factor inhibiting HIF1α (FIH) hydroxylases are active and hydroxylate HIFs, this hydroxyl groups create a binding site for von Hippel-Lindau protein (pVHL), develop an E3 ubiquitin ligase complex and targets HIFα for proteasomal degradation. **b**. Under hypoxic condition, PHDs are inactive and HIF α subunit is stable and translocates into the cell nucleus where it heterodimerises with the beta subunit inducing binding to the DNA of target genes carrying a hypoxia-response element (HRE). Contact with the co-activator CBP/p300 starts the repression or induction of large number of genes e.g apoptosis, angiogenesis, erythropoiesis, cell proliferation and survival, glucose metabolism, pH regulation, proteolysis and anaerobic glycolysis [38].

In hypoxia, oxygen deprived conditions inhibit the hydroxylation of the HIF-α subunit, as a result of this no binding site for pVHL is formed and HIF-α is free to combine with HIF-1β and form heterodimer, which activates the conformational change and transfer into the nucleus [39].

Epethelial-Mesenchymal Transition (EMT)

During tumor progression, cancer cells invade and destroy distinct anatomical sites and form metastasis (from micrometastasis to macrometastasis) [40]. It happens when cancer cells start losing their cell-cell adhesion ability, intra cellular signaling functions and gain the ability to move freely and intravasate through lymphatic or blood stream, invade and destroy adjacent tissues [40]. Metastasis occurs during the loss of E-cadherin, which induces EMT. All this happens during EMT, when epithelial cell start losing their cell

polarity and epithelial cell junctions, and a gain of mesenchymal phenotype in addition with epithelial marker (E-Cadherin and gamma catenin are the epithelial cell markers and fibronectin and vimentin are mesenchymal markers) and transcription factors (Snail, slug and twist) [40]. It has been proposed that the transcriptional factors Snail and Slug (zinc finger proteins) work as direct repressors of E-cadherin both in vitro and in vivo. E-cadherin promoter interacts with COOH-terminal region with a 5¶-CACCTG-3¶ sequence, induce resistance to apoptosis and enhances tumor survival. Twist is also well known molecule to trigger EMT a highly conserved basic helix-loop-helix transcriptional factor that activates N-cadherin during Drosophila embryogenesis [41].

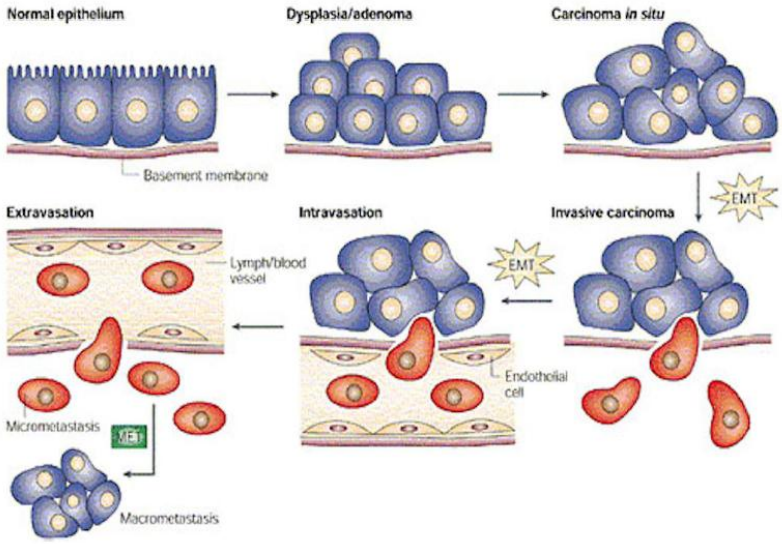

Figure 4. Epethelial-Mesenchymal transition (EMT) schematic view: During cancer metastasis loss of epithelial cell junctions and cell polarity and gain of mesechymal phenotype takes place, which helps the mesenchymal cell to form micro and later macremetastasis to secondary sites after intravasation through lymphatic or blood stream from primary site. [42]

AIMS

- To study if the frequency of the proposed CSC population is altered by oxygen conditions.
  - Optimize the methods for detection and study in cell lines cultured under normoxic and hypoxic conditions
  - Transfer the optimized methods to primary cells from tumors and secondary sites i.e. pleural effusions
- To set up and optimize method for cell sorting and retrieval of CSCs based on at first CD44/CD24 phenotype.
- To compare the phenotype of stem and non-stem cancer cells (first cell lines later primary cells).

Materials and methods

Breast Cancer Cell Lines included in this thesis

There are hundreds of cell lines of breast cancer and out of those we selected these 4 cell lines in order to include cells with and without ER expression and with different degrees of epithelial to mesenchymal characteristics.

1) MDA-MB-231 is a human epithelial cell line with adherent property. It was derived from pleural fluid (metastatic site) from a patient with mammary adenocarcinoma. It has receptors for e.g. epidermal growth factor (EGF) and transforming growth factor (TGFα) [43].

2) MCF-7 is a human breast cancer cell line with adherent nature. It was also established from the pleural fluid (metastatic site) from a patient with mammary adenocarcinoma. This cell line expresses ER and progesterone receptor [44].

3) T-47D is an adherent epithelial cell line derived from pleural fluid (metastatic site) from a patient who had ductal carcinoma of the breast. This cell line expresses receptors for e.g. calcitonin, androgen receptor, ER, progesterone receptor and glucocorticoid receptor [45].

4) Sum159 is extracted from a primary tumor from a patient that had breast anaplastic carcinoma. e.g. Prolactin receptors [46].

Cell culture

The cell lines MDA-MB-231, T-47D and MCF-7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SUM159 was a kind of gift from Associate Professor Sofia Gruvberger-Saal Lund University in collaboration with Professor SP Ethier.

Cells of the above mentioned cell lines were routinely cultured as monolayer in 100 mm diameter tissue culture dishes (BD-Falcon #353001) until semi confluent in a humidified incubator at $37^{o}C$ in 5% CO_2.

MDA-MB-231 and MCF-7 were cultured in growth medium consisting of DMEM: F12 (Invitrogen # 31330-038) supplemented with 10% Fetal Calf/Bovine Serum (FCS, Saveen & Werner # 5795), Penicillin (100 units/ml), Streptomycin (100 units/ml) (HyClone # J111431) and 1% Sodium Pyruvate Solution 100 mM (1 ul/100 ml, PAA the cell culture company). To MCF-7 cells, insulin (1x 105 IE/L) was added.

T-47D was cultured in RPMI, supplemented with FCS, Penicillin, Streptomycin, Sodium Pyruvate Solution and Sodium Bicarbonate as described above. The medium was also supplemented with glucose 7.2 g/L and insulin (1x 105 IE/L).

SUM159 was cultured in Ham's F12, FCS (5%), Penicillin, Streptomycin (as above), hydrocortisone (0.5 μg/L), and insulin (1x 105 IE/L).

For hypoxic incubations, cells were seeded and allowed to adhere to the plastic at 37^{o}C in 5% CO_2. Cells were thereafter transferred to the hypoxic work station (VWR International AB-DW Scientific Ltd.) where cells grew at 37^{o}C in 5% CO_2 and 1% O_2 (oxygen depleted environment) for the indicated time interval.

Cell harvest for Protein or RNA Analysis:

For cell harvest, cells were detached by EDTA (10 mM) treatment for 12-15 minutes in 37^{o}C, unless otherwise stated. Cells were suspended in PBS and centrifuged at 15000 g for 1 min at 4^{o}C. The sample was immediately frozen in liquid nitrogen and stored at -80^{o}C.

Western Blotting

Preparation of total cell protein extract was done by adding lysis buffer (RIPA, containing Protease Inhibitor Cocktail (Complete)) to the cell pellet. Protein determination was performed by Bradford assay. 80 μg protein was added per lane on a 6% poly-acrylamide gel. Proteins were separated by electrophoresis (Bio Rad), and electrophoretically transferred onto a nitrocellulose membrane (H-Bond C). General protein detection was done by Ponceau staining. Protein detection was by anti-Hif-1α (Millipore, polyclonal rabbit antibody) and anti-HIF-2α (ep19 Novum Biologicals, mouse monoclonal antibody), loading control was done by SDHA (Ms mAb to SDHA (ZE3), 100 μg (1 mg/ml) ab14715 abcam, LOT# 888711) and secondary antibodies were HRP conjugated anti-mouse (from sheep, NA931V) /rabbit (from donkey, NA934V) (GE Health Care UK Limited). Fluorescence detection was by EZ-ECL-Chemiluminescence (Biological Industries). For protein size reference Page RulerTM prestained protein ladder (part # 26616, Thermo Scientific) was used.

Fluorescence Activated Cell Sorting (FACS)

To analyze/detect the TIC/CSC phenotype (i.e. CD44+/CD24-/EpCam+) flow cytometry (FACS caliber, BD) was performed on the different cell lines e.g. SUM159, MDA-MB-231, T-47D and MCF-7. Cells were harvested by adding EDTA; incubate for 10-15 min at 37^{0}C. Stained harvested cells with CD24-PE, CD44-APC and EpCAM-FITC antibodies and then examined with flow cytometry.

Cell Sorting with magnetic beads

CD44+/CD24- were the marker expression of the cell population of our interest. Since all cell lines in this work had expressed high levels of the CD44 marker, negative selection was done with CD24 coupled magnetic beads. First cells were marked with CD24-biotin (MACS, Miltenyi Biotech) and then anti-biotin antibody conjugated to magnetic beads was added. The cell suspension was passed through LC columns (MACS Separation

column, Miltenyi Biotech) in a magnetic stand. Cells bound to the magnetic beads (i.e. CD24+) were retained on the LC column while CD24- cells passed through. CD24+ cells were retrieved by use of a plunger after removal of the column from the magnetic stand. Both CD24 positive and negative cell fractions were stained with antibody cocktail; CD24-PE, CD44-APC and EpCAM-FITC, analyzed results by flow cytometry methods.

Staining was performed, first cells were marked with CD24-biotin and then anti-biotin antibody conjugated to magnetic beads was added, stained cells were bound with magnet when cell suspension passed through the LC column. All unstained cells were eluted out and all CD24 stained stayed in the magnetic column, until use of plunger to collect those cells forcefully, then we stained all collected cells with antibody cocktail (CD24-PE, CD44-APC and E-Cadherin-FITC) and investigated cell population markers by flow cytometry. Negative selection was done, since all MDA-MB-231 and SUM159 were all CD44 positive

Aldefluor Assay

An alternative way of selecting, isolating and evaluating stem and progenitor cells, which express high level of Aldehyde dehydrogenase (ALDH) activity by aldefluor flow cytometry-based assay. FACS analysis of MDA-MB-231 and MCF-7, 72 hours incubated, normoxic and hypoxic cells using the Aldefluor assay, cells were incubated with ALDH substrate, BAAA, which was then converted by intracellular ALDH1 into a negatively charged reaction product BODIPYaminoacetate. BODIPY-aminoacetate was sustained inside cells positively expressing ALDH1, giving the bright fluorscent. Brightly fluorescent ALDH1-expressing cells (ALDH1-positive cells) were analyzed in the green fluorescence channel by using flow cytometry. Cells stained with BAAA and a specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), were used as a as a negative control or cutoff fluorescence of these cells. Cell populations were analyzed by flow cytometry (FACS caliber, BD).

Gene Expression

RNA purification and isolation was done by Qiagen QIAshredder (#79654) and RNeasy mini kit (#74106) according to the instructions of the manufacturer. RNA concentrations were measured by spectrophotometer (Nanodrop ND-1000). DNA was removed by RNase-free DNase treatment (1 U/µl, Promega #6101) and the RNA was concentrated and washed on Microncon-100 columns (Amicon #42413). The analysis of relative gene expression of genes of interest was done by reverse transcriptase real-time quantitative poly-chain-reaction (RT qPCR) based on two-step SYBR green chemistry. Generation of cDNA from the purified RNA was done by using PTC-100TM programmable thermal controller (MJ Research) and High-Capacity cDNA Reverse Transcription kit (Applied Biosystem #4368813), which has random primers and multiscribe reverse transcriptase.

The qPCR reaction was performed (7300 Real time PCR system, Applied Biosciences) by loading the mixture of 5 µl cDNA, and 20 µl SYBR green PCR mastermix (Applied biosystems (#4309155)) containing forward and reverse primers (below in table 1 and 2) for the gene of interest, per sample. Normalization of target gene expression was done

with the help of the geo-mean of 3 house-keeping genes (SDHA, UBC and YWHAZ) [47].

Table 1 Internal control genes used in RT-qPCR for normalization procedure

Gene	Forward primer	Reverse primer
UBC	5′-ATTTGGGTCGCGGTTCTTG-3′	5′TGCCTTGACATTCTCGATGGT-3′
YWHAZ	5′ACTTTTGGTACATTGTGTGGCTTCAA-3′	5′CCGCCAGGACAAACCAGTAT-3′
SDHA	5′TGGGAACAAGAGGGCATCTG-3′	5′CCACCACTGCATCAAATTCATG-3′

Table 2 Genes of interest used in RT-qPCR and primer sequences

Gene	Forward primer	Reverse primer
HIF-1α	5′TTCCAGTTACGTTCCTTCGATCA-3′	5′TTTGAGGACTTGCGCTTTCA-3′
HIF-2α	5′GCTCTCCCACGGCCTGTA-3′	5′TTGTCACACCTATGGCATATCACC-3′
VEGF	5′AGGAGGAGGGCAGAATCATCA-3′	5′CTCGATTGGATGGCAGTAGCT
OCT4	5′GGAAGGTATTCAGCCAAACGACCA3′	5′CTCACTCGGTTCTCGATACTGGTT-3′
DEC1	5'GGCGGCAACAAGCGCAAGAAGTCCAAG-3'	5'TGTCTGAAATGCGTCGCCCTCGCCATCCTCTGGTGCTG-3'

Results

Optimization of cell harvest method

Cells have proteins on its surface, which help them to bind with other cells or ligands to perform various works, tasks and function [48]. Harvesting treatments (chemically or physically) can cleave these proteins from the surface. By using different methods of harvesting it was observed that different chemicals and physical techniques put some effect on the epitopes. The best method was observed under FACS was EDTA, which put lesser effects on epitopes, cell membrane and cell viability. For all further flow cytometry analyses cells were harvested by EDTA treatment.

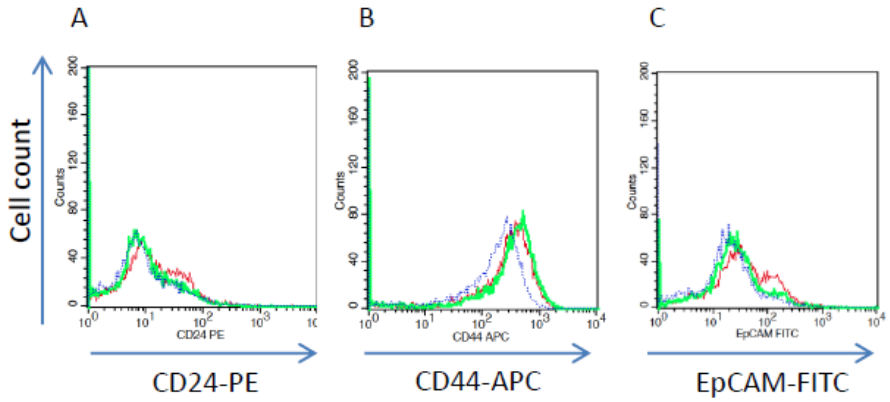

Figure 5. Cell harvest: Different methods (Scraping, EDTA and trypsin) were used to optimize the harvesting method. A, B and C show different epitope binding affinity or ability or presence of cell surface markers to bind with.

Red= Scrape

Green = 10mM EDTA
Blue= Trypsin

Expression of HIFs in hypoxic condition

To investigate if the hypoxic condition affected the HIFs protein stability, western blot was done to analyze the expression of our protein of interest (HIF-1 α and HIF-2 α) and control protein SDHA in normoxic and hypoxic conditions. Since HIFs are stabilized in oxygen deprived condition, these proteins are hypoxia inducible proteins, and therefore the hypoxic (H) cells are showing more prominent expression than the normoxic/control (C) cells. (Fig. 6)

MDA-MB-231, SUM159, T47D and MCF-7 were seeded and incubated for 72 hours in normoxic (21% O_2) and hypoxic (1% O_2) conditions. Cells were harvested and western blot was performed. It was observed that the hypoxic condition to all cell lines gave high accumulation of HIF-1α since as a positive control T-47D cells treated with 200 μM of the prolyl hydroxylase inhibitor 2,2'-dipyridyl were included. (Fig. 6)

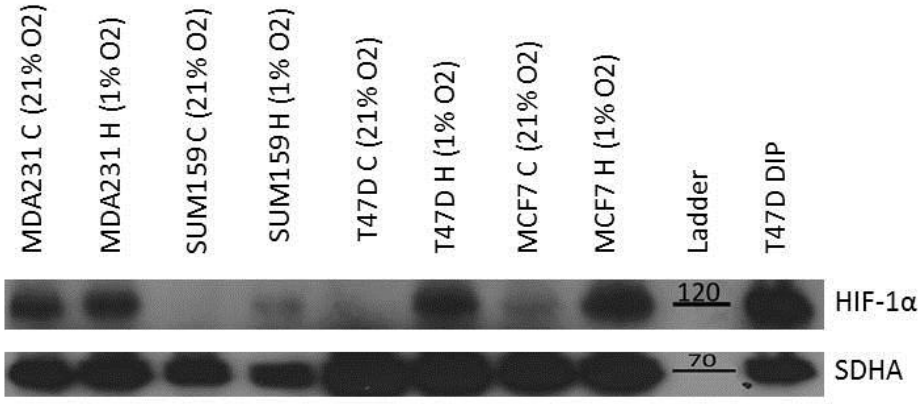

Figure 6. Western blot: Protein levels of HIF-1a and loading control SDHA, under normoxic (21% O_2) and hypoxic (1% O_2) conditions after 72 hours incubation. DIP treated sample was used as a positive control, DIP stabilizes the HIF-alpha subunits, since it is an iron chealator and makes the cell environment oxygen depleted so proteosomal degradation of HIFs was inhibited. All cell lines were showing more protein expression in normoxic conditions, except MDA-MB-231.

DIP treatment was done as a positive control, DIP, an iron-chelator inhibits prolyl hydroxylase domain (PHD) and factor inhibiting HIF1 (FIH) hydroxylases are inactive and then pVHL is not able to bind to take HIF- α towards proteolytic degradation. It stabilizes the HIF proteins and thus can be used as a reference of HIF α protein. All hypoxic cells (e.g. MDA-MB-231, SUM159, MCF7 and T47D) and DIP treated cells are showing the HIF-1 α protein, but none or low in normoxic cells. (Fig. 7)

Gene expression in normoxic and hypoxic breast cancer cell lines

The relative gene expression of the hypoxia and HIF regulated gene vascular endothelial growth factor (VEGF) was analyzed in hypoxic and normoxic cells. Up-regulation of VEGF was observed in hypoxic cells. A tendency towards down-regulation of E-

Cadherin expression was detected in MCF-7 hypoxic cells. There was no expression of E-cadherin in MDA-MB-231 and SUM159, which was in line with their mesenchymal-like phenotype. (Fig. 7, these results are preliminary and will be repeated)

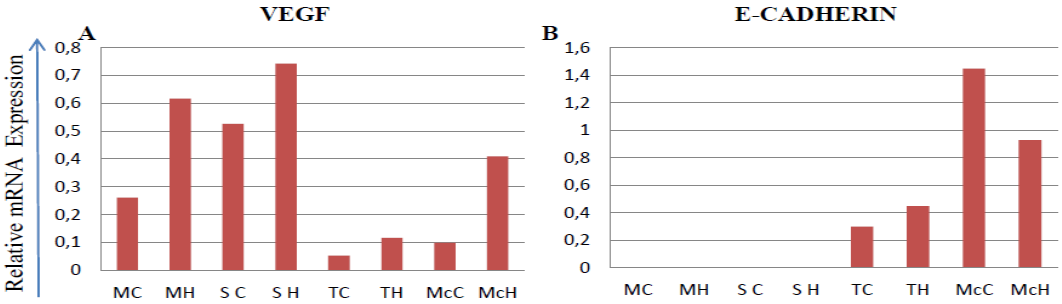

Figure 7. Relative mRNA expression of MDA-MB-231 (MC and MH), SUM159 (SC and SH), T47D (TC and TH) and MCF-7 (McC and McH), (where C stands for normoxia and H stands for hypoxia), cell line under normoxic (21% O_2) and hypoxic (1% O_2) conditions after 72 hours of incubation. A. Relative expression of VEGF analyzed by qPCR after 72h culture under normoxic © or hypoxic (H) conditions. B. Relative expression of E-Cadherin analyzed by qPCR after 72h culture under normoxic © or hypoxic (H) conditions.. Normalization of target genes was done with the help of the geo-mean of 3 house-keeping genes (SDHA, UBC and YWHAZ).

$CD44^{+}/CD24^{-/low}$ population in normoxia and hypoxia

Breast cancer cells with the $CD44^{+}/CD24^{-/low}/lineage^{-}$ phenotype has been proposed to harbor cells of the TICs/CSCs population, only few cells of this phenotype are CSCs (ref Al-Hajj 2003). We analyzed the four breast cancer cell lines regarding their $CD44^{+}/CD24^{-/low}/^{EpCAM}$ and E-cadherin expression after normoxic and hypoxic culture for 72 hours. In MCF-7 cell line, no difference was observed in CD44/CD24 expression in hypoxic treated after 72 hours incubation (gate 13 (R13) (Fig. 8 B and C). But, interestingly a significant increment in E-Cadherin negative cells was shown in hypoxic cells as compare to normoxic cells (Fig. 8). Three independent repeats of this experiment showed a significant increase in the E-cadherin-low population after hypoxic culture, we tried it several time and had t-test value p=0.02 meant that significant result was observed Since loss of E-cadherin is a hall mark of EMT, we propose that these cells have undergone hypoxia induced EMT, though functional studies are necessary to confirm this hypothesis.

In contrast to MCF-7, no difference was observed in either CD44/CD24 or E-cadherin low cell population in MDA-MB-231, SUM159 and T47D in flow cytometry. (Fig. 9, 10 and 11). However, a very small shift was observed in CD44 positive cells in MDA-MB-231and SUM159. (Fig. 9 and 10)

FACS data indicates that MDA-MB-231and SUM159 cells are showing fluctuations in $CD44^{+}/CD24^{-/low}$ cells population in hypoxic and normoxic conditions, but not in T47D and MCF-7 cells, however with this kind of shift or drift in the whole cell population it is difficult to show biological or statistical significance.

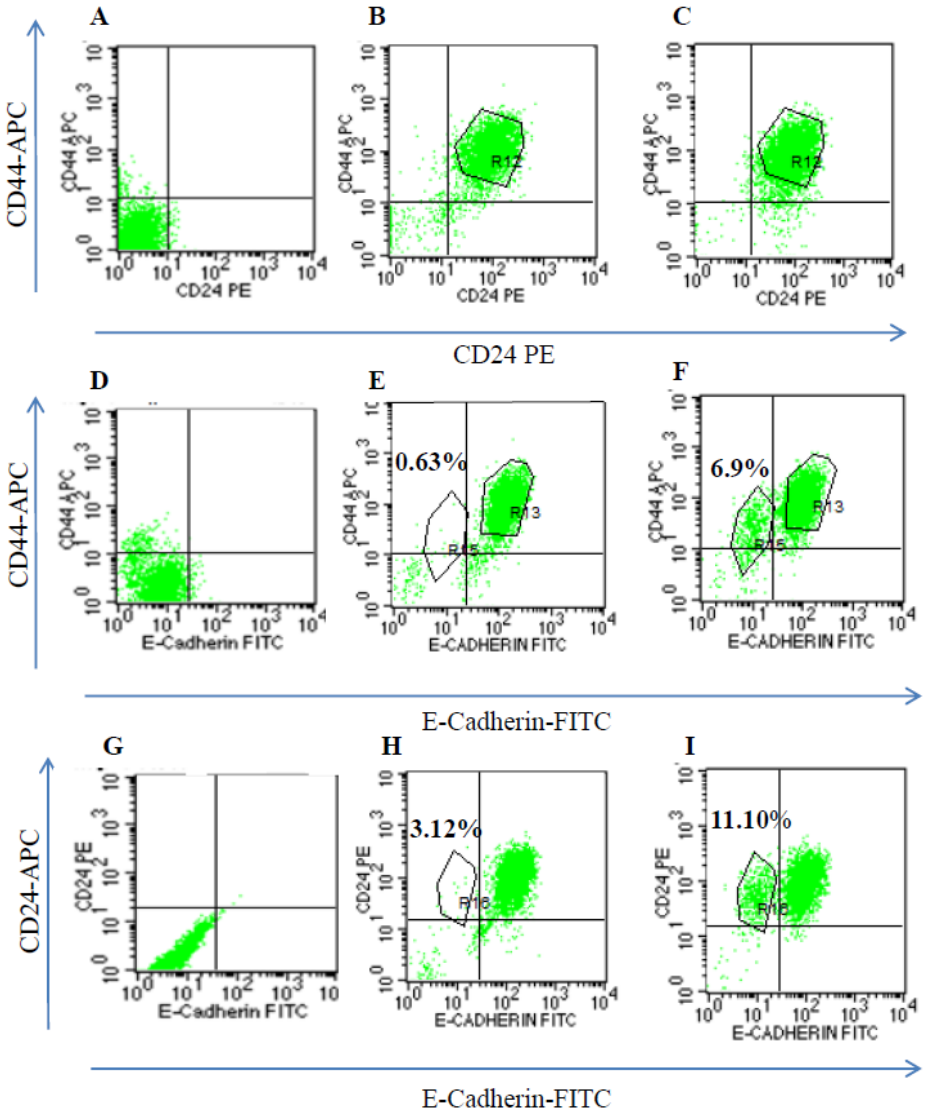

Figure 8. Flow cytometry analysis of MCF-7 cells after culture in normoxic (21% O_2) (B, E and H) and hypoxic conditions (1% O_2) (C, F and I). Cells were seeded and incubated for 72 hours, stained with antibody cocktail (CD24-PE, CD44-APC and E-Cadherin-FITC) to examine the surface markers with cell population of E-CADHERIN/CD44+/CD24- cell. IgG was used as a negative control (A, D and G). E, 21% O_2 is showing only 0.63% of E-cadherin negative cells, while, on the other hand, F. showing 6.9% of E-cadherin negative cells. Same in H and I.

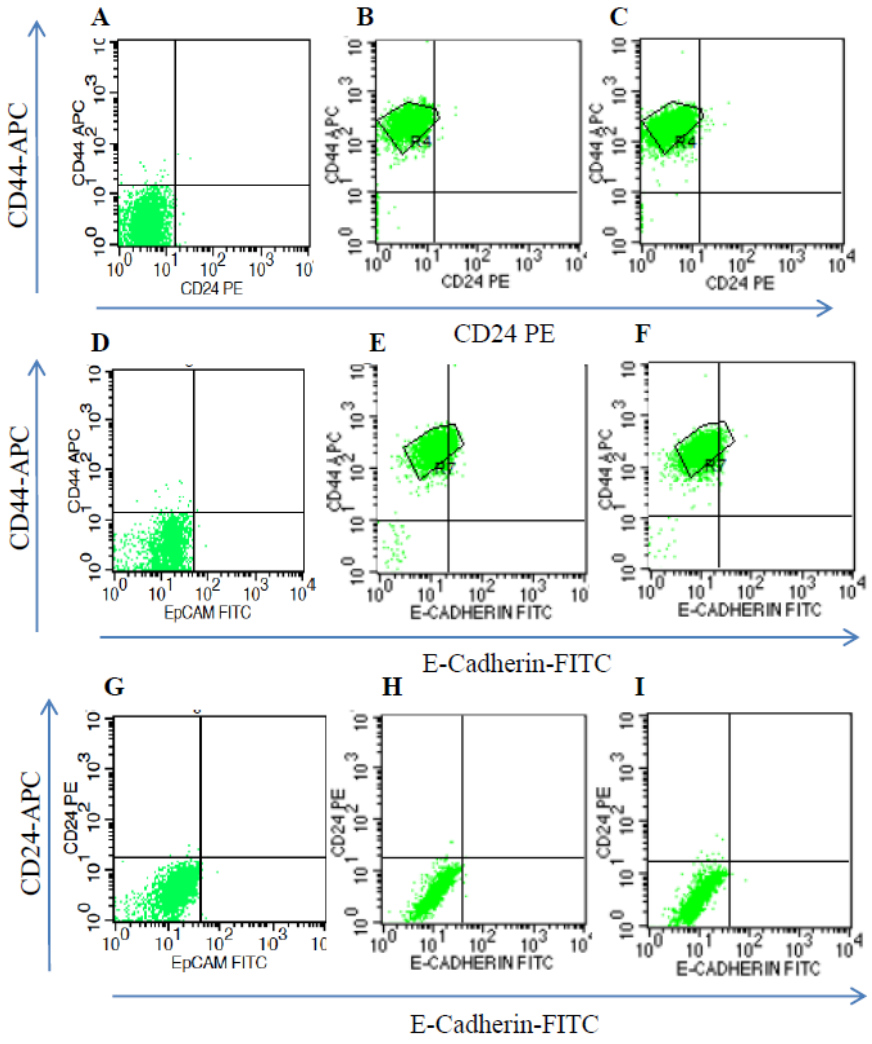

Figure 9. Flow cytometry analysis of MDA231 cells in normoxic (21% O_2) (B, E and H) and hypoxic conditions (1% O_2) (C, F and I). Cells were seeded and incubated for 72 hours, stained with antibody cocktail (CD24-PE, CD44-APC and E-Cadherin-FITC) to examine the surface markers with cell population of EpCAM/CD44+/CD24- cell. IgG was used as a negative control (A, D and G),

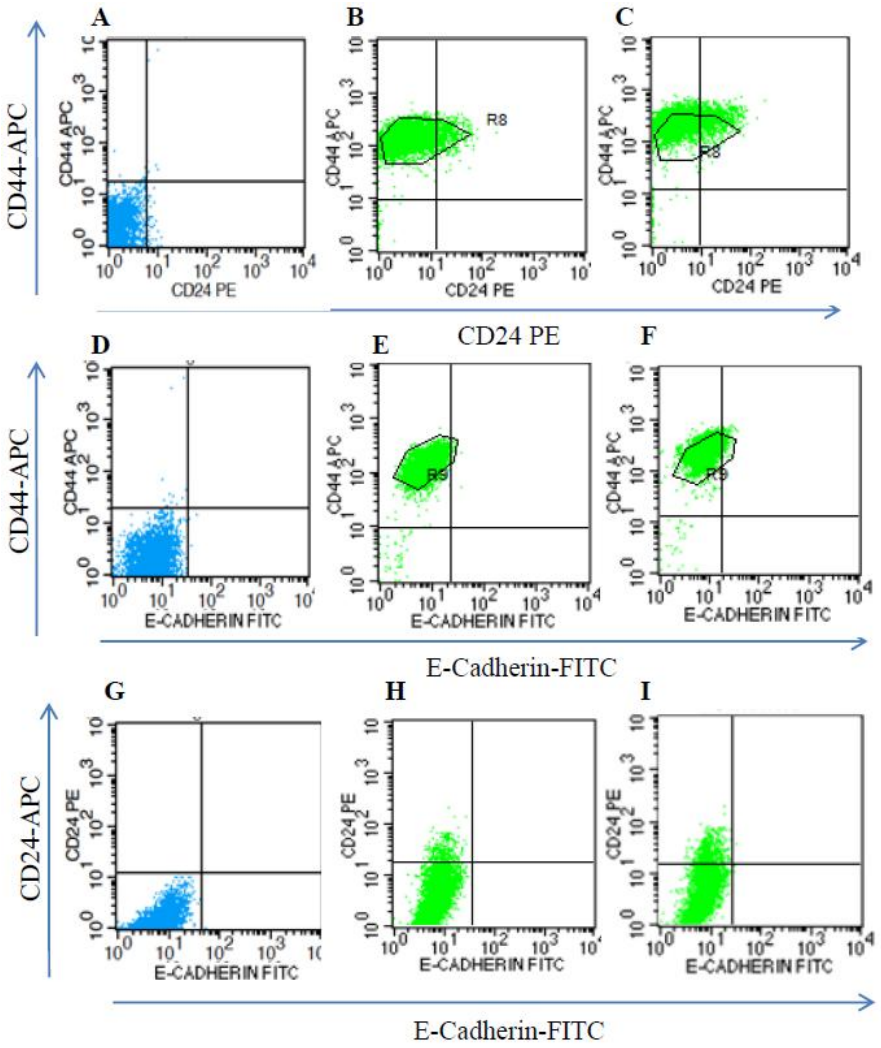

Figure 10. Flow cytometry analysis of SUM159 cells in normoxic (21% O_2) (B, E and H) and hypoxic conditions (1% O_2) (C, F and I). Cells were seeded and incubated for 72 hours, stained with antibody cocktail (CD24-PE, CD44-APC and E-Cadherin-FITC) to examine the surface markers with cell population of EpCAM/CD44+/CD24- cell. IgG was used as a negative control (A, D and G).

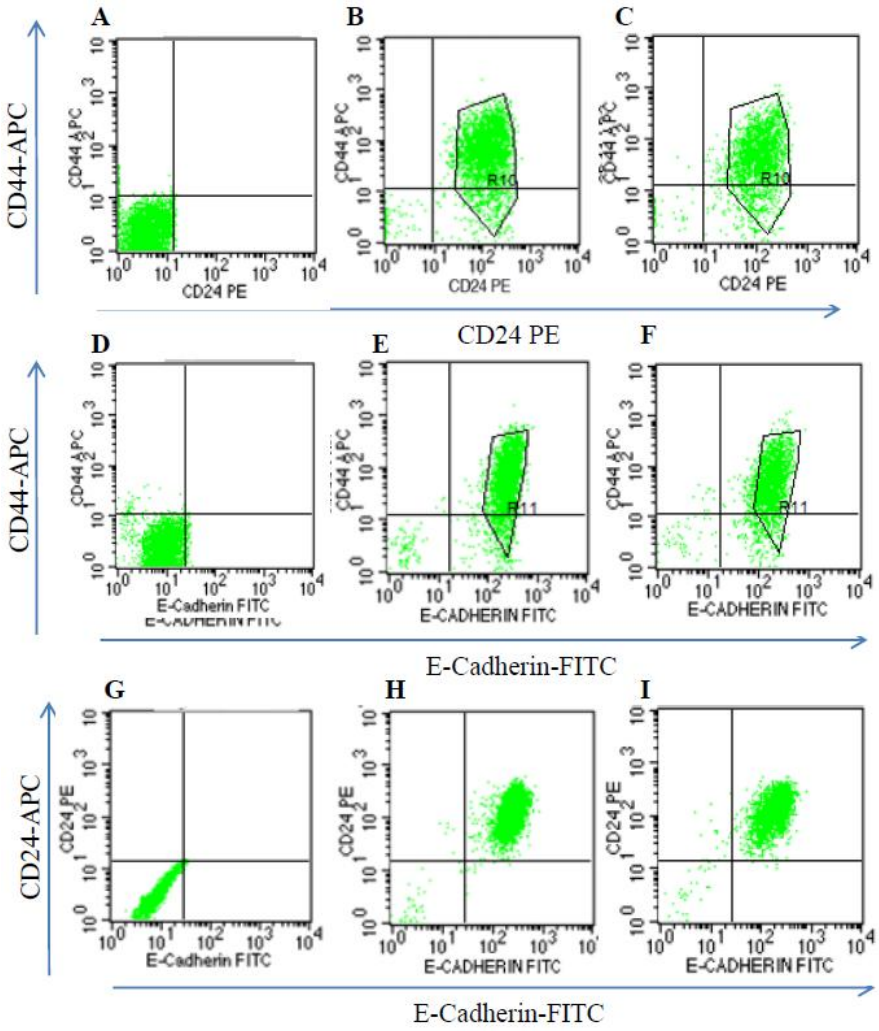

Figure 11. Flow cytometry analysis of T47D cells in normoxic (21% O_2) (B, E and H) and hypoxic conditions (1% O_2) (C, F and I). Cells were seeded and incubated for 72 hours, stained with antibody cocktail (CD24-PE, CD44-APC and E-Cadherin-FITC) to examine the surface markers with cell population of EpCAM/CD44+/CD24- cell. IgG was used as a negative control (A, D and G),

Cells from one primary tumor were stained with antibody cocktail (CD24-PE, CD44-APC and EpCAM-FITC) to see what it look like in flow cytometry analysis and here due to different many cells like epithelial, fibroblast, lymphocytes and so on, it is showing different picture in comparison with cell lines. No difference is observed in hypoxic and normoxic condition. (Fig. 12)

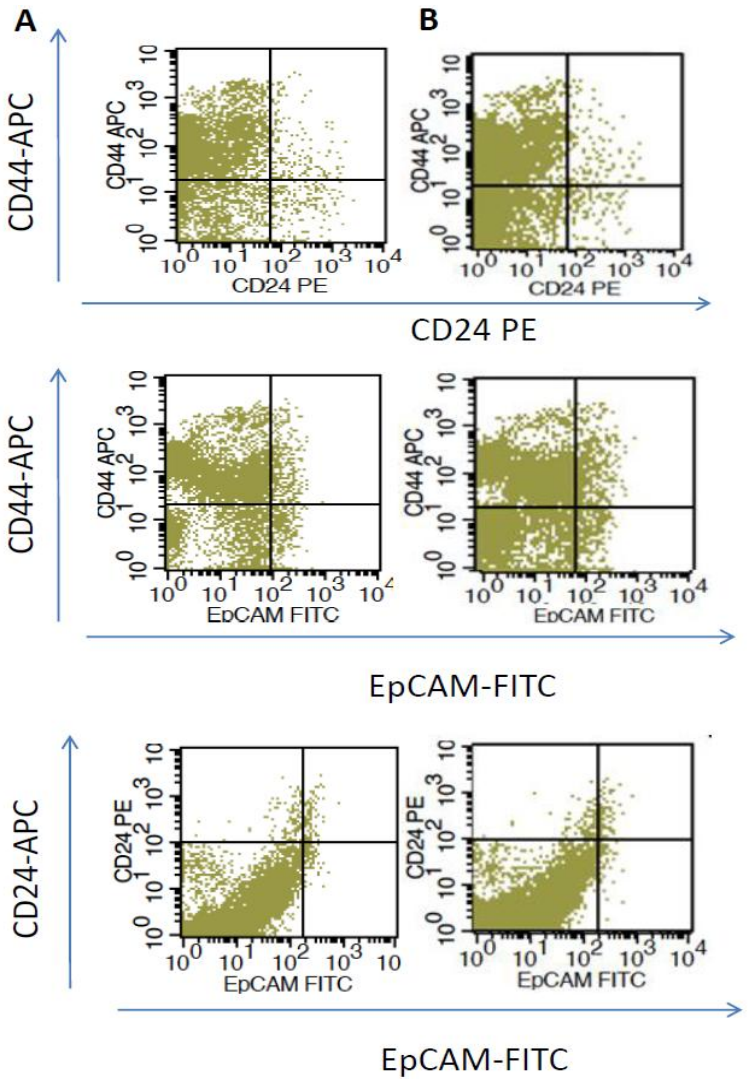

Figure 12. Flow cytometry analysis of primary cells in normoxic (21% O2) (A, C and E) and hypoxic conditions (1% O2) (B, D and F). Cells were seeded and incubated for 72 hours, stained with antibody cocktail (CD24-PE, CD44-APC and E-Cadherin-FITC) to examine the surface markers with cell population of EpCAM/CD44+/CD24- cell.

Specific retrieval of the CD24$^{-/low}$ cell population

In order to separate and separately investigate the CD44^{+}/CD24^{-} population, magnetic cell sorting (MACS) was done. Cells were bound with CD24-coupled magnetic beads and passed through a magnetic column where the magnetic beads were retained in the column, while CD24 negative cells were effluent out. To measure the specificity of the sorting procedure, sorted cells were incubated with antibody cocktail (CD24-PE, CD44-APC and EpCAM-FITC) and analyzed in flow cytometry. (Fig. 13)

To check and investigate the sorting procedure, SUM159 was used. Normally, in SUM159 it has both CD24 positive and negative cells. Same method was performed on MDA-MB-231, but this time more washing of the column was performed and when we

use plunger for positive cells unspecifically we had CD24- cells with CD24+ cells, which we don't know why? may be due to unspecific binding of the cells or due to the column or too little washing of the column.

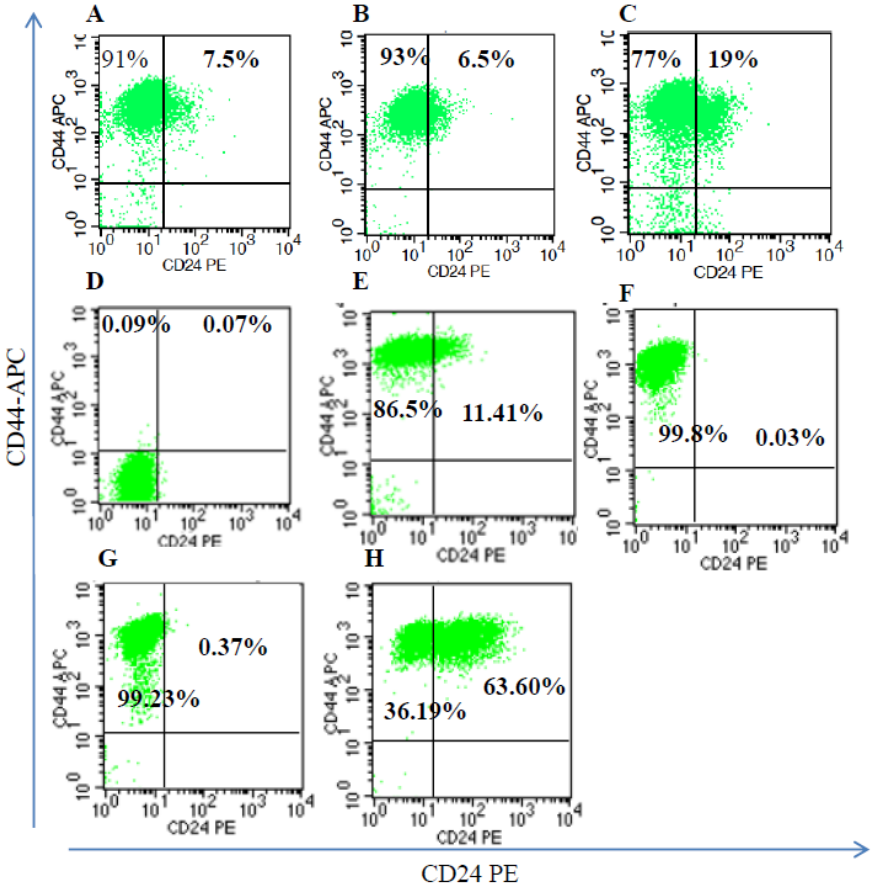

Figure 13. FACS analysis of MDA-MB-231 (A, B and C) and SUM159 (D, E, F, G and H) cell lines by magnetic beads sorting process. A. All cell population without staining with magnetic bead rather antibody cocktail, showing 91% cells are CD24-/CD44+ and only 7.5% are CD24+/CD4+. B. Non retained cells, after magnetic staining, which didn't bind with the magnet, indicating 93% CD24-/CD44+ and 6.5% CD24+/CD44+. C. Plunge retained cell, bounded with the magnetic bead and retained in the column, explaining 77% CD24-/CD44+ cells and 19% CD24+/CD44+. D. SUM159 cell population without staining, showing all cells are in negative quadrant. E. All cell population without staining with magnetic bead rather antibody cocktail, indicating 86.5% cells are CD24-/CD44+ and 11.41% are CD24+/CD44+. F. Non retained cells, after magnetic staining, which didn't bind with the magnet, showing almost 100% CD24-/CD44+. G. Magnetic column were removed from the magnet and collection of cells were taken place, still showing almost 100% CD24-/CD44+. H. Plunge retained cell with the help of plunger, bounded with the magnetic bead and retained in the column, showing 36.19% CD24-/CD44+ cells and 63.60% CD24+/CD44+. Left quadrant is showing negative population while right quadrant is showing positive cell population.

Conclusion of beads sorting

CD24 positive cells were successfully retained by the beads, CD24 negative cells were passed, but additional washing is necessary for those cells, which were lying in the CD24 negative quadrant in flow cytometry analysis.

Sorting of Cancer Stem Cells with Aldefluor assay

To detect the CSCs, the Aldefluor positive cells are proposed as another marker. In MDA-MB-231 and MCF-7 cells, investigation was performed to analyze the difference between normoxic and hypoxic cells to sort out ALDH positive cells by using Aldefluor assay for detecting ALDH1 activity followed by FACS analysis.

The DEAB treated portion of each sample was used to set the gate so that less than 1% of cells were in the gate representing Aldefluor positive cells in the DEAB containing samples (Gates R7, R5, R8 and R6 respectively. Gates are the cutoff value in reference with control for the positivity of the cells). In MDA-MB-231 no difference was observed in hypoxia treated cells compared with normoxia, while more ALDH positivity was observed in MCF-7 normoxic cells as compare to hypoxic. (Fig. 14)

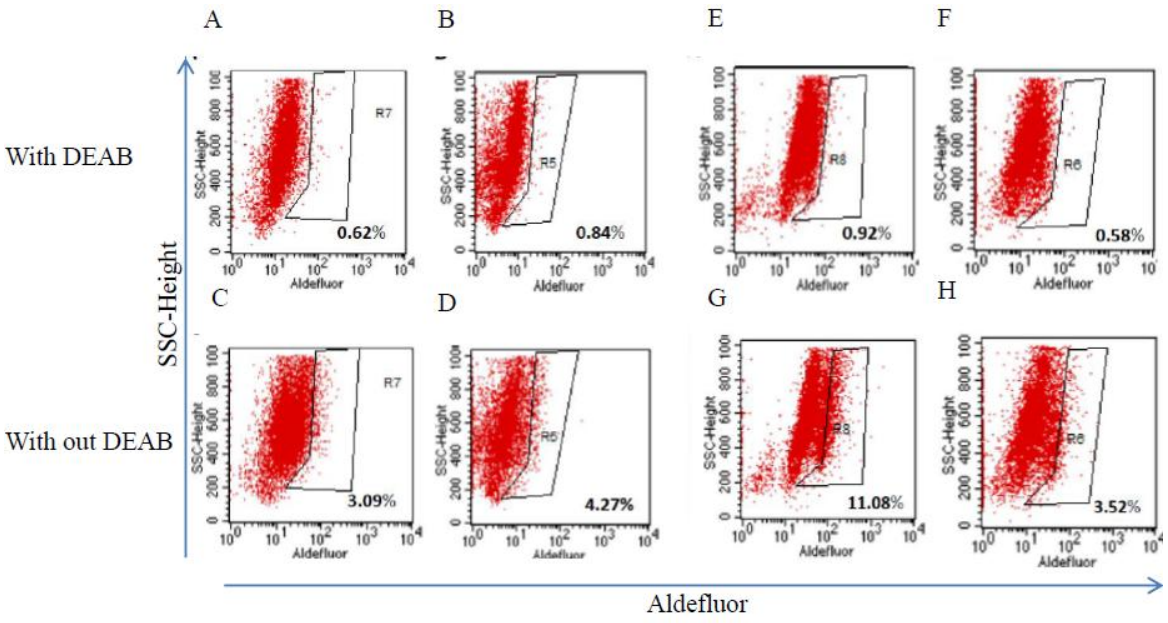

Figure 14. FACS analysis of MDA-MB-231 normoxic (21% O2) (A and C), where in A, DEAB treated cells, negative control, gate R7 wass showing 0.62% cell population, whereas in the same gate 3.09% positive cells were there in C, and hypoxic (1% O2) (B and D), where in B, DEAB treated cells, negative control, gate R7 was showing 0.84% cell population, whereas in the same gate 4.27% positive cells were there in D. MCF-7 normoxic (21% O2) (E and G), where in E, DEAB treated cells, negative control, gate R8 was showing 0.92% cell population, whereas in the same gate 11.08% positive cells were there in G, and hypoxic (1% O2) (F and H), where in F, DEAB treated cells, negative control, gate R6 was showing 0.58% cell population, whereas in the same gate 3.52% positive cells were there in H, cells were done by the Aldefluor assay, cells were stained with ALDH substrate, BAAA. Diethylaminobenzaldehyde (DEAB), were used as a cutoff fluorescence of these cells (A, B, C and D) (R7, R5, R8 and R6) and to define the Aldefluor (ALDH1)-positive region (C, D, G and H) (R7, R5, R8 and R6).

Double detection of Aldefluor activity and E-Cadherin$^{-/low}$ expression

We decided to analyze the relation between ALDH activity and E-Cadherin expression, whether E-Cadherin low cells were detected had ALDH activity or not. Double staining was performed for E-Cadherin and Aldefluor activity.

We found that the cell population, which is E-Cadherin $^{+/high,}$ has the same activity of ALDH as in E-Cadherin$^{-/low}$ cells. This is a preliminary result because we only performed this analysis once and are still working on it. Our data shows that in normoxic and hypoxic conditions, the E-Cadherin$^{-/low}$ cells and E-Cadherin$^{+/high}$ cells are same on ALDH activity scale, though EMT population increased in hypoxic conditions as we discussed earlier so the EMT cell types did not show any relation with the other proposed stemness markers. (Fig. 15)

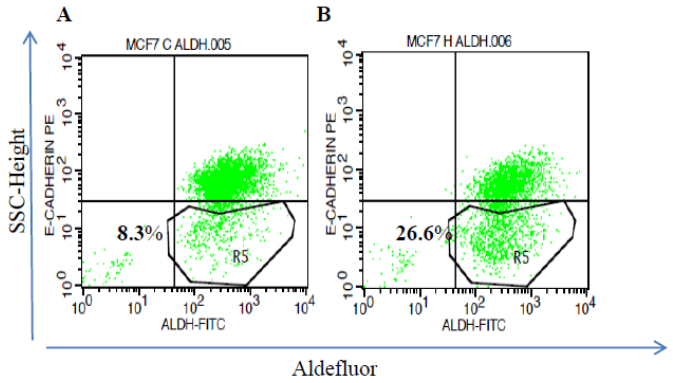

Figure 15. Flow cytometry analysis of MCF-7 cells, double staining was performed first stained with ALDH and later with E-Cadherin in A. normoxic (21% O2), showing 8.3% E-Cadherin low or negative cells and B. hypoxic conditions (1% O2), indicating 26.6% E-Cadherin low or negative cells, incubated for 72 hours.

Discussion

Study has shown that as tumor size increases, the concentration of oxygen in the intra-tumor environment is decreasing and this is termed hypoxia [32].

Nowadays, many evidences say that many human cancers are driven and maintained by CSC [49], which induces tumor metastasis and shows resistance to radiation therapy and some chemotherapy and later on tumor recurrence may take place [49]. According to the cancer stem cell hypothesis, only a specific subpopulation of cancer cells is able to propagate the cancer and form metastases. CSCs specifically have an ability to give rise to all cell types found in the same tumor [7].

In this study, we have tried different procedures and markers to detect and sort out CSCs from breast cancer cell lines. It has been proposed that hypoxia is associated with poor prognosis in cancer [32] and cell adaptation to hypoxic conditions is mainly directed by the activity of two hypoxia induced transcription factors, HIF-1α and HIF-2α that are accumulated under low oxygen conditions [32]. It has also been suggested that hypoxic

condition is essential in maintaining the cancer stem/initiating cell population [50]. To analyze the CSC population difference in hypoxic and normoxic treated cells, analysis of HIFs protein expression was monitored. We successfully showed that after 72 hours incubation the hypoxic cells expressed HIF-1 α protein expression while normoxic did not (except MDA-MB-231).

In order to investigate whether hypoxic cells has more population of $CD44^{+/high}/CD24^{-/low}$ cell as compare to normoxic cells or not, almost no difference was observed in $CD44^{+/high}/CD24^{-/low}$ cell population in normoxia and hypoxia.

It has been evident that the epithelial-to-mesenchymal-transition (EMT) is a prerequisite for tumor spread and this process has been proposed to give rise to cancer stem cells. The process of EMT has been proposed to give rise to cancer stem cells. [4]Interestingly, we successfully detected EMT cells (E-Cadherin low) in MCF-7 hypoxic cell as compare to normoxic after 72 hours of incubation. We cannot know whether these cells come from the small E-Cadherin negative population seen in normoxia, which perhaps proliferated more in hypoxic condition or whether these cells increase as a consequence of loss of E-cadherin expression in cells that previously expressed E-cadherin. We plan to isolate this cell population and analyze it for stem cell traits i.e. non-adherent culture growth and tumor induction in immune-deficient mice.

We used another proposed marker to detect CSC. Reports showed that the CSC has high activity of ALDH compared to non-CSC. We found a small population of ALDH high fluorescent cells, but no surety can be given whether this small population of cell is CSC or not. To prove its stemness property, more work and efforts would be performed, first to sort out the ALDH positive cells and later grow in non-adherent culture and also inject sorted cells in immune-deficient mice

We performed double detection for E-Cadherin and Aldefluor activity to analyze if the cells that were negative in E-Cadherin had any relation with ALDH activity positive cells, but no difference was found, both E-Cadherin positive and negative cells have the same activity of ALDH. (Fig. 15)

Finally to sort out specifically CSC ($CD44^{+}/CD24^{-}$) population, magnetic bead sorting (MACS) analysis procedure was used. We showed results successfully for cell sorting, but to determine if the selected populations contain the CSCs functional assay confirmations are required. We would like to optimize this method more and plan to analyze the ability of these cells to grow in non-adherent cultures and also inject cells in immune-deficient mice. If sorted cells contain the CSC population they should be able to grow in non-adherent culture and to give rise to all cell type of tumor and also to induce tumor in mice. It then remains for us to investigate whether these cells express more of the HIFs than the non-CSC population.

In conclusion, significant increase was observed in E-Cadherin low cells population in MCF7 under hypoxic conditions, since it has been proposed that hypoxic condition is essential to maintain CSCs population. We also showed the success full sorting of stem cells type phenotype marker ($CD44^{+/high}CD24^{-/low}$). We also analyzed that in hypoxic

condition; HIF protein is stabilized and highly expressed in all cell lines as compare to normoxic conditions. Our results indicated that Aldefluor assay showed small population of positive cells when compare with DEAB treated cells. But, almost no difference is observed in ($CD44^{+/high}CD24^{-/low}$) population in normoxic and hypoxic treated cells.

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