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The Role of Hypoxia Inducible Factors in Hypoxia Induced Epithelial to Mesenchymal Transition and Chemoresistance in Breast Cancer

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Abstract: *Hypoxia, a reduction of oxygen levels insufficient to meet the metabolic requirement is a common feature of solid tumours and to ensure survival and maintenance of oxygen homeostasis in tumours during chronic hypoxic conditions, several cellular responses are triggered such as metabolic reprogramming, activation of hypoxia-inducible factors (HIFs), de novo synthesis of new blood vessels or the activation of epithelial to mesenchymal transition (EMT) to escape the hostile hypoxic environment and metastasis to colonize a different environment. The hypoxia-inducible genes are implicated in many different cellular functions such as cell survival, cell proliferation, apoptosis, glucose metabolism, and angiogenesis. Hence, this study was performed to evaluate the response of these overexpressing HIF1α and HIF2α to anticancer drugs and to determine if they affect invasion and migration in cancer cell lines. Data from MTT analysis, western blot, invasion and migration analysis and fluorescence-activated cell sorting (FASC) showed that cells overexpressing HIF1α and HIF2α induced CSC-like characteristics in breast cancer cell line; the cells were significantly resistance to several chemotherapeutic drugs and cells displayed high migratory and invasive potentials, therefore hypoxia inducible factors induces these CSC-like phenotype leading to chemoresistance.*

Key Words: *Hypoxia, HIF1α and HIF2α, Cancer Stem Cell, Chemoresistance.*

1. INTRODUCTION:

Many human cancers have significantly lower O_2 concentration when compared to surrounding normal tissues. This persistent reduction in O_2 availability induces the hypoxia Inducible Factors (HIFs), Notch and nuclear Factor Kappa B (NFκB) pathways which regulates several cancer biological processes such as cell immortalization and stem cell maintenance, glucose and energy metabolism, vascularization, genetic instability, invasion and metastasis and resistance to therapy (1,2). A study reported that there are regions of hypoxia or anoxia within sold tumours caused by poor or altered vascularization and deterioration of diffusion geometry which results in limited oxygen diffusion and delivery (3). This decrease in oxygen tension may be detrimental to some tumour cells however hypoxia can contribute to a selection of cells that are more aggressive and promote tumour growth (2, 4), leading to poor prognostic outcome and an increased risk for metastasis that may escape therapy (5). Neoplastic cells surviving in hypoxia display increased invasive tendency suggesting that hypoxia may favour cancer progression (6, 7).

2. LITERATURE REVIEW:

Neoplastic cells respond to hypoxic microenvironments through the activation of hypoxia-inducible factors (HIFs). HIFs are DNA-binding transcription factors that respond to changes in oxygen levels specifically during hypoxia. They are the key regulator of hypoxia-inducible genes implicated in many different cellular functions such as cell survival, cell proliferation, apoptosis, glucose metabolism, and angiogenesis. HIF is a heterodimer consisting of an oxygen-labile α-subunit (HIFα) closely regulated by PHDs (prolyl-hydroxylases) and a constitutively expressed β-subunit (HIFβ or ARNT) (8). Three HIF alphas isoforms (HIF1α, HIF2α and HIF3α) have been identified in humans with HIF1α and HIF2α been the most established of the HIF family. Under hypoxia, several transcription factors are activated, one of such is the hypoxia-inducible factors (HIF-1 and HIF-2) referred to as the master regulators for hypoxia. HIF1α and HIF2α are commonly over expressed in malignant cells and is greatly associated with poor prognosis in many breast cancers (9, 10).

2.1 Rationale and aims of this study

This study was carried out to investigate the role of HIF1α and HIF2α in hypoxia induced epithelial to mesenchymal transition (EMT) and related stemness and to find out if HIF1α or HIF2α functions as the driving force behind chemoresistant CSCs in TNBC.

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3. MATERIALS:

Cell lines and reagents:

TNBC cell lines MDA-MB 231 was purchased from ATCC, Middlesex, UK. HypoxyprobeTM-1 plus Kit (Burlington, MA, USA) Sox2 (3578), Oct4 (2890) and Nanog (3580)(Cell Signalling, Herts, UK). HIF1α (NB100-479) and HIF2α antibody (NB100-122) (Novus Biologicals, CO, USA). Gemcitabine (dFdC), Paclitaxel (PTX), Cisplatin (CDDP), Vincristine (VCR), Doxorubicin (DOX) were purchased from Sigma.

4. METHODOLOGY:

Stable transfection of MDA-MB 231 cell line with HIF1α and HIF2α

MDA-MB 231 cells were cultured ($1x10^6$ cells/well) in 6 well plates without antibiotics overnight. 4µg of empty vector pcMV6 and recombinant vector with HIF1α and HIF2α were introduced into the cells separately using Lipofectamine™ 2000 reagent. The transfected cells were incubated at 37^0C for 24 hours selected for 7-10 days in a selective medium containing G418 150ug/mL for HIF1α culture and 200ug/mL for HIF2α cultured cells. Colonies of cells were picked up HIF1α (clone 3 and 4) and HIF2α (clone 4 and 6), enlarged and screened for over-expression of target gene in comparison with mock transfected clones using western blot.

MTT cytotoxicity assay in HIF1α and HIF2α transfected cells

The overnight cultured cells (Mock and HIF1α; HIF2α) (5000 per well) in 96-well flat-bottomed microtiter plates were exposed were exposed to anticancer drugs 72 hours paclitaxel (PTX), 48 hours vincristine (VCR), 120hours doxorubicin (DOX), cisplatin (CDDP) 120 hours and gemcitabine (dFdC) 72 hours and MTT assay performed. (Plumb et al, 1989).

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.

Determination of EMT characteristics in HIF1α and HIF2α transfected cells

To examine the epithelial to mesenchymal transition (EMT) in HIF1α and HIF2α transfected cells, EMT markers vimentin, E-Cadherin and N-cadherin were determined by western blot. *In vitro* wound healing assay (scratch assay) for migration and transwell invasion assay (Boyden chamber assay) were also performed to determine the invasive and migratory properties of the cells.

5. DISCUSSION:

Cellular adaptation to environmental stresses such as hypoxia promotes the activation of several signalling pathways to ensure survival. Primary mediators of the hypoxic response of cells HIF1α and 2α are activated under hypoxic stress. Evidence from several reports demonstrated that HIF activation is associated with an undifferentiated phenotype in several cancers; in primary pancreatic carcinomas, nuclear accumulation of HIF1α protein was found mainly in poorly differentiated tumour cells and is responsible for the maintenance of the cancer stem cell (CSC) phenotype and its functions (11, 12). Similar observations were seen in neuroblastomas, renal cancer and non-small lung cancer as elevated levels of HIF1α and HIF2α proteins were found in stem cell-like populations (13, 14). Covello *et al.,* (15) confirmed that Oct4, a stem cell transcription factor is a target for HIF2α and HIF2α activated Oct4 regulates self-renewal and differentiation in stem cells. Flow cytometry data analysis from our study showed that both HIF1α and HIF2α overexpression induced higher expression of CSC marker (ALDH and CD133) compared to the mock cells (Figure 2 and 3) suggesting that HIF protein may induce these stem-like characters in cells. Embryonic stem cell markers indicating pluripotent phenotype (Nanog, Oct4, Sox2) were also overexpressed in the HIF transfected cells (Figure 4). These results are consistent with other reports which showed that elevated levels of these stem cell markers occurs in many somatic carcinomas (16, 17).

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HIF the master regulator of hypoxia is not normally expressed in breast tissues but is highly expressed in malignant breast cancer (18). Hypoxia promotes EMT in BC cells via the induction of notch and twist pathways which are direct transcription target of HIF1α (19). HIF2α was shown to induce EMT by overexpression of 'snail' and 'vimentin' in mouse lung model (13). Our results (Figure 6) showed that both HIF1α and 2α overexpressing clones have higher expression of vimentin and N-cadherin indicating a mesenchymal phenotype with associated loss of E-cadherin a hallmark of epithelial phenotypes. The migratory and invasive potential from these HIF overexpressing clones were also increased (Figure 7 and 8) suggestive that HIF1α and 2α may play important roles in the activation of EMT.

It was hypothesized that the induction of CSCs-like traits by HIF may be responsible for this drug resistance. Study by Teicher *et al.,* (20) demonstrated that breast cancer cells acquire resistance to doxorubicin under low-oxygen-induced hypoxia and $CoCl_2$-induced chemical hypoxia indicative that HIF play significant role in hypoxia induced chemoresistance. The focus of this study was to examine the effects HIF1α and 2α overexpression on chemoresistance and possibility understand the mechanism involved in this drug resistance. The results (Figure 5A and 5B) showed that both HIF1α and 2α overexpressing cells were significantly resistant to the 5 conventional anticancer drugs tested (Cisplatin, paclitaxel, doxorubicin, vincristine and gemcitabine) which is in line with previously published study (21). Recent studies show that cancer stem-like cells play key roles in TNBC chemoresistance (22, 23). Bhola *et al.,* (22) demonstrated that HIF-1α is a central determinant of triple negative cancer stem-like cell chemo-resistance. These finding along with results obtained from this study strongly suggest that HIF are important for induction of CSC traits in BC with associated EMT and chemoresistance.

6. RESULTS:

Stable transfection HIF1α in MDA-MB 231BC cell line

Mock cells and two clones (C3 and 4) with the highest expression of HIF1α cultured in media containing 150µg/ml of G418 were chosen for further testing. Mock cells and two clones (C4 and 6) with the highest expression of HIF2α cultured in media containing 200µg/ml of G418 were chosen for further testing Western blot results (Figure 1) show that clones 3 and 4 expressed higher levels of HIF1α and clones 4 and 6 expressed higher levels of HIF2α in whole cell lysates and nuclear protein extracts.

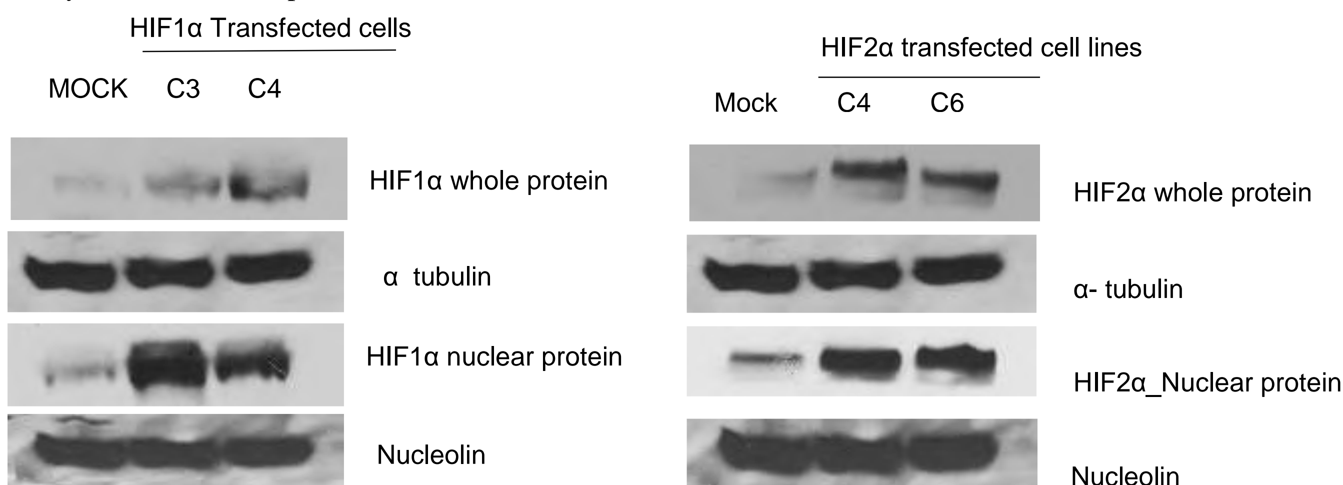

Figure 1: Representative Western Blots of Mock and HIF1α and HIF2α transfection cells:
Tubulin and Nucleolin were used as loading control.

Overexpression HIF1α and HIF2α in breast cancer cells induces higher expression of CSC markers.

Fluorescence Activated Cell Sorting FASC results (Figure 2) shows ALDH expression in Mock and HIF1α transfected clones (C3 and C4); HIF2 α transfected clones (C4 and C6) with and without treatment with DEAB (30µM). HIF transfected clones expressed high percentage of $ALDH^+$ compared to the mock cells before treatment with DEAB. Histogram (median ± interquartile range) displays the statistically significant increase in the $ALDH^+$ activity in HIF transfected clones in comparison to mock cells (Kruskal-Wallis test, $^{**}p=<0.0001, n=9$). After treatment with DEAB, there was no statistical difference in ALDH+ cell from both cultures.

FASC data (Figure 3) shows CD133 expression in Mock and HIF1α transfected clones (C3 and C4); HIF2 α transfected clones (C4 and C6). There was an increase in the expression of CD133 in the HIF overexpressing clones

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(C3 and C4) compared to mock cells. Histograms (median ± interquartile range) displays the statistically significant increase in the CD133 expression in HIF positive clones in comparison to mock transfected cells (Kruskal-Wallis test, ***p=<0.0001, n=9*).

The embryonic stem cell markers (Sox2, Oct4 and Nanog) were also elevated in the transfected cells when compared to the mock (Figure 4). (Kruskal-Wallis test, ***p=<0.0001, n=9*).

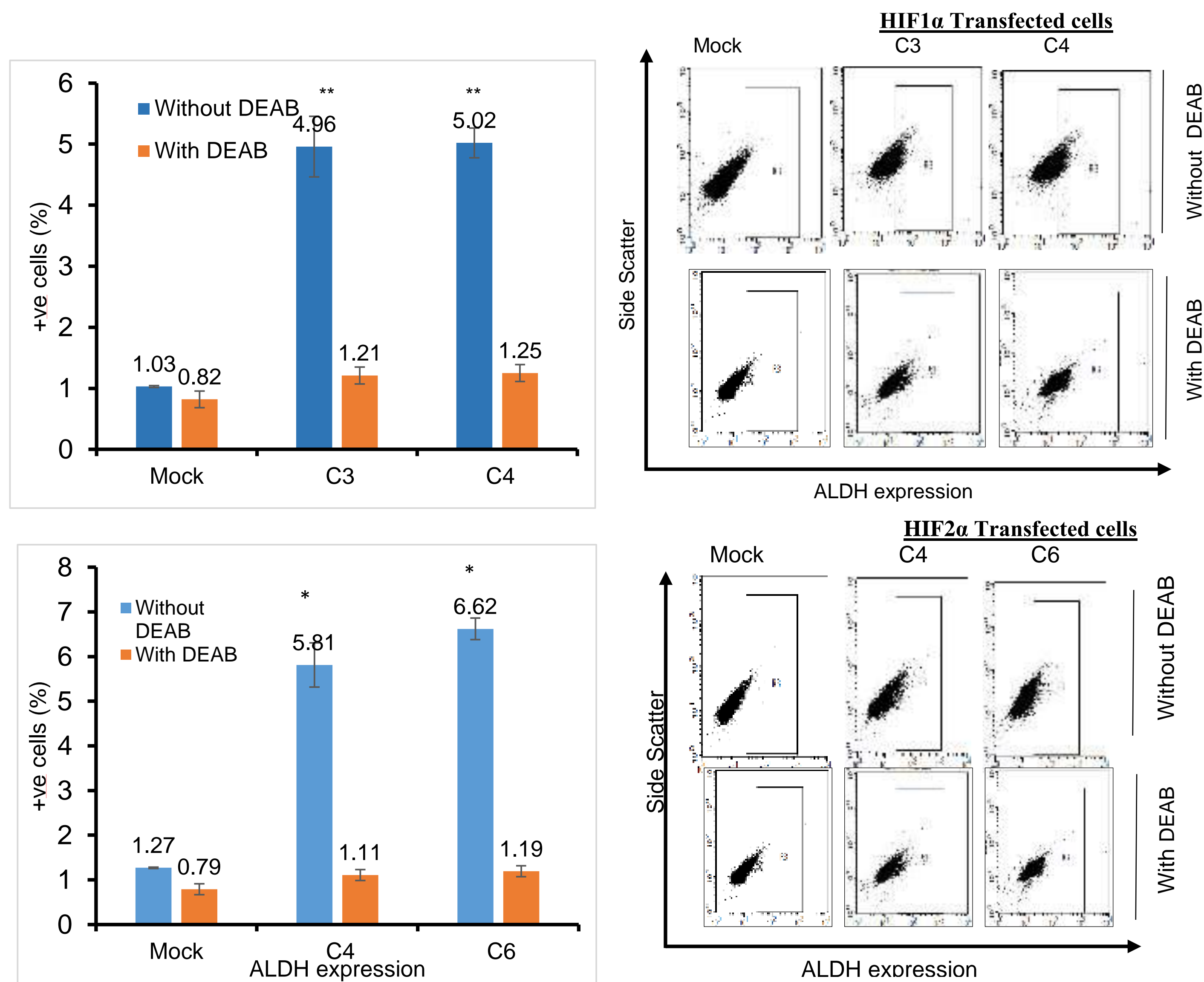


Figure 2: Representative FASC Plots of ALDH expression in MDA-MB 231 HIF1α and HIF2α **transfected cells measured by ALDEFLUOR assay.** (Abbreviations: DEAB: Diethylaminobenzaldehyde; ALDH: Aldehyde dehydrogenase active)

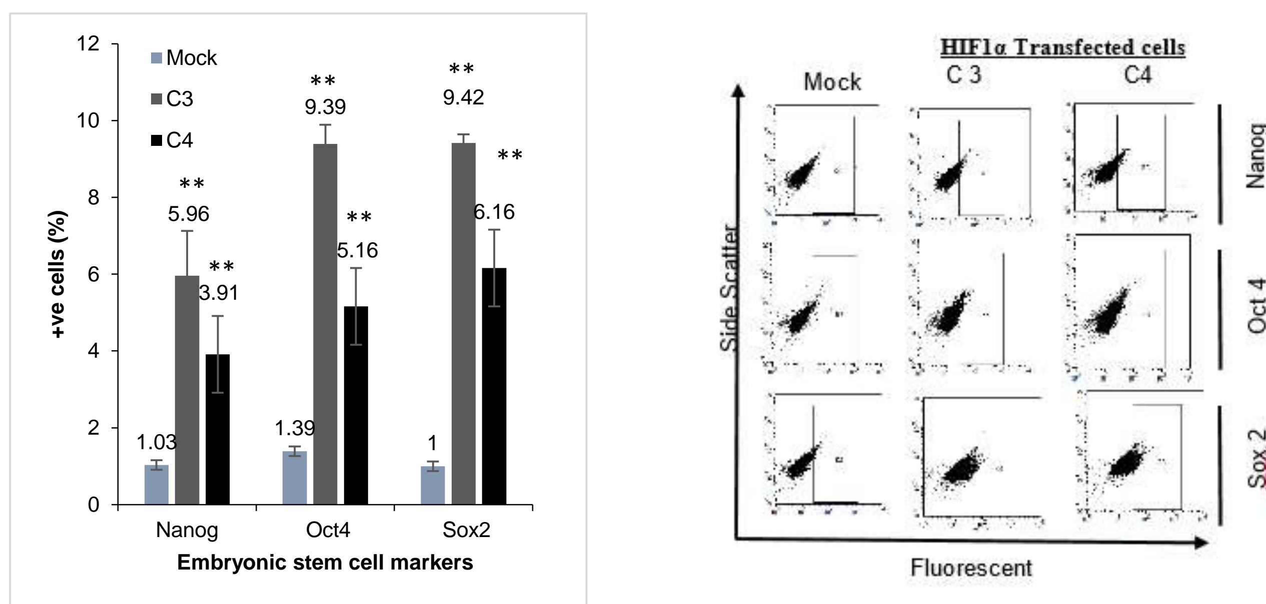
+ve cells (%)
3.00 2.50 2.00 1.50 1.00 0.50 0.00
1.07 ** 2.40 ** 1.54
MOCK C3 C4
CD133 expression

HIF1α Transfected cells
Mock C3 C4
Side Scatter
CD133 expression

+ve cells (%)
6.00 5.00 4.00 3.00 2.00 1.00 0.00
1.07 3.51 ** ** 4.97
Mock C4 C6

HIF2α Transfected cells
Mock C4 C6
Side Scatter
CD133 expression

Figure 3: Representative FASC Plots of CD133 expression in MDA-MB 231 mock and HIF (1α and 2α) transfected cells measured by PE-CD133 immunostaining assay.



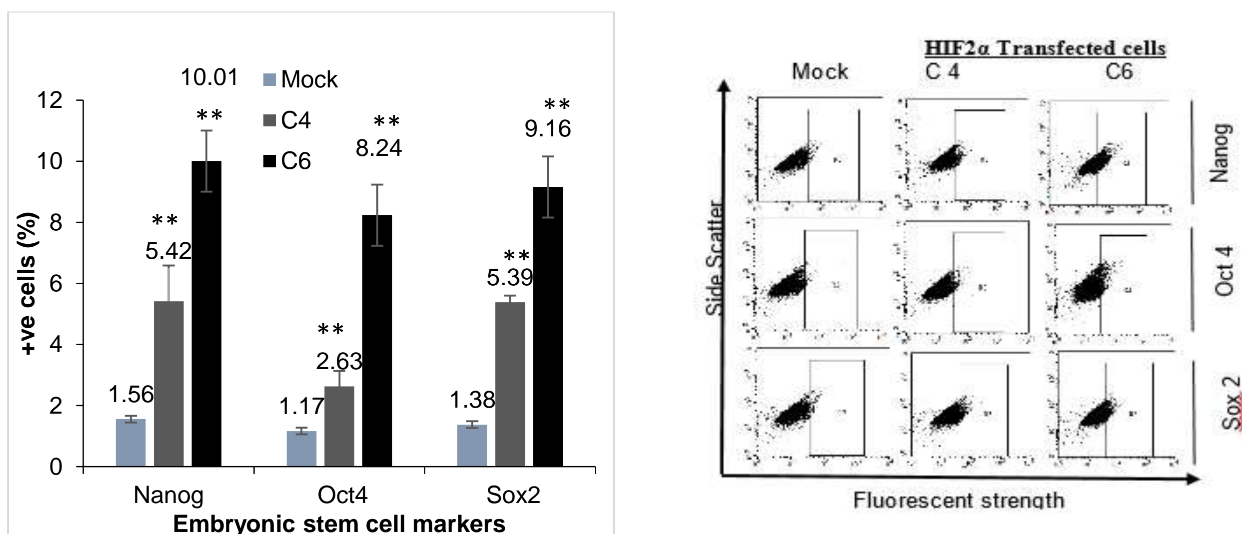
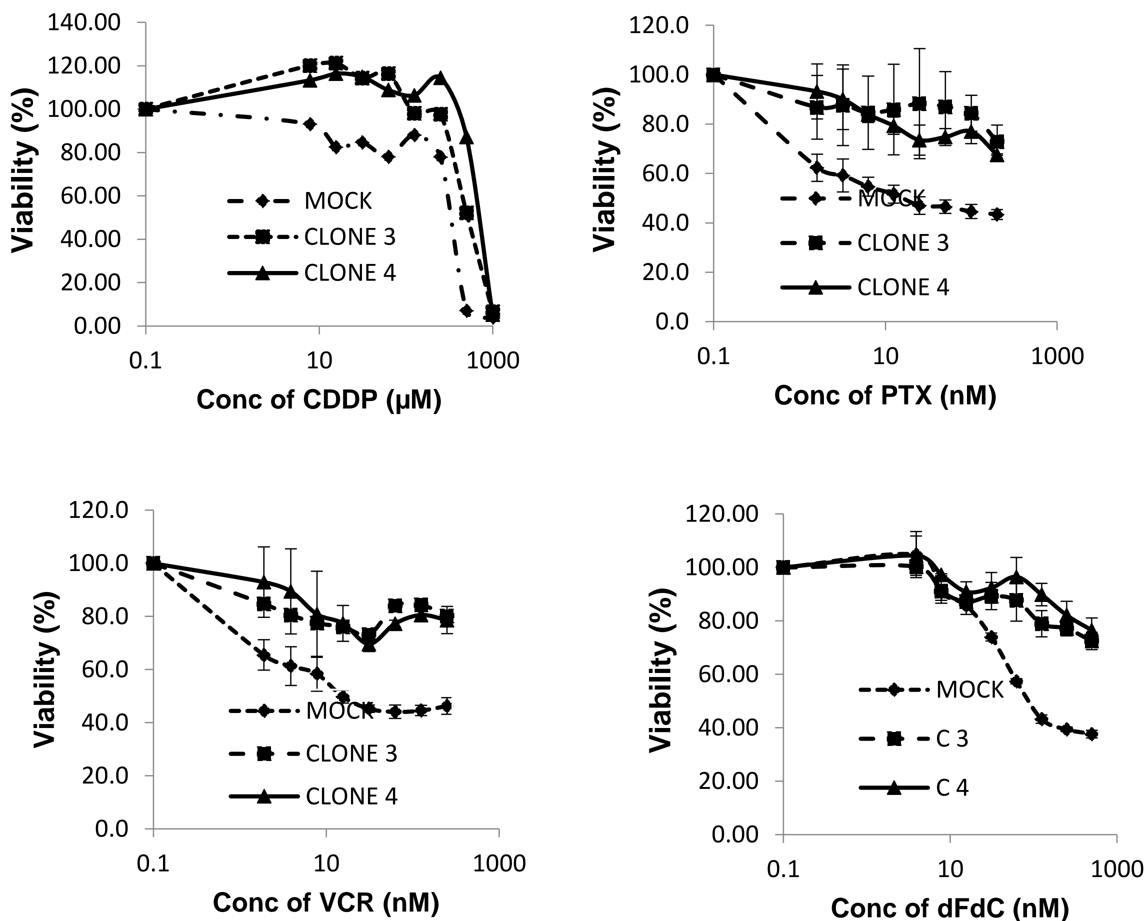


Figure 4: Representative FASC Plots of Embryonic Stem cell markers expression in MDA-MB 231 HIF (1α and 2α) transfected cell.

Increase in HIF (1α and 2α) activities induces resistance to anticancer drugs

MTT cytotoxicity results showed that HIF1α positive clones (C3 and C4) and HIF2α (C4 and C6) were extremely resistant to all five anticancer drugs (178isplatin, doxorubicin, vincristine, paclitaxel and gemcitabine) tested compared to the Mock cells. Figure 5A and B shows the cell viability curves and Table 1 and 2 for the IC_{50} values for the five anticancer drugs indicating that increase HIF1α and HIF2α proteins respectively is able to drug resistance in cells.



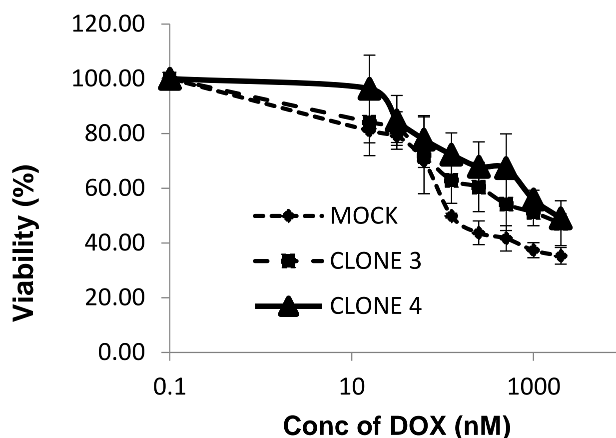


Figure 5A: Representative Drug Concentration Response Curves of Mock and HIF1 α transfected cells after treatment with anticancer drugs CDDP, VCR, PTX, dFdC and DOX. (Abbreviations; CDDP: 179isplatin, VCR: vincristine, PTX paclitaxel, dFdC: 179isplatin179e and Dox: doxorubicin).

Table 1 IC_{50} values of some Anticancer Drugs in Mock and HIF1α Transfected Cells

Anti –cancer drug	IC_{50} Mock	IC_{50} HIF1α C3	IC_{50} HIF1α C4	H- value	p-value
Cisplatin (μM)	201± 20.61	> 500	> 500	19.53	0.002**
Paclitaxel (Nm)	20.56 ± 10.67	> 200	> 200	26.32	0.0001**
Vincristine (Nm)	16.89 ± 5.33	> 150	>150	22.67	0.0001**
Gemcitabine (Nm)	30.49 ± 9.81	> 500	> 500	38.78	0.0001**
Doxorubicin (Nm)	300.11 ± 8.73	> 1000	> 1000	27.10	0.0001**

Table 1 displays the results (median ± interquartile range) of half maximum inhibitory concentration (IC_{50}) values of HIF1 α transfected cells and mock cells. (Kruskal-Wallis test, ** p<0.05, n=9).

Table 2: IC_{50} values of 5 Anticancer Drugs in Mock and HIF2α Transfected cells

Anti –cancer drug	IC_{50} Mock	IC_{50} HIF2α C4	IC_{50} HIF2α C6	H- value	p-value
Cisplatin (μM)	201 ± 40.32	> 500	> 500	17.83	0.001**
Paclitaxel (Nm)	20.56 ± 10.67	> 200	> 200	24.42	0.0001**
Vincristine (Nm)	16.89 ± 5.33	> 150	>150	27.57	0.0001**
Gemcitabine (Nm)	30.49 ± 9.81	> 500	> 500	36.18	0.0001**
Doxorubicin (Nm)	300.11 ± 8.73	> 1000	> 1000	29.90	0.0001**

Table 2 displays the results (median ± interquartile range) of half maximum inhibitory concentration (IC_{50}) values of HIF2 α transfected cells (C4 and C6) and mock cells. (Kruskal-Wallis test, ** p<0.05, n=9).

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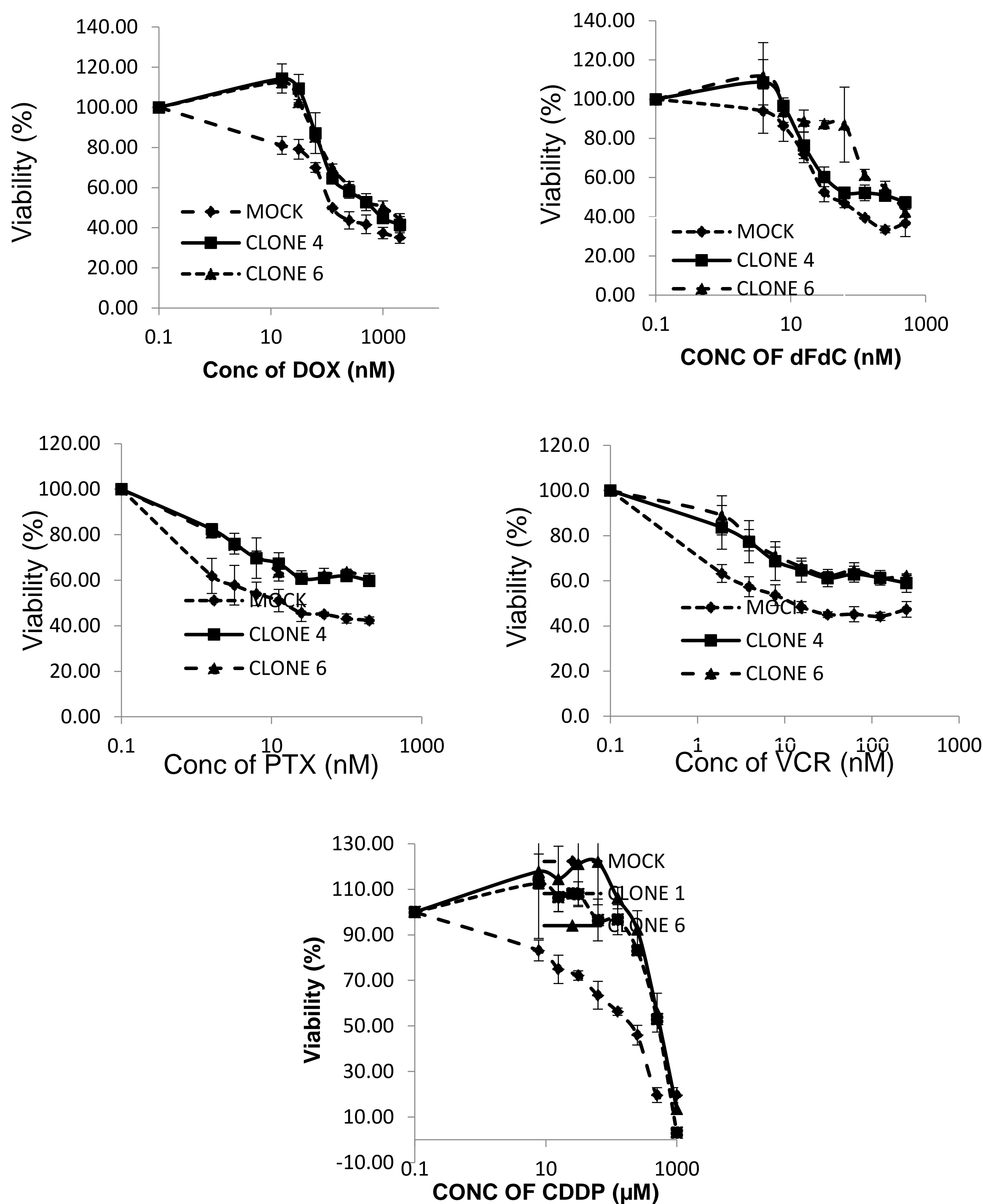

Figure 5B: Representative Drug Concentration Response Curves of Mock and HIF2α transfected cells after treatment with CDDP, VCR, PTX, dFdC and DOX. (Cisplatin: CDDP, vincristine: VCR, paclitaxel: PTX, gemcitabine (dFdC) and doxorubicin (DOX).

Cells with increase HIF1α and 2α activities displayed mesenchymal properties.

Expression of mesenchymal proteins vimentin and N-cadherin in HIF1α (Clones 3 and 4) and HIF2α (clones 4 and 6) positive clones were observed as shown from western blot analysis in Figure 6, with a loss of the epithelial marker E-cadherin. In the mock cells the E-cadherin expression was high with decreased expression of vimentin and N-cadherin suggesting an epithelial phenotype in these cells.

Images (Magnification 20X) taken by light microscope from wound healing scratch assay (Figures 7) and matrigel invasion (Figures 8) after 3 days show that increase in HIF activities increased invasive properties of HIF1α (Clones 3 and 4) and HIF2α (clones 4 and 6) in comparison to Mock cells indicating EMT activation

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and mesenchymal properties These results show that HIF overexpression in cells is able to induce a more mesenchymal phenotype.

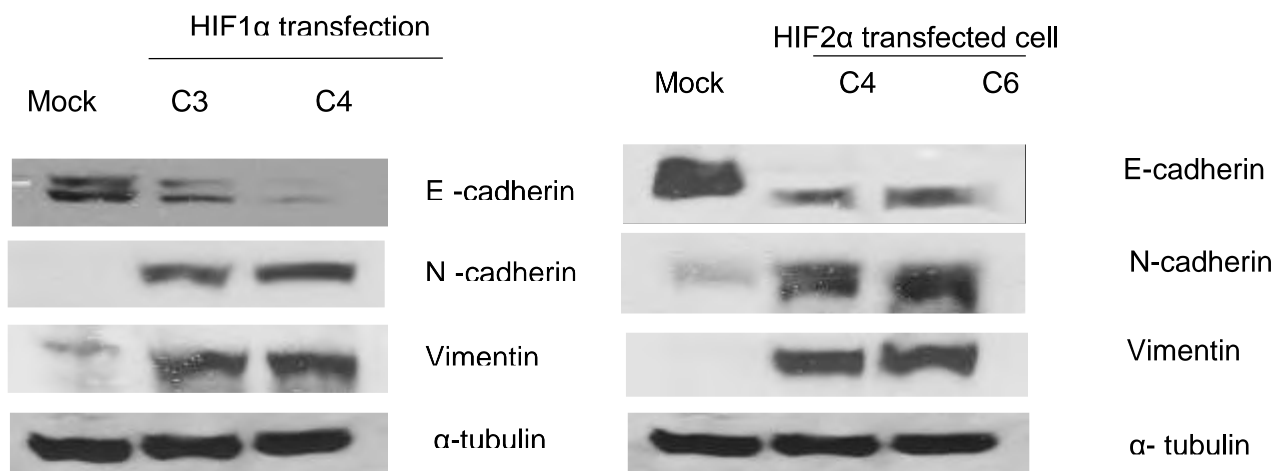

Figure 6: Representative Western Blots of EMT markers in Mock and HIF1α and HIF2α transfected cells using whole cell lysates. Tubulin was used as loading control.

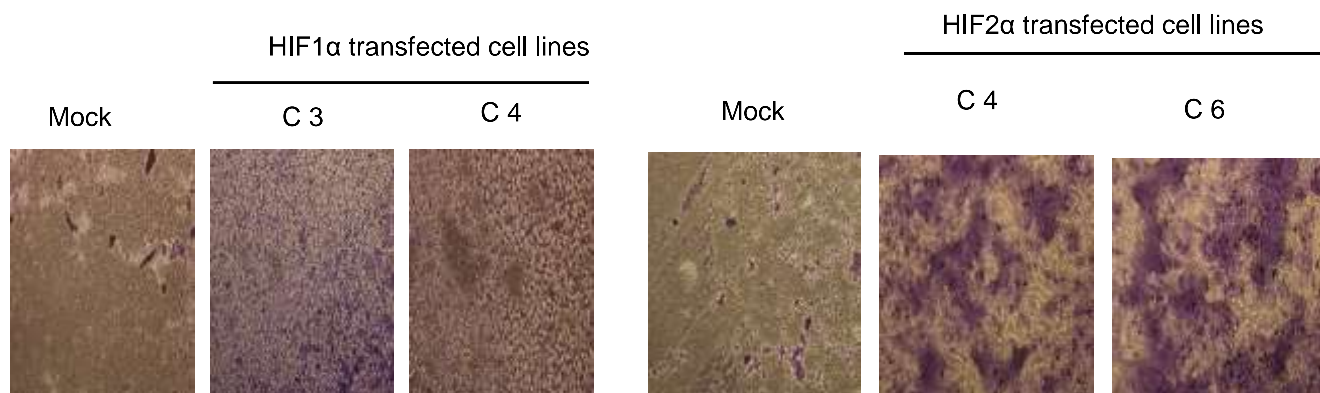

Figure 7: Representative Matrigel Invasion assay Images of Mock and **HIF1α and HIF2α transfected cells.**

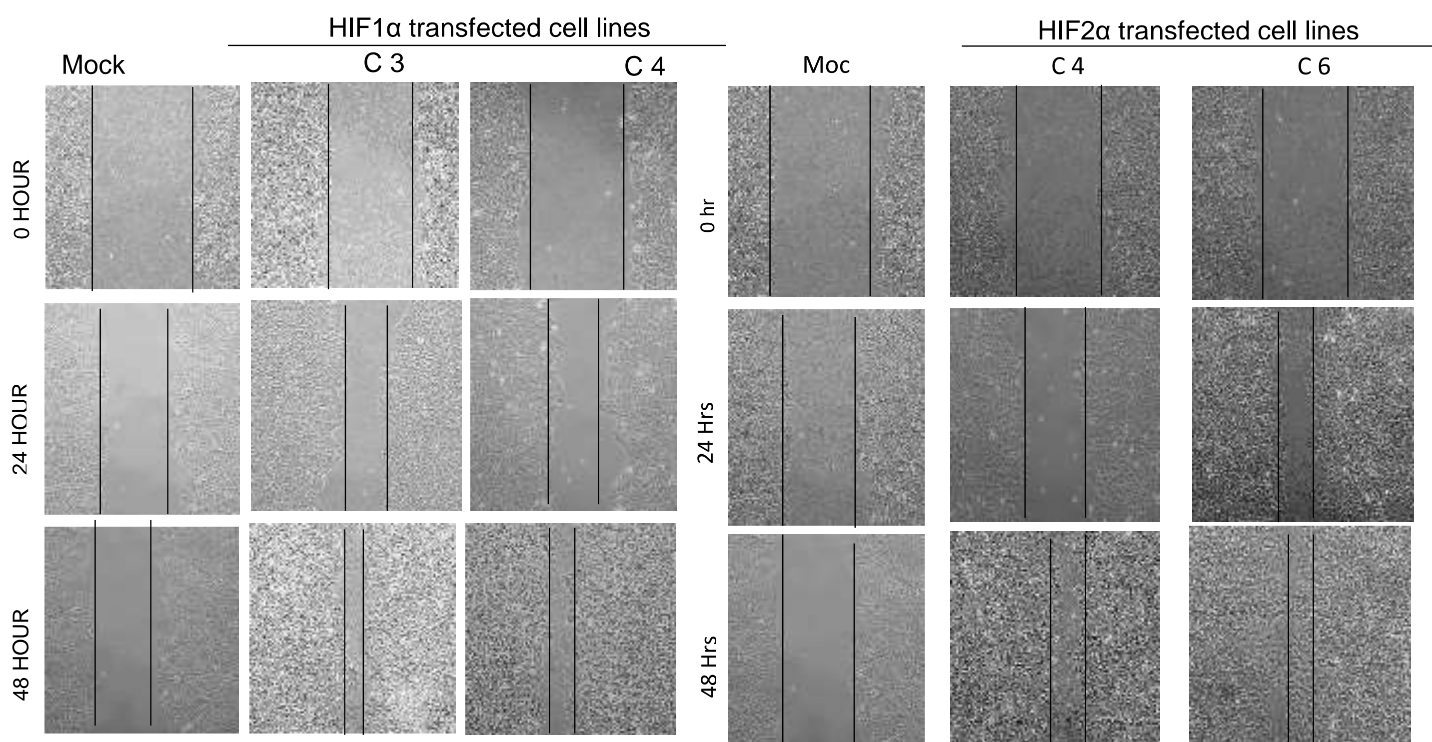

Figure 8: Representative Migration (wound healing) assay Images of Mock and HIF1α and HIF2α transfected cells.

7. CONCLUSION:

The main aim of this study was to examine the role of HIF1α and 2α proteins in hypoxia-induced EMT and consequent chemoresistance. Data from our results showed that both HIF1α and 2α can induce EMT and CSC traits. Therefore, development of pharmacological agents to inhibit these factors may hold promise for patients with TNBC as they lack targetable receptors.

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