SJIF Impact Factor: 6.260| ISI I.F.Value:1.241| Journal DOI: 10.36713/epra2016 ISSN: 2455-7838(Online)
EPRA International Journal of Research and Development (IJRD)
Volume: 5 | Issue: 3 | March 2020 - Peer Reviewed Journal

HYPOXIA INDUCES CANCER STEM CELL CHARACTERISTICS, CHEMORESISTANCE AND MIGRATION/INVASION TRAITS IN TRIPLE NEGATIVE BREAST CANCER CELL LINES

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ABSTRACT
Cancer is rapidly becoming a global pandemic. Even with the improvement in overall survival of Breast Cancer (BC) patients due to early detection and advancement with systematic therapy, triple negative breast cancer (TNBC), an aggressive subtype of BC still remains a major challenge as it lacks targetable receptors. Chemotherapy is the main treatment for TNBC. This study was performed to investigate if hypoxia induces cancer stem cells (CSC)s traits in TNBC cell lines; to examine the response of these cells to conventional anticancer drugs and to confirm if hypoxia induced epithelial to mesenchymal transition (EMT) which determines the process of cell metastasis. Data from MTT analysis, western blot and fluorescence-activated cell sorting (FASC) showed that hypoxia induces CSC-like characteristics in breast cancer cell line and the cells were significantly resistance to several chemotherapeutic drugs. The hypoxic cells show slow proliferation rate but displayed high migratory and invasive potentials, therefore hypoxia induces these CSC-like phenotype leading to chemoresistance.

KEY WORDS: *Hypoxia, Cancer stem cells, TNBC breast cancer cell line, cross-resistance, chemotherapy*

INTRODUCTION

Triple negative breast cancer (TNBC) is the subtype of breast cancer that does not express genes for oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Curigliano and Goldhirsch, 2011; Penault-Llorca and Viale, 2012). Scientific advancement in Breast Cancer (BC) research have improved BC management with resultant increase in survival rate, however TNBC is still a difficult cancer to treat because of the lack of targetable receptors and is an assemblage of different breast cancer subtypes.

Stem cell ''niche'' a specialized microenvironment within tumours maintains the properties of cancer stem cells (CSCs) self-renewal and multipotency (Hill *et al.,* 2009). The stem cell niche contains CSCs and these cells are mostly in a hypoxic state. Reports by Ezashi *et al.*, (2005) showed that the low O_2 tension (1%) in the niche decreased cell proliferation and maintained pluripotency while higher oxygen tension (3-5%) maintained pluripotentency of cells but had no effect on their proliferation rate. These results suggest that the hypoxic niche maintains slow-cycling proliferative rate and quiescence in relation to the oxygen tension within tumours. Hypoxia Inducible Factor (HIF) protein activated under hypoxia also regulates signalling pathways that maintain stem cell self-renewal and multipotency. $CD133^+$ CSCs from gliomas were found to overexpresses HIF2α and other HIF regulated genes compared to non-stem cells or normal neural progenitor cells, inhibition of HIF2α reduced self-renewal, proliferation and survival *in vivo* with diminished tumour initiation potential of glioma CSCs (Li *et al.,* 2009). This finding was also confirmed by Franovic *et al.* (2009) in which

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inhibition of HIF2α in colorectal, non-small cell lung cancer and gliobastoma that harboured CSCs population prevents *in vivo* growth and tumourigenesis irrespective of the mutational status and tissue origin (Franovic *et al.,* 2009).

HYPOXIA INDUCED EMT

High invasion and metastasis are the principal factors causing poor prognosis of patients with TNBC. Approximately 90% of cancer related death results from the metastatic spread of primary tumours (Christofori, 2006). Hypoxic response regulated by the HIF protein particularly HIF1α activation correlates with tumour metastasis by promoting tumour cell metastatic potential. HIF1α is suggested to meditate the repression of E-cadherin as overexpression of HIF1α upregulates repression of E-cadherin repressors twist, Snail and SIP1 with associated loss of E-cadherin and overexpression of N-cadherin and Vimentin which promotes epithelial to mesenchymal transition (EMT) and metastatic phenotypes in human cancer cells (Evans *et al.,* 2007).

CSCs are widely accepted to be responsible for multidrug resistance in cancer cell and are believed to be one of the reasons for chemotherapeutic failure and cancer relapse. Previous studies have shown that hypoxia play key role in the induction and maintenance of CSCs traits, chemoresistance and metastasis in cancer cells (Li *et al.,* 2009, Franovic et al., 2009).

Rationale and aims of the study

This study was carried out to confirm if hypoxia is able to induce CSCs traits in triple negative breast cancer (TNBC) cell lines, and to examine the response of these cells to conventional anticancer drugs. Hypoxia induced EMT was also examined to determine the process of cell metastasis.

METHODOLOGY

Cell lines and reagents:

TNBC cell lines MDA-MB 231 and BT 549 were purchased from ATCC, Middlesex, UK. HypoxyprobeTM-1 plus Kit (Burlington, MA, USA). Gemcitabine (dFdC), Paclitaxel (PTX), Cisplatin (CDDP), Vincristine (VCR), Doxorubicin (DOX) were purchased from Sigma.

Culturing cells under hypoxic conditions

Monolayer TNBC breast cancer cell lines MDA-MB 231 and BT 549 were grown under hypoxic conditions using STEMCELL Technologies Hypoxia incubator chamber.

Detection of hypoxia in hypoxic cell cultures

The hypoxic status of MDA-MB 231 and BT 549 cells grown in hypoxic conditions was determined using HypoxyprobeTM-1 plus Kit. Additional confirmation of the hypoxia status was done by western blot analysis of hypoxia-inducible factors (HIFs).

Determination of EMT properties of cells

Hypoxia is believed to induce epithelial to mesenchymal transition (EMT). *In vitro* wound healing assay (scratch assay) for migration and transwell invasion assay (Boyden chamber assay) were performed in hypoxic and normoxic cultures to determine the invasive and migratory properties of the cells.

Detection of ALDH positive population

The Aldehyde dehydrogenase (ALDH) positive population was detected by ALDEFLUOR kit (StemCell Tech., Durham, NC, USA) following the supplier's instruction. The cells (2.5 × 105) were analyzed after stained in ALDH substrate containing assay buffer for 30 min at 37°C. The negative control was treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor.

Flow cytometric analysis of CD 133

The adherent were trypsinised and passed through a 25G needle. The cells (2.5 × 105) were incubated with CD 133 antibody (BD Pharmingen, Oxford, UK) for 20 min at 4°C. Unbound antibodies were washed off with 2% fetal calf serum (FCS) HBSS (Sigma) and the cells (10,000 events) were examined no longer than 1 hour after staining on a BD Facscalibur.

Immunofluorescent flow cytometric analysis of embryonic stem cell markers

The expression of Nanog, Oct4 and Sox2 was determined by immunofluoresent flow cytometry. The cultured cells were collected by trypsinization. The cells fixed by acetone/methanol and permeabilized by 0.1% triton-X100. After blocked with 3% BSA for 1 hour the cells were stained with primary (1:50 dilution) and FITC-conjugated secondary antibodies respectively for 1 hour at RT. The positively stained population was detected using a FACS Calibur flow cytometer with 488-nm blue laser and standard FITC 530/30 nm band pass filter.

MTT cytotoxicity assay for hypoxic cell cultures

The overnight cultured cells (5000 per well) in 96-well flat-bottomed microtiter plates were exposed to drugs for 72 hours (PAC), dFdC 72hours, Vincristine (VCR) 48hours, DOX 72hours and 120 hours (CDDP) and subjected to a standard MTT assay (Plumb et al, 1989).

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Western blotting analysis: The protein expression levels were determined by staining with primary antibodies and relevant HRP conjugated secondary antibodies. Primary antibodies N-caderin, E-caderin, Vimentin, hypoxia-inducible factors (HIFs 1 and 2) supplied by Santa Cruz, Dallas, TX, USA.

Growth curves and doubling time analysis

To examine the growth profile and proliferative rates of hypoxic cells, growth curve and doubling time analysis was performed on MDA-MB 231 and BT 549 cell lines.

Statistical Analysis

SPSS 13.0 Student's *t* test and one way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test were used to calculate the differences. Data were expressed as mean ± SD. P≤0.05 was considered as significantly change.

RESULTS

Increase proportion of hypoxic cells in hypoxic culture

Monolayer breast cancer (BC) cells lines grown in hypoxic culture were compared with those grown under normoxia using hypoxyprobe. The results obtained from *Fluorescence Activated Cell Sorting (*FACS) analysis showed that a substantial number of cells grown in hypoxic culture had an increase in proportion of hypoxic cell (%) compared to those cultured in normoxia (Figure 1). Further analysis for the confirmation of hypoxia was done by western blot and the results show an increase and nuclear translocation in HIFs in the hypoxic culture when compared to the normoxia culture of BC cell lines (Figure 2)

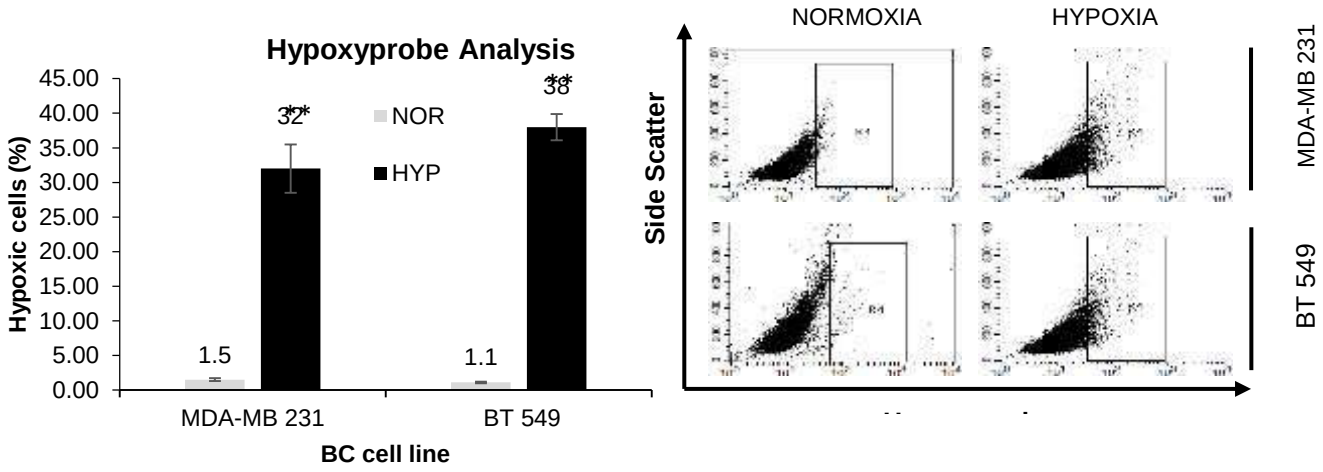

Figure1: Representative FACS Plots of expression and Bar chart representation of hypoxic and normoxic populations in BC cell lines (Mann and Whitney U test, ***p=<0.05, n=9*).

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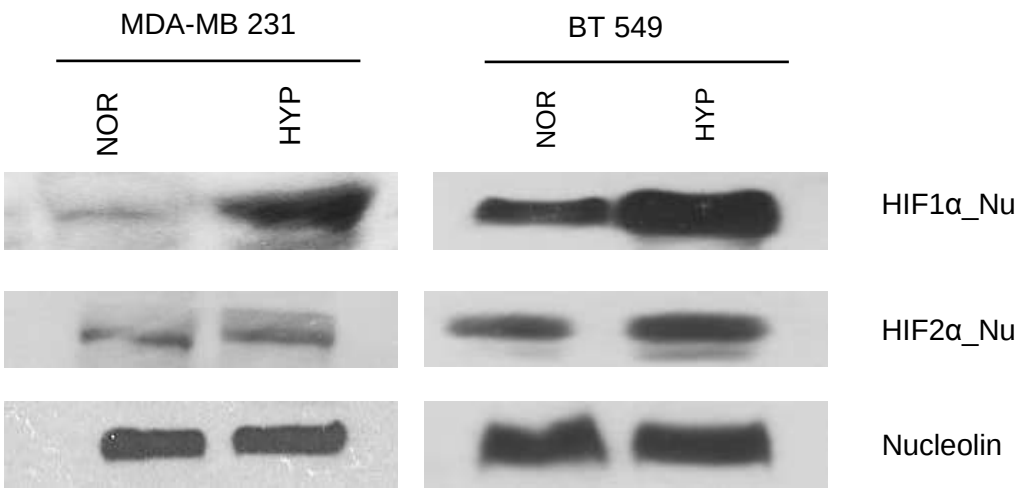

Figure 2. Representative Western blots of hypoxia-inducible factors (HIFs) in BC cell lines: (NOR: normoxic culture, HYP: hypoxic culture, HIF: hypoxia-inducible factor).

Hypoxic cells significantly resistant to anticancer drugs

To study the effect of hypoxia in the induction of chemoresistance, cisplatin (CDDP), a first line drug for TNBC and four conventional anticancer drugs paclitaxel (PTX), doxorubicin (Dox), vincristine (VCR) and gemcitabine (dFdC) were tested on cells cultured in both hypoxic and normoxic conditions. MTT cytotoxicity results showed that the cells grown in hypoxic conditions were resistant to all five anticancer drugs tested, while the cells cultured in normoxic conditions were killed at low concentrations of the drugs. The hypoxic cells were able to survive at even high concentrations of anticancer drugs used. The half maximal inhibitory concentration (IC_{50}) values indicates how much of the anticancer drug is needed to inhibit cell growth expressed as cell viability. Figure 3 (A) and (B) shows that cell viability curves and bar charts (IC_{50} values) for MDA-MB 231 and BT 549 breast cell lines respectively grown in hypoxia were significantly higher than their normoxic culture.

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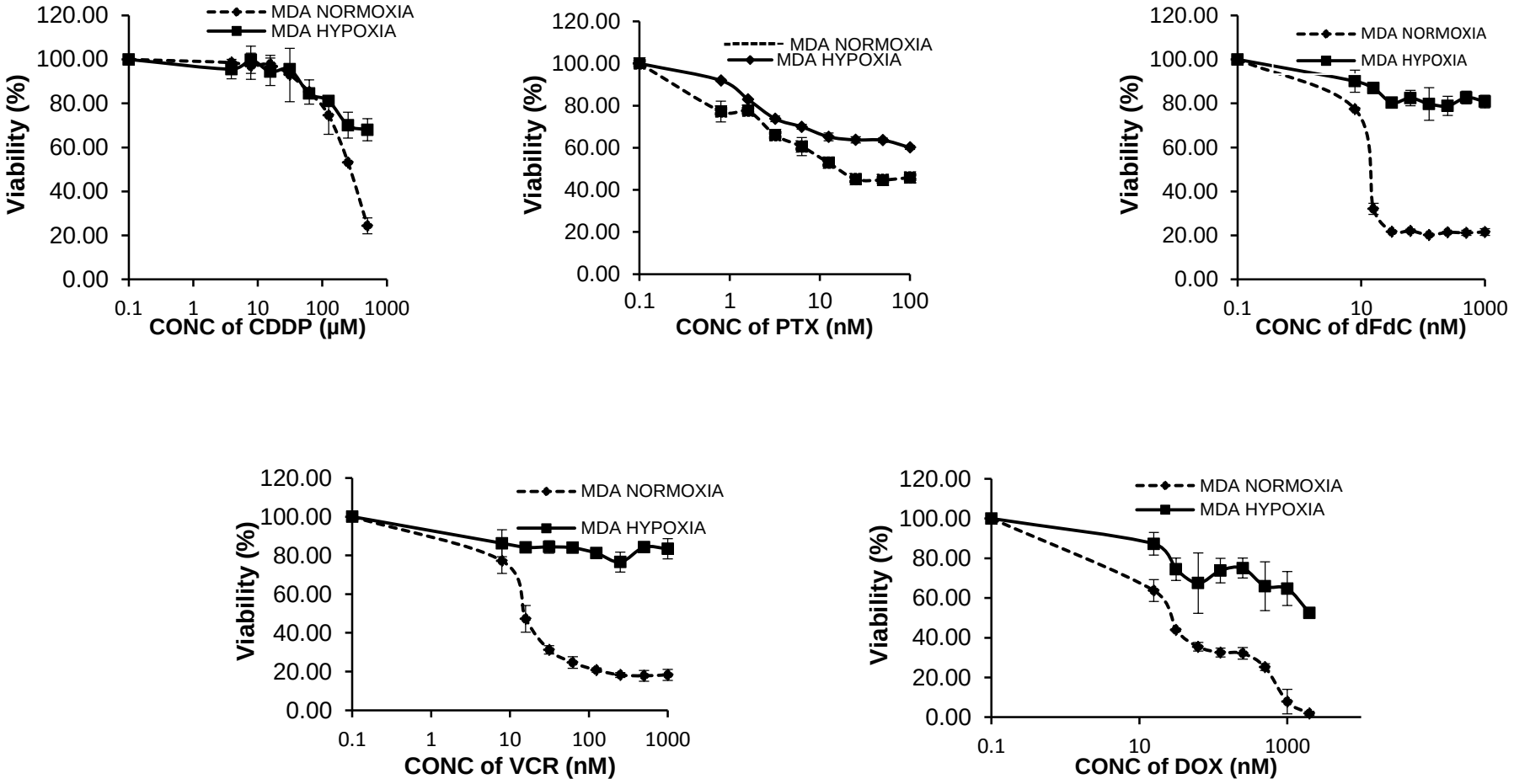

Figure 3(A1). Representative Drug Concentration Response Curves of MDA-MB 231 in normoxic and hypoxic culture. Cell viability curve shows difference in dose response between normoxia and hypoxia cultures. The hypoxic cells were highly resistant to the CDDP, DOX, PTX, dFdC and VCR while cell death was induced in the normoxic cells at much lower concentrations of these anticancer drugs. (MDA: MDA-MB 231 cell line, Cisplatin (CDDP), paclitaxel (PTX), doxorubicin (Dox), vincristine (VCR) and gemcitabine (dFdC))

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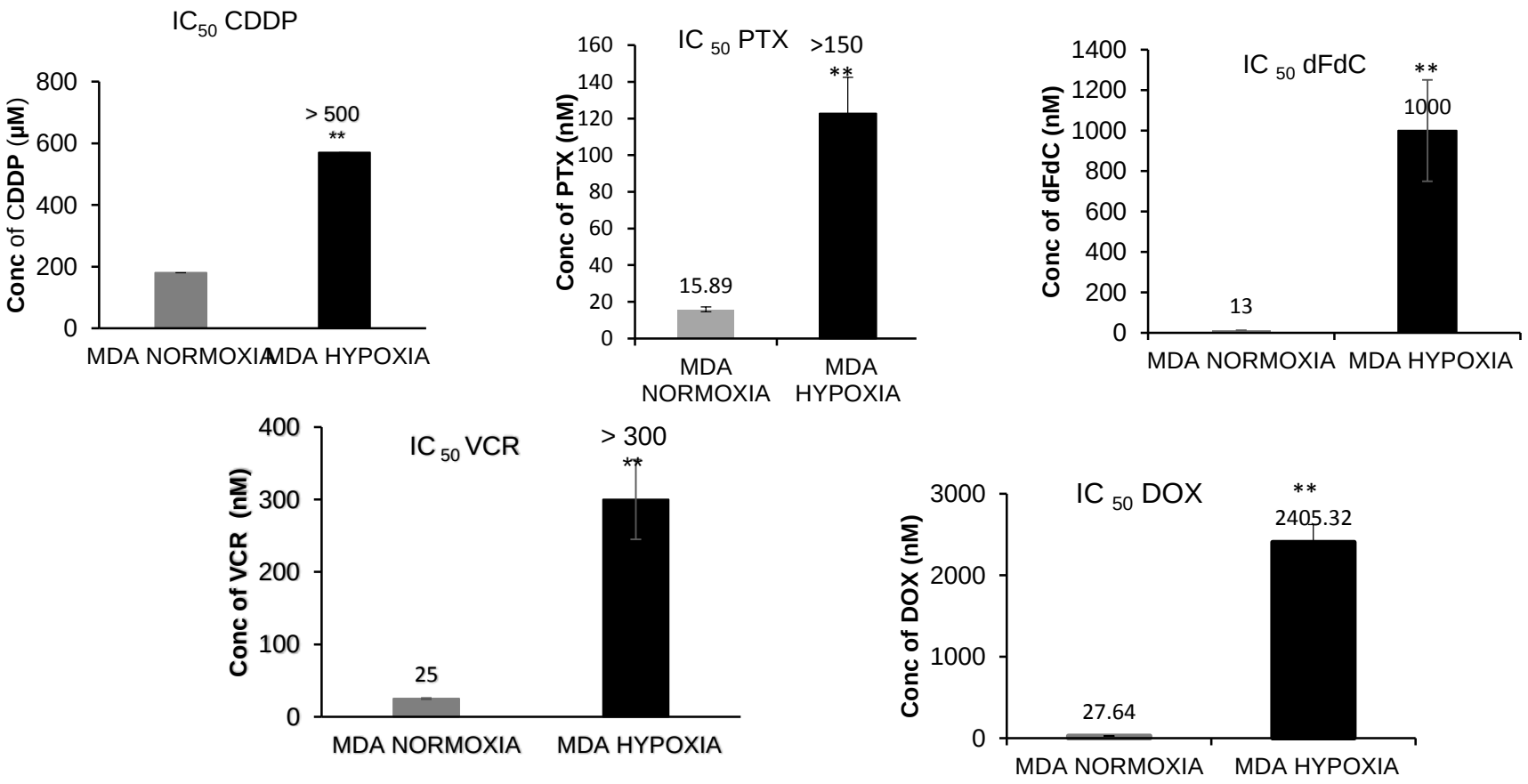

Figure 3(A2). Bar chart representative of IC_{50} values of normoxic and hypoxic cells treated with anticancer drugs. The histograms (median ± interquartile range) shows the elevated half maximal inhibitory concentration (IC_{50}) values for hypoxic cells indicating increased drug to 5 anticancer drug tested cisplatin (CDDP), paclitaxel (PTX), doxorubicin (Dox), vincristine (VCR) and gemcitabine (dFdC), (Mann and Whitney U test, **p=<0.001, n=9).

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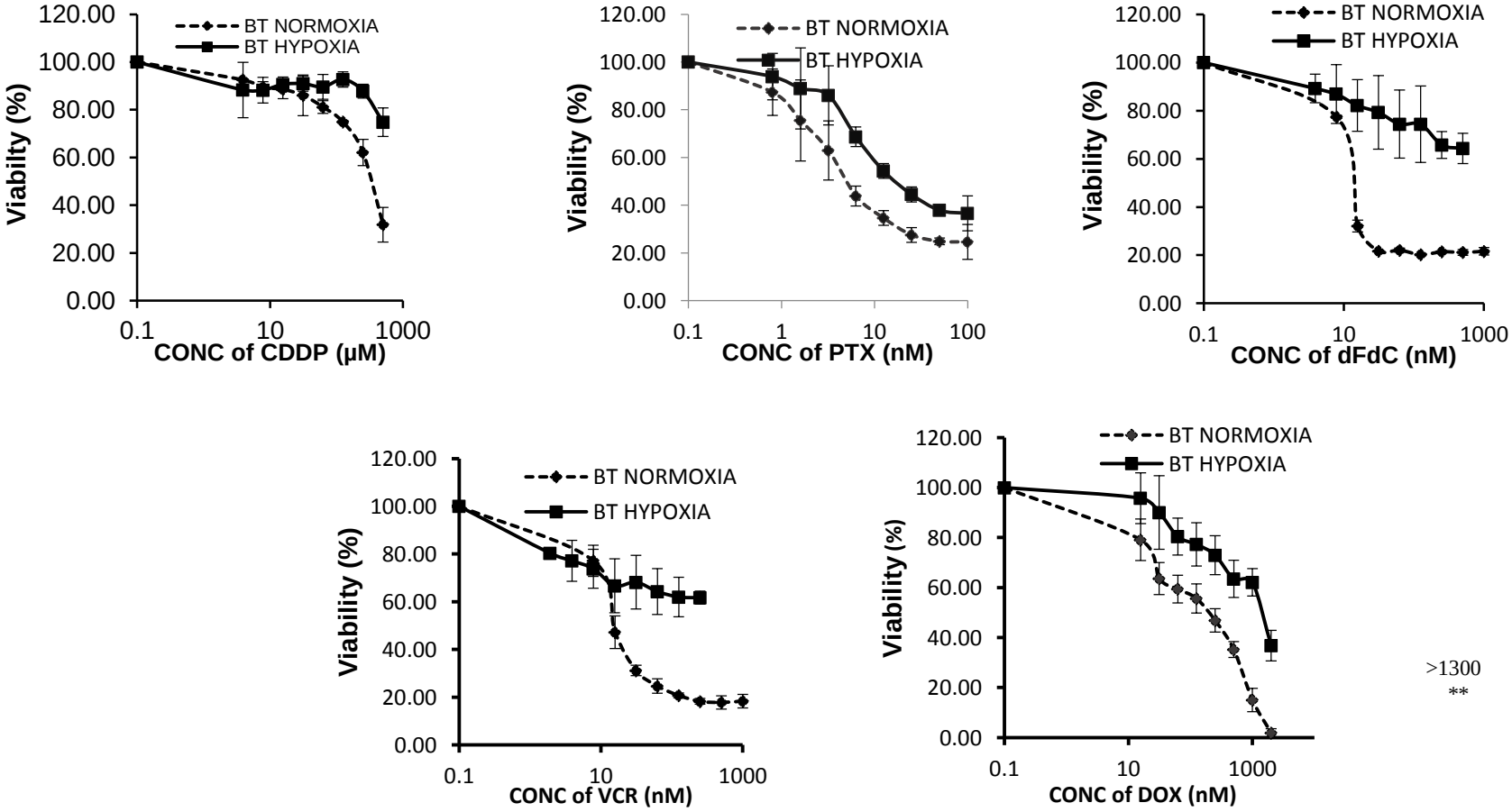

Figure 3.(B1). Representative Drug Concentration Response Curves of BT 549 in normoxic and hypoxic culture. Cell viability curve shows difference in dose response between normoxia and hypoxia cultures. The hypoxic cells were highly resistant to the CDDP, DOX, PTX, dFdC and VCR while cell death was induced in the normoxic cells at much lower concentrations of these anticancer drugs. (BT: BT 549 cell line, Cisplatin (CDDP), paclitaxel (PTX), doxorubicin (Dox), vincristine (VCR) and gemcitabine (dFdC)).

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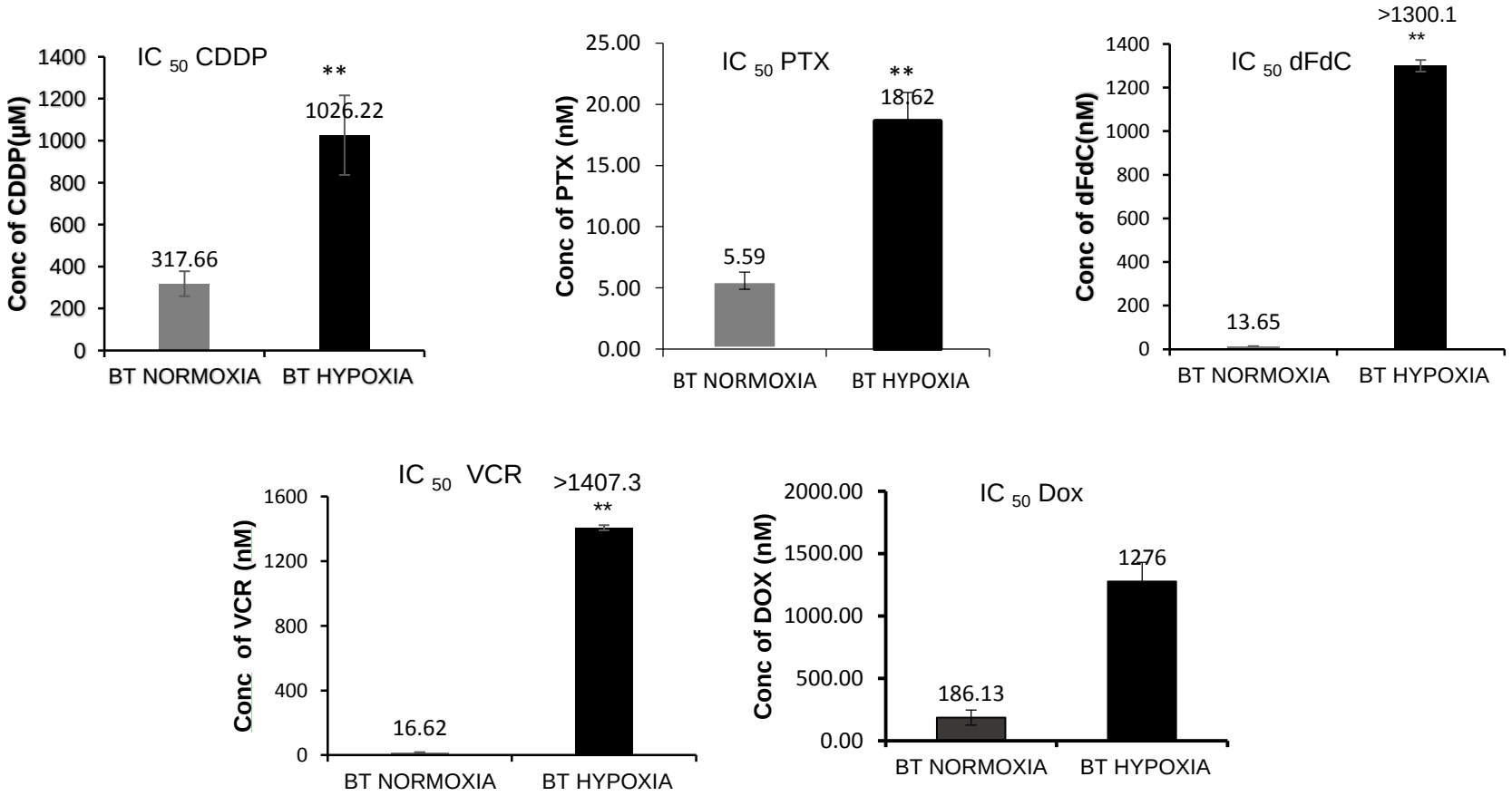

Figure 3(B2) Bar chart representative of IC_{50} values of normoxic and hypoxic cells treated with anticancer drugs. Histogram (median ± interquartile range) shows elevated half maximal inhibitory concentration (IC_{50}) values for hypoxic cells indicating increased drug resistance to 5 anticancer drug cisplatin (CDDP), paclitaxel (PTX), doxorubicin (Dox), vincristine (VCR) and gemcitabine (dFdC) (Mann and Whitney U test, resistance, **p=<0.001, n=9).

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Hypoxic cells express high CSCs markers

Fluorescence-activated cell sorting (FASC) analysis showed that BC cells grown under hypoxia had higher expression of CSC markers ($ALDH^+$, CD133) FASC results shows ALDH activity in hypoxic and normoxia cells with and without treatment with DEAB (30µM). Histograms (median ± interquartile range) displays statistically significant increase in the $ALDH^+$ activity in hypoxic cultures in comparison to their respective normoxic cultures (Mann and Whitney U test, ***p=<0.0001, n=9*). After treatment with DEAB, there was no statistical difference in ALDH+ cell from both cultures (Mann and Whitney U test, p>0.05). (Figure 4 A and B), when compared to cells cultured in normoxic conditions. The expression of CD 133 (Figure 4C); embryonic stem cell proteins Sox2, Oct4 and Nanog which regulates stemness, self-renewal and pluripotency of cells were found to be significantly increased in hypoxia in both MDA-MB 231 (Figure 5) and BT 549 (Figure 6). Growth curve experiments were also conducted and the results (Figure 7) obtained demonstrated that hypoxic cells had a significant decrease in growth rate and doubling time indicating quiescence which is an important stem cell feature.

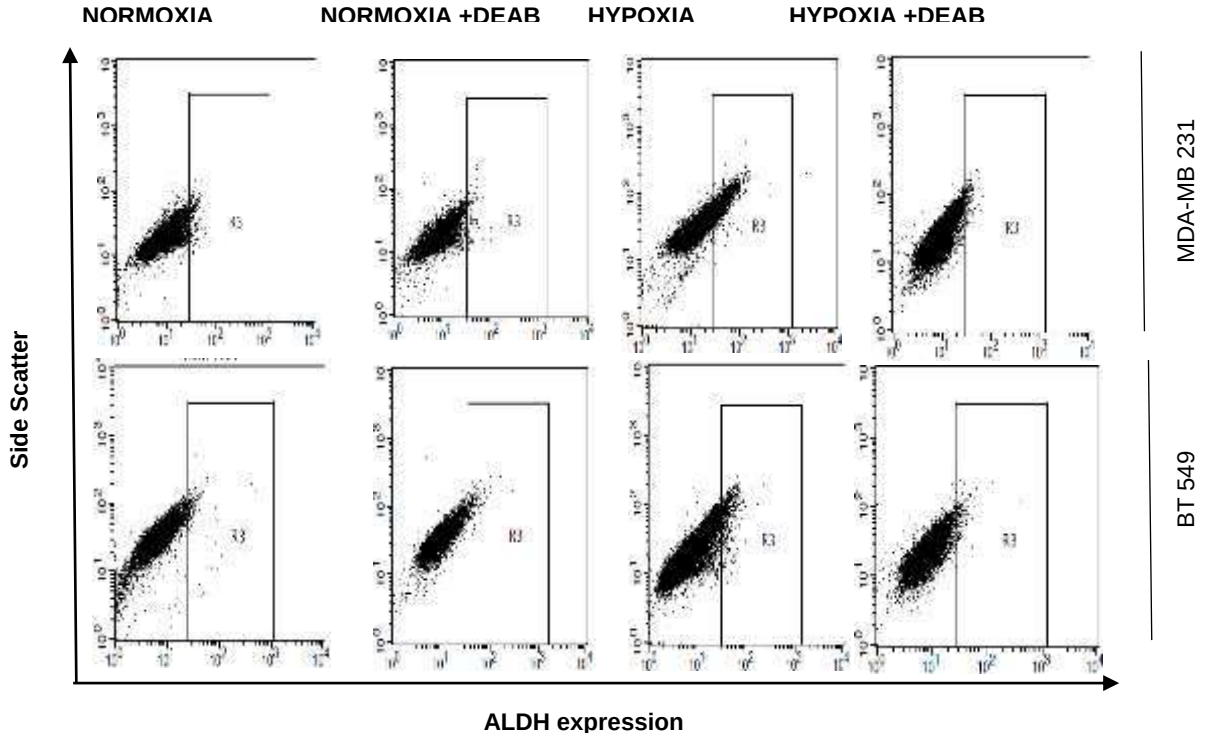

Figure 4(A1). Representative FASC Plots of ALDH expression in normoxic and hypoxic cultures measured by ALDEFLUOR assay. (Abbreviations: DEAB: Diethylaminobenzaldehyde; ALDH: Aldehyde dehydrogenase activity, FASC: *Fluorescence Activated Cell Sorting).*

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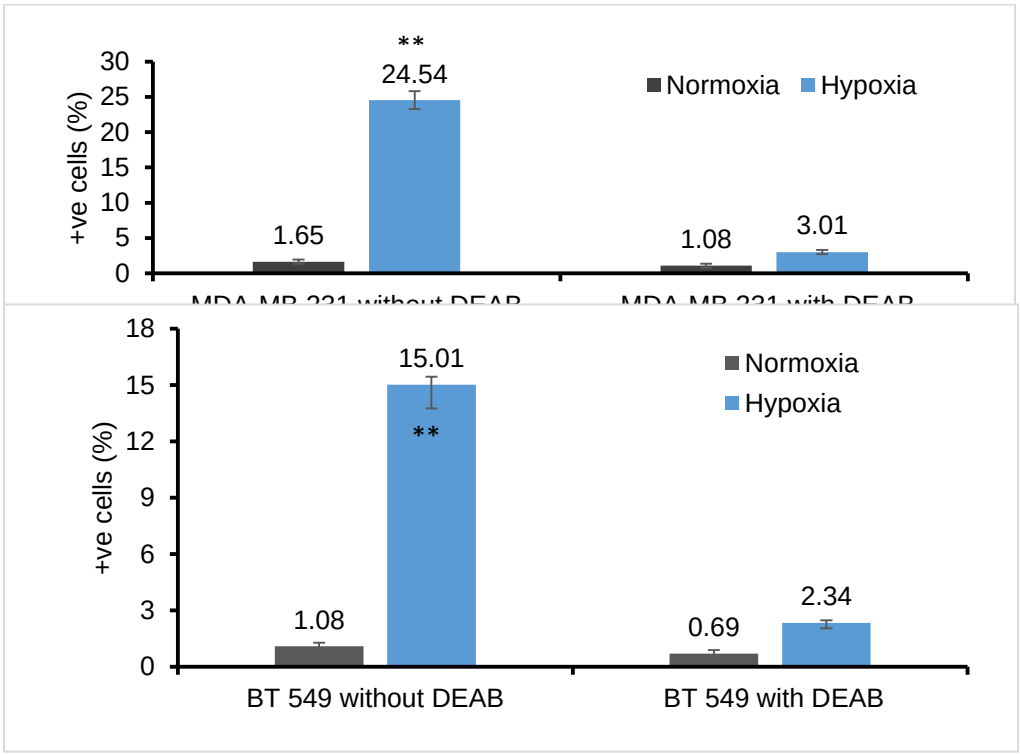

Figure 4B. Bar chart representation of ALDH+ cells in hypoxic and normoxic populations in BC cell lines. (Abbreviations ALDH: aldehyde dehydrogenase, DEAB: diethylaminobenzaldehyde).

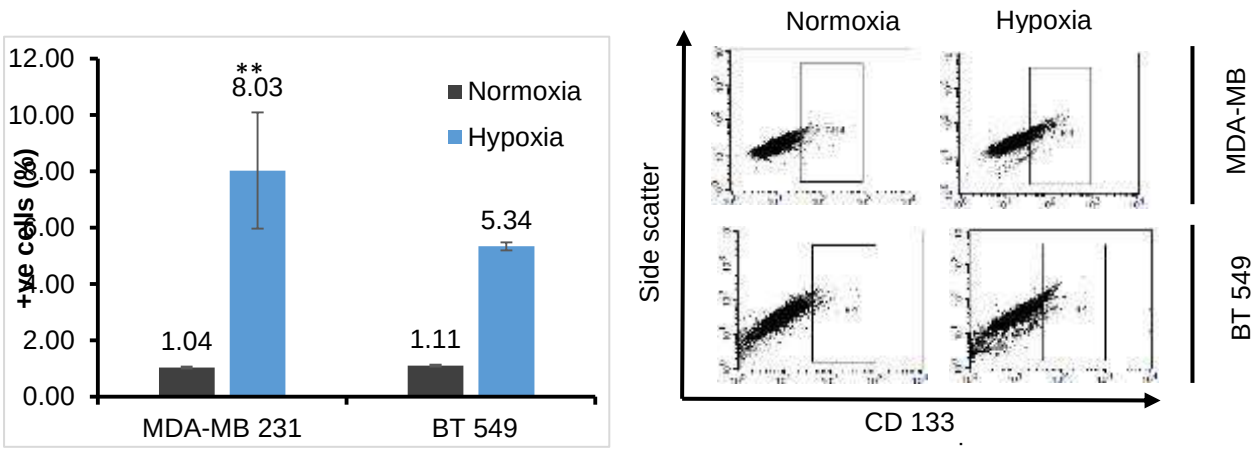

Figure 4 (C) Representative FASC Plots and Bar Chart of CD133 expression in normoxic and hypoxic cultures measured using CD133-PE conjugated antibody. (Mann and Whitney U test, **p=<0.001, n=9*). (Abbreviations FASC: *Fluorescence Activated Cell Sorting).*

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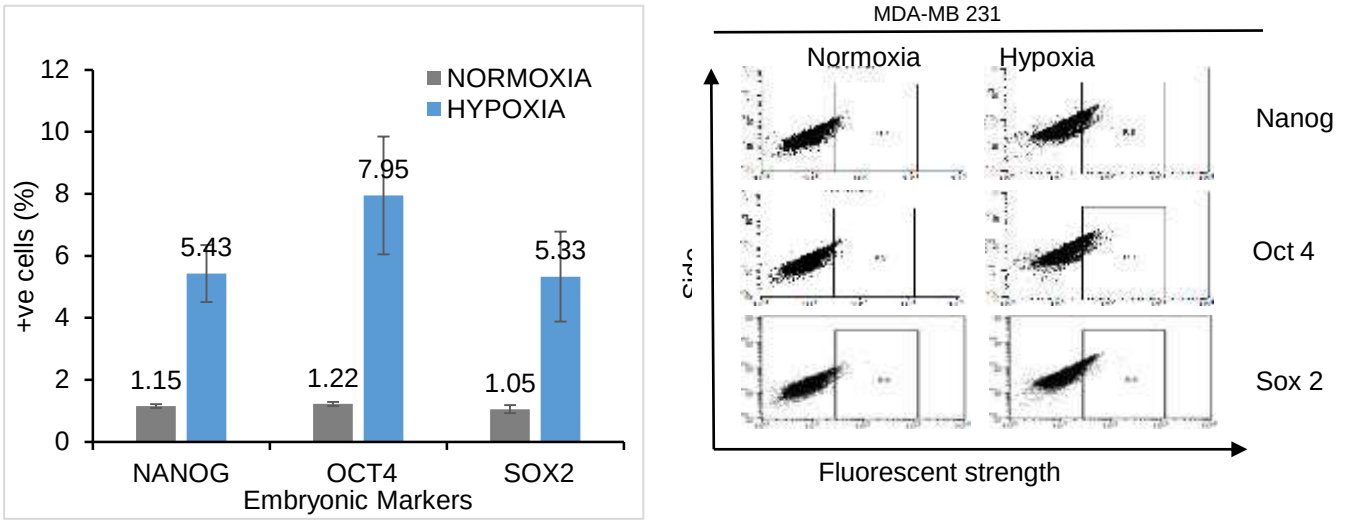

Figure 5. Representative Bar Chart and FASC Plots of Embryonic CSC markers Expression in normoxic and hypoxic cultures of MDA-MB 231 cell line

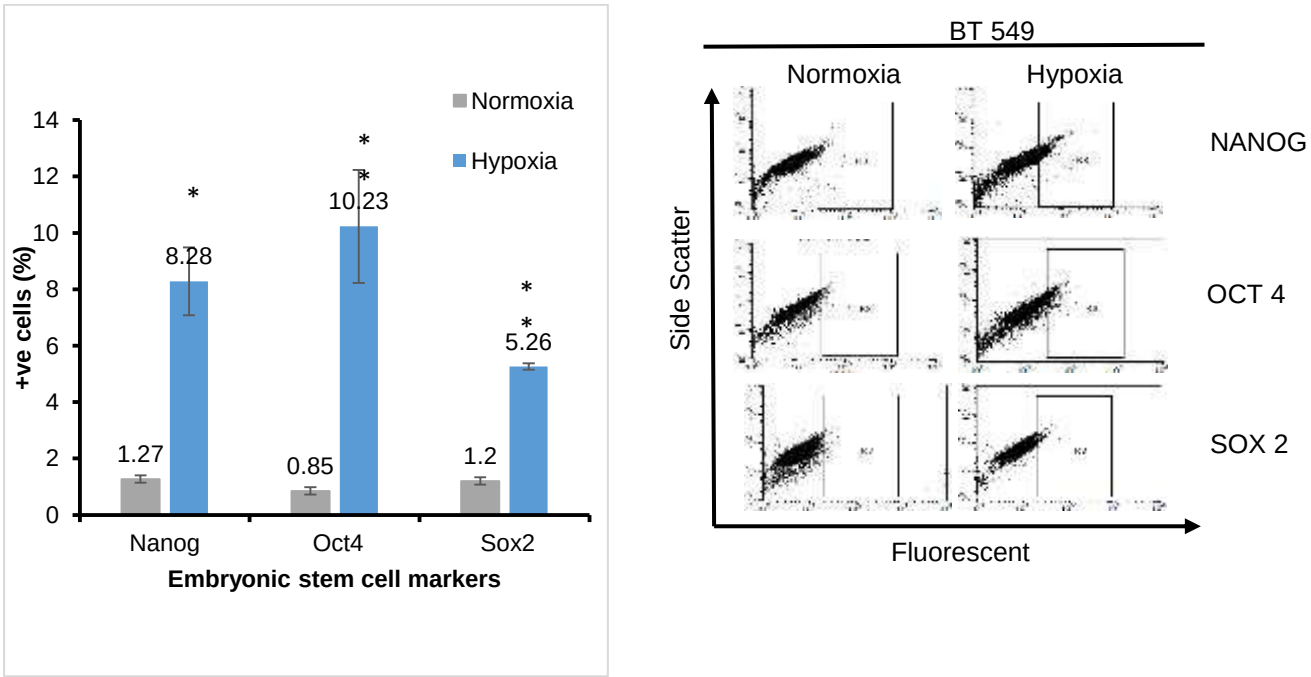

Figure 6. Representative FASC Plots of Embryonic CSC markers Expression in normoxic and hypoxic cultures of BT 549 cell line.

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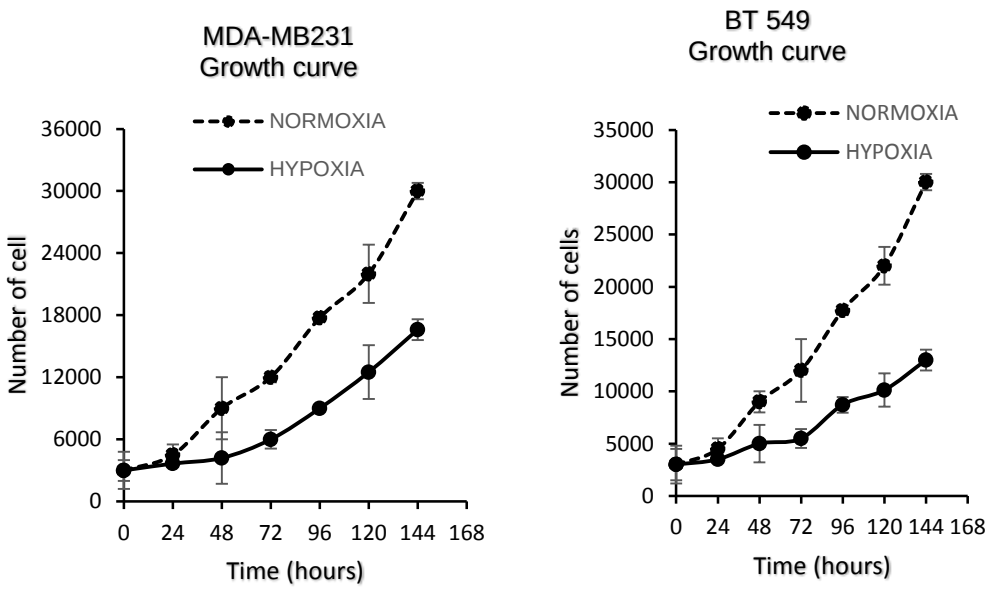

Figure 7. Representative Growth curves of MDA-MB 231 and BT549 cancer cell lines cultured in normoxia and hypoxia. (Mean± SD, n=6).

Hypoxia induces EMT in BC cell lines

Results from western blot protein analysis showed that cells grown in hypoxia were found to have higher expression of EMT markers such as increase in N-cadherin and decrease in E-cadherin expressions, this switch indicates the cells obtained a more mesenchymal characteristic. Vimentin expression was also increased (Figure 8). Whereas in cells grown under normoxia there was no loss of E-cadherin and hence they remain epithelial in nature. These results suggest that hypoxia can transform the cells into a more mesenchymal phenotype and can be reversed to epithelial nature if the cells were to return to normoxia.

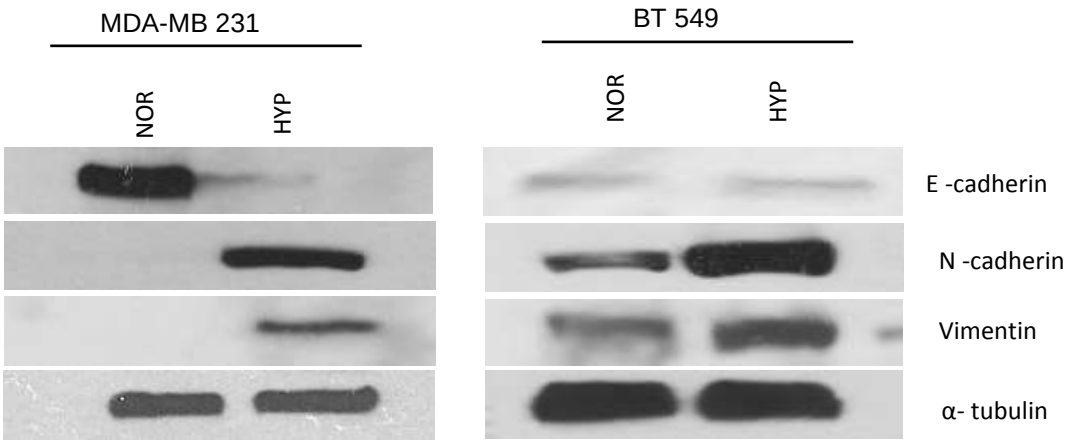

Figure 8. Representative Western Blots of EMT markers in hypoxic cells using whole cell lysates of NOR and HYP cultures of breast cell lines. (NOR-normoxic culture, HYP: hypoxic culture).

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Hypoxic cells display increase migration and invasive potential

Further confirmation of hypoxia-induced epithelial to mesenchymal transition (EMT) and transformation of cells into mesenchymal phenotype was done by analysing the migratory potential of cells under hypoxia. The images taken using the inverted microscope for Boyden chamber migration assay and the optical density (OD) measured from the experiment enabled determination of percentage migration between normoxic and hypoxic cell cultures. Results from the analysis (Fig.9) showed significant increase in the migratory potential of cells grown under hypoxic conditions. Histograms (median ± interquartile range) shows statistically significant invasive rate of hypoxic cells (Mann and Whitney U test, $**p=<0.0001, n=6$). Mesenchymal phenotypes are also known to have increased invasion potential that can be measured by transwell matrigel invasion assay. Cells with mesenchymal characteristics have the potential to produce matrix metalloproteinases that can digest the extracellular membrane or substance like matrigel that enables them to invade the gel and move out of the matrix towards chemoattractants like serum or growth factors. Images taken from the above experiment shown in Figure 10 indicate a remarkable increase in the invasion potential of cells grown under hypoxia. The results given as invasion index (%) confirm the increased invasion potential of hypoxic cell cultures which is statistically significant.

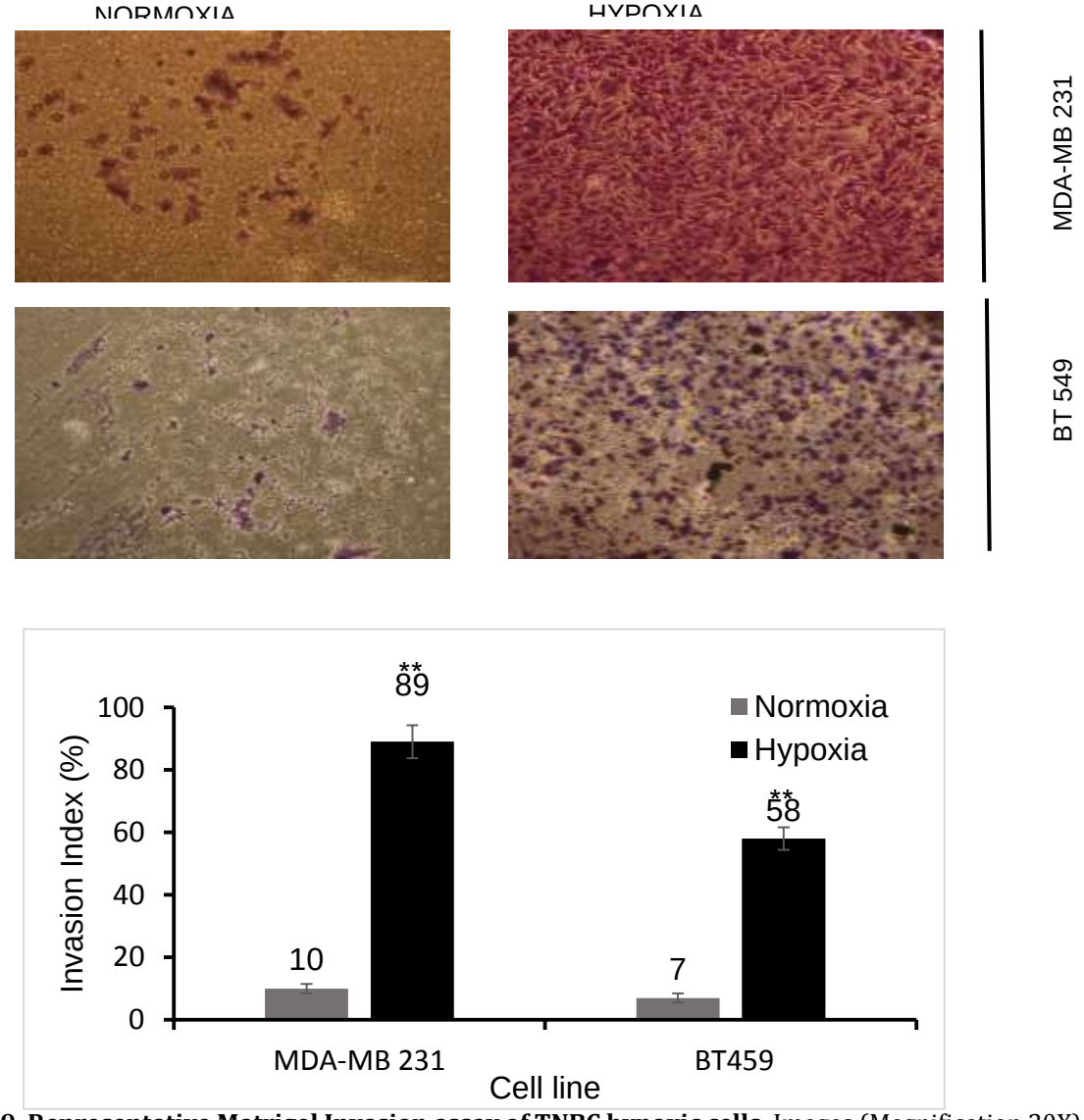

Figure 9. Representative Matrigel Invasion assay of TNBC hypoxic cells. Images (Magnification 20X) (Abbreviations Triple negative breast cancer (TNBC). Epithelial to mesenchymal transition (EMT)).

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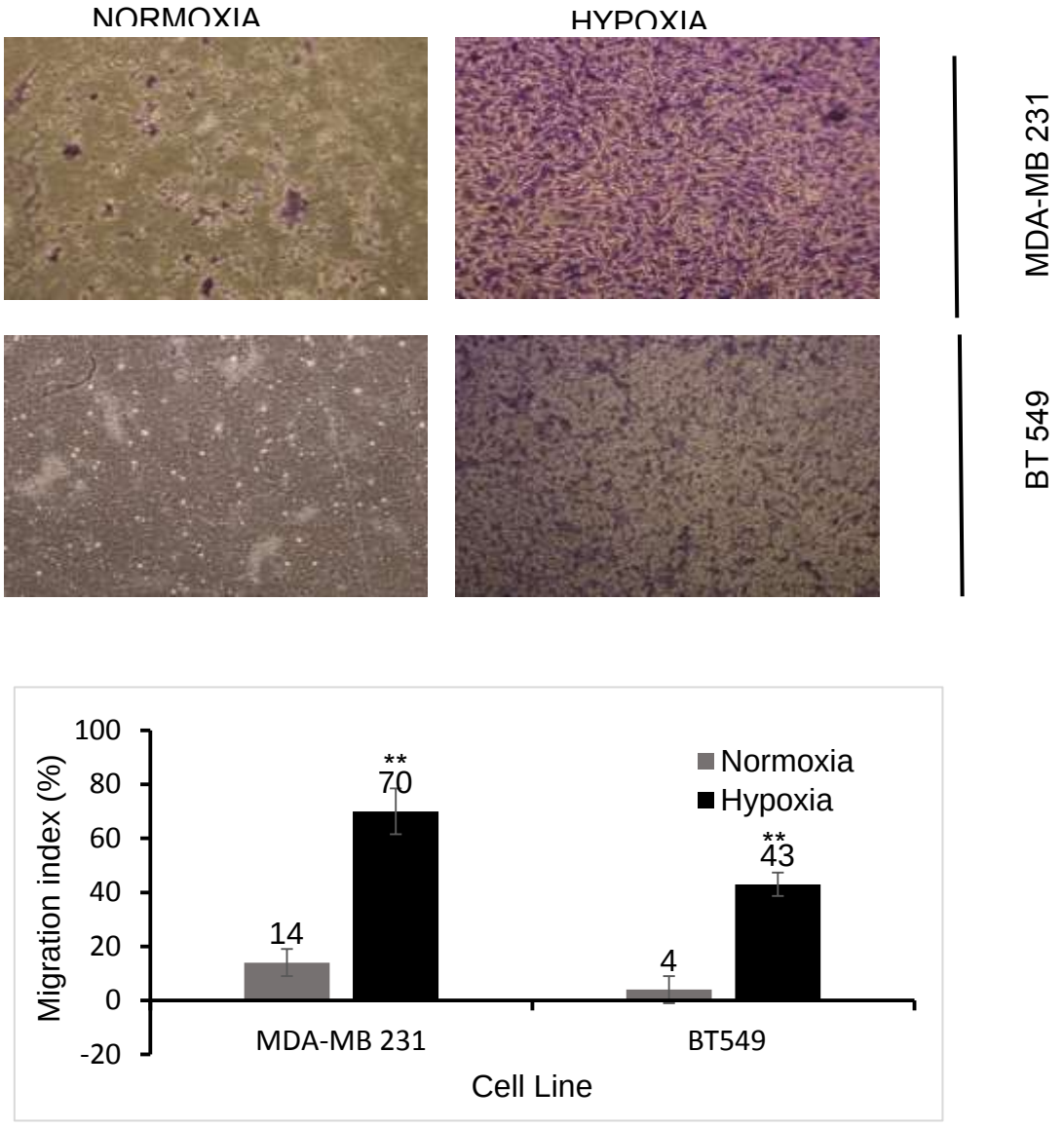

Figure 10. Representative Matrigel Migration assay of TNBC hypoxic cells. Images (Magnification 20X) (Mann and Whitney U test, $^{**}p=<0.0001, n=6$). (Abbreviations Triple negative breast cancer (TNBC). Epithelial to mesenchymal transition (EMT)).

DISCUSSION

Hypoxia, a reduction in tissue oxygen tension is a common feature associated with solid tumours (Vaupel and Mayer, 2007). Tumours adapt to these hypoxic changes by switching on genetic pathways that promote tumour aggressiveness, metastasis and chemoresistance; patients with hypoxic tumours generally have a poorer prognosis (Semenza, 2007; Keith and Simon, 2007). Increasing evidence show that cancer is a disease that involves stem cells as cells with stem-like features have been isolated from many solid tumours including the breast, brain, colon, liver and pancreatic cancers (Li *et al.,* 2007; Prince *et al.,* 2007). Increased expression of certain cancer stem cell markers (CSC) markers such as $ALDH^+$ and overexpression of embryonic stem markers (Sox2, Oct4, Nanog) have been used to identify stemness in BC cells (Tirino *et al.*, 2013).

Das *et al.,* (2008) reported that hypoxic tumour cells are poorly differentiated and express stem cell markers. Other studies also postulate that hypoxia may be responsible for the persistence of CSCs in tumours and the hypoxic niche provides a suitable microenvironment for the maintenance of these CSCs

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which are involved in promoting therapeutic failure and tumour relapse (Lin and Yun, 2010). With these facts in mind, it was hypothesized that hypoxia may be involved in the induction of CSCs traits and maintenance of CSCs in cancer cells. Breast cancer cell lines MDA-MB 231 and BT549 cultured in hypoxic chamber were observed to have higher population of hypoxic cells compared to those cells cultured in normoxia. HIF nuclear translocation was also increased in the hypoxic cultured cells (Figure 1 and 2). Data from this study (Figure 4, 5, 6) also showed that monolayer TNBC cells grown under hypoxia had higher expression of CSCs markers ($ALDH^+$, CD133) and embryonic markers (Sox2, Oct4, Nanog) compared to cells grown in normoxia, suggestive that hypoxia induces these CSC traits.

Breast cancer metastasis is one of the primary cause for cancer related deaths in TNBC patients (Gupta and Massagu, 2006). Patients with primary tumours that contain high population of hypoxic cells have been reported to have a reduction in overall survival rate after surgical resection of the primary tumour (Vergis *et al.,* 2008), these suggests that hypoxia is able to promote a more aggressive tumour with metastatic phenotype. According to the report by Badve and Nakshatri, (2012) CSC phenotype represents tumours that are aggressive with inherent ability to adapt, lie dormant or rapidly proliferate and these CSC phenotype represents the plasticity of tumours to undergo EMT giving rise to metastasis. The results from this study shows that cells under hypoxia displayed an increase in vimentin and N-cadherin expression indicating a switching to a mesenchymal phenotype while losing E-cadherin which is the trademark of epithelial phenotype (Figure 8). Further confirmation from the invasion and migration assay (Figure 9 and 10) also demonstrated that cells cultured under hypoxia have significant increased migratory and invasion potentials compared to cells in normoxic culture indicating that there is a close relationship between stem cell induction and EMT phenotype in cancer cell lines.

Exposure of cells and tissues to hypoxia stimulates stress response pathways that enable self-preservation and anti-apoptotic phenotype in cells. There is strong association between tumour hypoxia and poor prognosis and resistance to therapies (Li *et al.,* 2009). Data from MTT analysis (Figure 3 A and B) reveal that cells grown under hypoxia were significantly resistant to the 5 conventional anticancer drugs (Cisplatin, vincristine, paclitaxel, Doxorubicin and gemcitabine) tested suggestive that hypoxia may be involved in the induction of these drug resistance indicative that hypoxia induces stemness of cells with resultant drug resistance.

CONCLUSION

Even with the advancement in treatment and outcome of patients in recent times, drug resistance of tumour cells is a major factor limiting the effectiveness of chemotherapeutic treatment in most human tumours. Hypoxia induces CSC-like characteristics in breast cancer cell line. The hypoxic cells show slow proliferation rate but displayed high migratory and invasive potentials. It is concluded that hypoxia induces these CSC-like phenotype leading to chemoresistance, therefore further understanding of the mechanisms of hypoxia-induced EMT will be beneficial in targeting these hypoxic stem cell.

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SJIF Impact Factor: 6.260| ISI I.F.Value:1.241| Journal DOI: 10.36713/epra2016 ISSN: 2455-7838(Online)
EPRA International Journal of Research and Development (IJRD)
Volume: 5 | Issue: 3 | March 2020 - Peer Reviewed Journal

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